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Modification of receptor selectivity and functional activity of cyclic cholecystokinin analogues

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Abstract – We reported earlier on the synthesis and biological activity at the CCK-B receptor of cyclized derivatives of CCK. These peptides, in which the positions 28 and 31 were replaced by lysine residues, were bridged by a succinyl moiety. To determine the importance of the nature and size of the cyclic structure, cyclic analogues were synthesized in which: (i) the lysine residues were replaced by ornithine and diaminobutyric acid and (ii) the succinic moiety was replaced by a malonic, adipic and glutaric moiety. They were tested for their ability to inhibit the specific binding of ^{125}I-BH-CCK-8 to CCK receptors in rat pancreatic acini and guinea pig brain membranes. They were also evaluated for their ability to stimulate amylase secretion from rat pancreatic acini. The potency and selectivity of these analogues were compared with those obtained with CCK-4 and compound JMV320, a potent and selective CCK-B receptor ligand synthesized earlier in our laboratory. © Elsevier, Paris

amylase release / CCK receptor / CCK-receptor antagonist / cyclic CCK analogue / partial agonist

1. Introduction

Cholecystokinin (CCK) is a peptide hormone of 33 amino acid residues that functions as a digestive hormone in the periphery and as a neurotransmitter in the central nervous system [1, 2]. The multiple peripheral actions of this peptide include gall bladder contraction and pancreatic amylase release whereas in the central nervous system CCK may modulate the dopaminergic system [3]. Although several molecular forms of CCK have been identified, the C-terminal octapeptide CCK-8 is the smallest naturally occurring fragment that retains the whole range of biological activities of the entire molecule [4]. Extensive biochemical studies have shown that these different actions are mediated by two CCK receptor subtypes designated as CCK-A (alimentary) and CCK-B (brain) receptors [5], both of which are found in the CNS and periphery. They differ in structure and ligand specificity [6].

Constrained potent ligands, highly specific for either class of receptors are therefore essential to understand the physiological role of CCK. Since each type of CCK-receptor (CCK-A or CCK-B) interacts with distinct biological active conformation of the native peptide, many structure–activity studies have been done to investigate the effects of side-chain and backbone modifications of CCK-8 or CCK-7 derivatives for binding to CCK-A and CCK-B receptors [7]. The existence of different types of CCK receptors supports the idea of different biologically active conformations of the native peptide. Thus, introduction of conformational constraints [8] could facilitate recognition by a single class of binding site. Constrained cyclic peptides are useful probes in the exploration of the conformational features necessary for binding to receptors and to obtain selective ligands for various receptor types of peptide hormones. This approach has been successfully undertaken in the CCK series, leading to selective CCK-B ligands [9].

Previously described conformational studies of CCK-8 [10] have shown that its backbone is highly folded, and that the side chains of methionine in position 28 and 31 are pointing outwards. These residues are consequently good candidates for cyclizations and should stabilize this conformation. We have previously described compound JMV320

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Ac-Tyr-Lys-Gly-Trp-Lys-Asp-Phe-NH_2

$CO(CH_2)_2CO$

obtained by introduction of lysine residues in position 28 and 31 whose side chains are linked by a succinyl moiety [11]. It is well known that structural alterations of the C-terminal tetrapeptide of cholecystokinin lead to compounds showing a weak affinity for CCK-B receptors [12, 13]. However, compound JMV320 exhibits a high-affinity and selectivity for the CCK-B receptor with a loss of affinity for CCK-A binding sites [14]. To determine the importance of the size and the nature of the cyclic structure, we synthesized a series of cyclic CCK-7 analogues derived from compound JMV320. In these analogues, methionine residues in positions 28 and 31 were substituted by various amino acid residues (ornithine, lysine, diaminobutyric acid) and the succinyl bridge replaced by malonyl, adipyl or glutaryl moieties.

This paper describes the synthesis of these new cyclic peptide analogues, their apparent affinity on guinea pig brain membranes and pancreatic acini and their biological activity on pancreatic amylase secretion.

2. Chemistry

Syntheses of all the cyclic peptide derivatives were carried out in solution by fragment condensation and subsequent cyclization. The cyclization step was performed by taking advantage of the presence of a glycine residue in position 29.

The final cyclic compounds **52** and **59** were prepared according to the method previously described for the preparation of compound JMV320 [11] as illustrated in *figure 1*.

The fragment Z-A_2Bu(Fmoc)-Asp(OtBu)-Phe-NH_2 **16** was synthesized by coupling Z-A_2Bu(Fmoc)-OH

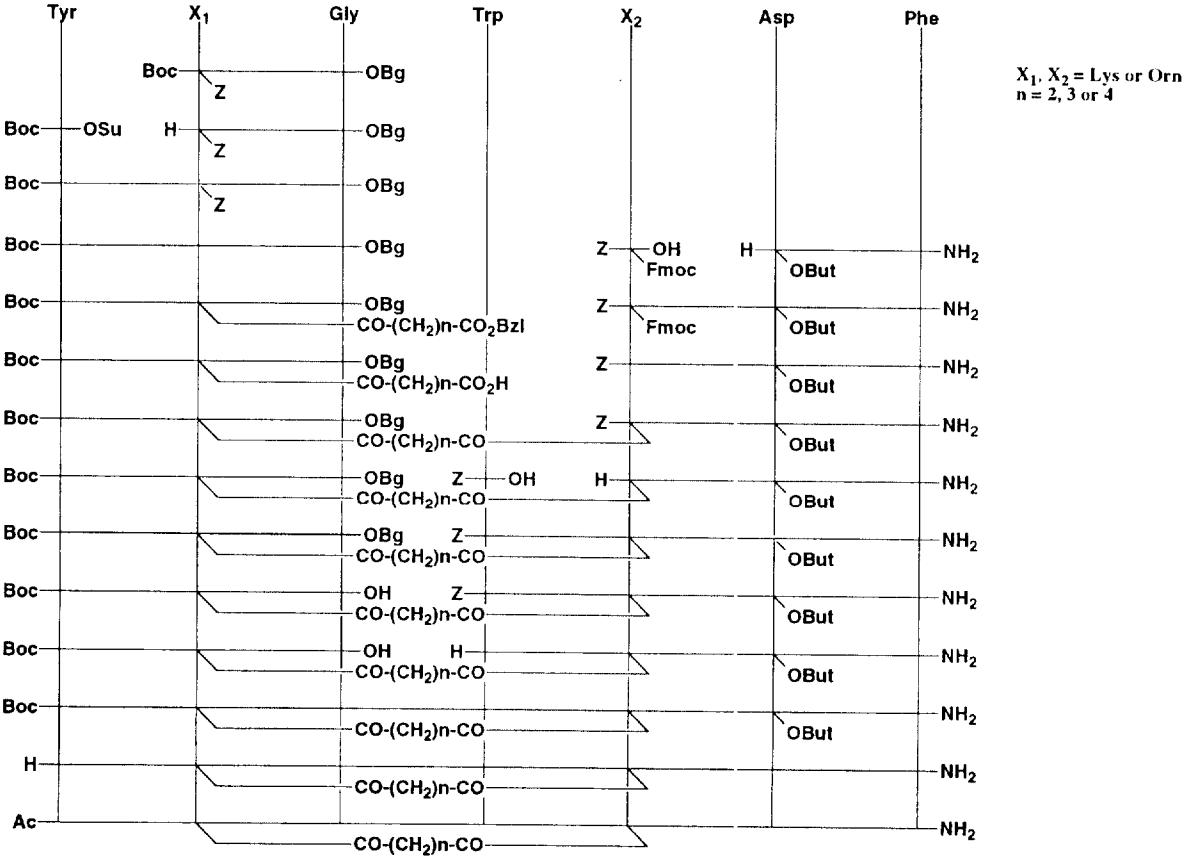

Figure 1. Synthesis of compounds **52**, **54–57**, **59**.

14 to the dipeptide H-Asp(OtBu)-Phe-NH_2 [11] in the presence of BOP [15] ((benzotriazolyloxy)tris-(dimethylamino)phosphonium hexafluorophosphate) as coupling reagent. Action of BTIB ([Bis(trifluoro-acetoxy)-iodo]-benzene) [16] on Z-Gln-OMe led to Z-A_2Bu-OMe which was reacted with Boc_2O ((di-tert-butyl)-dicarbonate) in dioxane to afford Z-A_2Bu(Boc)-OMe **12**. Saponification of the methyl ester and acidic removal of the tert-butyloxycarbonyl group followed by reaction with Fmoc-Cl produced Z-A_2Bu(Fmoc)-OH **14**. The fragment Boc-Tyr-A_2Bu(CO-CH_2-CH_2-COOH)-Gly-OBg **15** was synthesized by coupling Boc-Tyr-OSu with the dipeptide H-A_2Bu(CO-CH_2-CH_2-COOH)-Gly-OBg. Compound Boc-Gln-Gly-OBg **10** was prepared by condensation of Boc-Gln-OH with TFA-H-Gly-OBg [17] in the presence of isobutyl chloroformate. The peptide Boc-A_2Bu(CO-CH_2-CH_2-COOH)-Gly-OBg **11** was obtained by action of BTIB on Boc-Gln-Gly-OBg in acetonitrile followed by condensation with succinic anhydride. The total synthesis of the cyclic peptide **53** is described in *figure 2*.

The cyclic peptides **54**, **55**, **56** and **57** were synthesized in solution as outlined in *figure 1*. The different fragments Boc-Tyr-Lys-[CO(CH_2)$_n$-COOH]-Gly-OBg and Boc-Tyr-Orn-[CO(CH_2)$_n$-COOH]-Gly-OBg (n = 3, 4) were obtained by hydrogenation of Boc-Tyr-Lys(Z)-Gly-OBg [11] and Boc-Tyr-Orn(Z)-Gly-OBg **2** and subsequent reaction with adipic acid monobenzyl ester or glutaric anhydride to yield compounds **31**, **21**, **37**, and **26**. Hydrogenolysis of compounds **31** and **37** produced compounds **32** and **38**. The syntheses of the final peptides were performed as previously described.

Fragment Z-Lys(CO-CH_2-COOBzl)-Asp(OtBu)-Phe-NH_2 **47** was obtained by base-promoted cleavage of the Fmoc group of Z-Lys(Fmoc)-Asp(OtBu)-Phe-NH_2 followed by reaction with malonic acid monobenzyl ester. Hydrogenation of compound **47** followed by coupling with Z-Trp-OSu and subsequent reaction with the fragment Boc-Tyr-Lys-Gly-OBg led to compound **49**. Total synthesis of compound **58** was achieved as described in *figure 3*.

Physical and analytical data of the synthetic intermediates are reported in *table I*, those of the final compounds in *table II*. All compounds were identified by ^1H NMR at 360 MHz and mass spectrometry.

3. Biological results and discussion

All target compounds described above were tested for their ability to stimulate in vitro amylase secretion from rat pancreatic acini and to inhibit binding

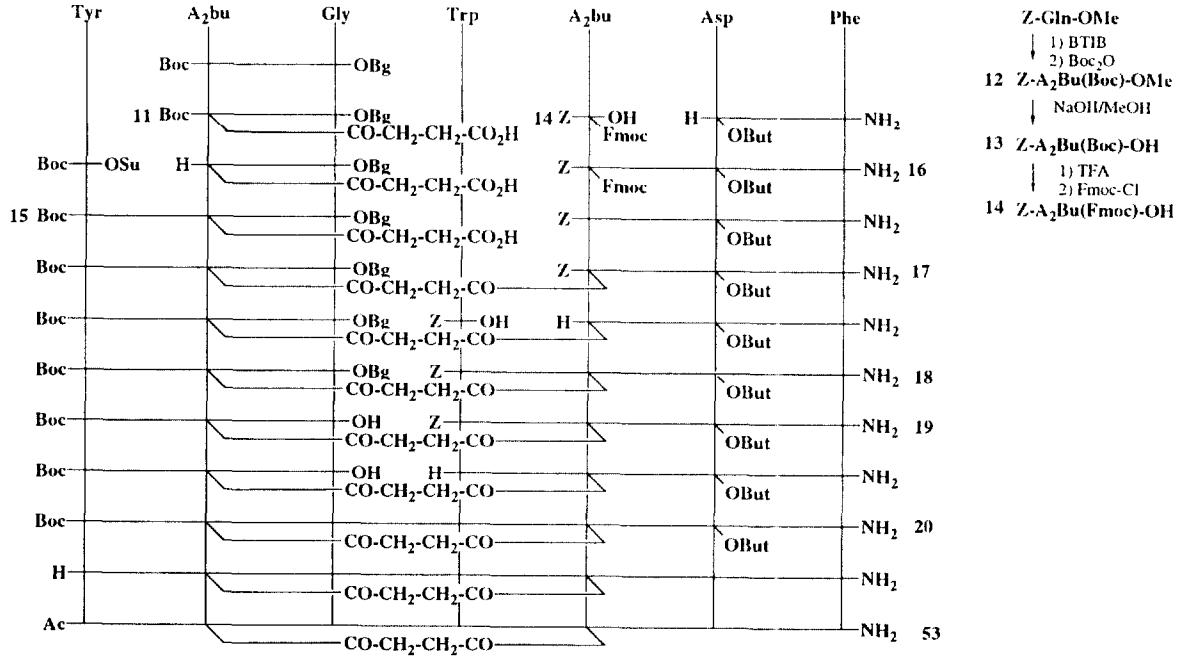

Figure 2. Synthesis of compound **53**.

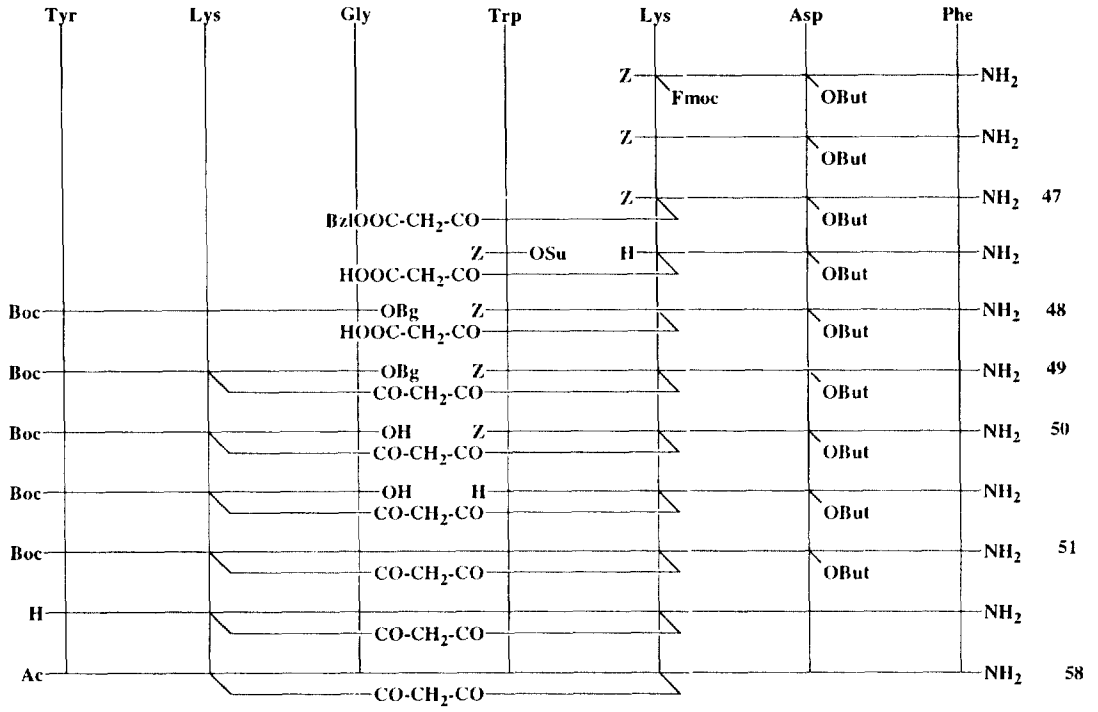

Figure 3. Synthesis of compound **58**.

of ^{125}I-BH-CCK-8 (Bolton–Hunter derivative of the C-terminal octapeptide of cholecystokinin) to isolated rat pancreatic acini (CCK-A receptors) and to guinea pig brain membrane CCK-receptors (CCK-B receptors) [18, 19]. They were compared in the same experiments with the potent CCK analogues Boc-[Nle^{28,31}]-CCK-7 [12, 20], Boc-Trp-Leu-Asp-Phe-NH_2 [21], and with compound JMV320, a potent and selective cyclic CCK-B receptor ligand. Results are summarized in *table III*.

Substitution of the succinyl moiety in compound JMV320 by a malonyl, adipyl or a glutaryl moiety led to compounds **58**, **56** and **54** respectively. Compound **58**, in which the link between the two lysines has been shortened, showed approximately 2-fold increased affinity for CCK-B receptors as compared to compound JMV320 but also significantly increased affinity for CCK-A receptors (about 100-fold). Lenghtening of the linker slightly decreased affinities of the analogues for CCK-B receptors (by approximately a factor of 20 or 100 for compounds **56** and **54** respectively) and increased affinity for CCK-A receptors. The efficacy and potency of these compounds to stimulate amylase release from pancreatic acini remained comparable to that of compound JMV320.

To study the length of the cyclic structures in compound JMV320 (i) the lysine residue in position 28 was substituted by an ornithine to lead to compound **59**, (ii) both lysine residues in position 28 and 31 were replaced by ornithine residues to lead to compound **52**, (iii) both lysine residues in position 28 and 31 were replaced by aminobutyric acid residues to lead to compound **53**. Compound **59** was able to bind to CCK-B receptors with a lower affinity than compound JMV 320 (by a factor of 90). The affinity for CCK-A receptors slightly increased but the biological response on amylase secretion remained almost unchanged. For compound **52** the affinity for CCK-B receptors decreased by a factor of more than 1000 while the affinity for CCK-A receptors remained unchanged. Surprisingly, the potency by which this compound was able to stimulate amylase secretion was decreased about 70-fold as compared to compound JMV320, while it exhibited the same efficacy. This difference in potency between the affinity for a given receptor calculated from binding experiments and the biological activity is often observed and still lacks, as far as we know, a rational explanation. Substitution of the lysine residues by two aminobutyric acid residues (compound **53**), which dramati-

Table I. Physical and analytical data of the synthetic intermediates.

Compound	Yields	Mp	R_f	$(\alpha)_D$ (c, DMF)	Compound	Yields	Mp	R_f	$(\alpha)_D$ (c, DMF)
1	89	dec. 40	A: 0.37; B: 0.60	−5 (0.93)	**30**	54	dec. > 220	P: 0.31, H: 0.41	−18 (1.06)
2	88	122–125	A: 0.1; B: 0.61	−8 (0.90)	**31**	84	97–99	C: 0.57; D: 0.85	−9 (0.97)
3	72	124–126	C: 0.25; D: 0.52	−6 (0.97)	**32**	99	dec. 80	C: 0.32; D: 0.55	−9 (1.33)
4	98	103–105	C: 0.61; D: 0.77	−2 (1.27)	**33**	75	142–144	C: 0.22; D: 0.53	−20 (1.02)
5	100	dec. 90	A: 0.59; B: 0.32	−13 (0.48)	**34**	77	dec. 78	C: 0.18; D: 0.38	−20 (0.97)
6	68	dec. 163	D: 0.68; C: 0.27	−18 (1.03)	**35**	52	dec. 140	F: 0.38; G: 0.87	−24 (0.89)
7	70	166–168	D: 0.62; C: 0.22	−20 (1.34)	**36**	51	dec. 128	F: 0.49; G: 0.89	−14 (0.94)
8	84	147–150	F: 0.39; H: 0.78	−21 (1.04)	**37**	75	dec. 90	D: 41; E: 0.46	−6 (1.28)
9	34	218–220	E: 0.36; F: 0.57	+27 (0.96)	**38**	79	dec. 74	E: 0.24; O: 0.49	−6 (1.01)
11	64	76–78	D: 0.23; E: 0.48	−9 (0.93)	**39**	80	156–158	O: 0.45; F: 0.80	−17 (1.15)
15	83	114–116	D: 0.49; E: 0.66	−14 (0.93)	**40**	95	dec. 100	E: 0.14; O: 0.36	−17 (0.99)
16	98	206–208	A: 0.45; B: 0.22	−31 (0.89)	**41**	78	dec. 138	F: 0.37; O: 0.14	−19 (1.18)
17	75	190–192	E: 0.37; F: 0.64	−26 (1.09)	**42**	88	dec. 158	G: 0.21; H: 0.39	−13 (0.96)
18	82	184–186	E: 0.48; F: 0.69	−30 (1.14)	**43**	82	195–197	P: 0.38; H: 0.49	−21 (0.97)
19	46	dec. 170	G: 0.18; H: 0.38	−36 (1.0)	**44**	93	192–194	P: 0.35; H: 0.46	−20 (0.98)
20	70	dec. 158	H: 0.34; I: 0.56	−31 (1.02)	**45**	68	188–190	H: 0.28; I: 0.43	−19 (0.94)
21	77	dec. 74	E: 0.56; O: 0.69	−10 (1.2)	**46**	65	dec. 195	H: 0.26; I: 0.52	−13 (0.95)
22	71	158–160	E: 0.57; O: 0.72	−21 (0.9)	**47**	82	dec. 155	D: 0.31; E: 0.62	−29 (1.01)
23	70	dec. 88	E: 0.22; F: 0.41	−19 (0.96)	**48**	79	dec. 145	F: 0.32; P: 0.58	−37 (1.26)
24	78	dec. 151	F: 0.15; P: 0.56	−23 (0.82)	**49**	84	dec. 120	E: 0.31; F: 0.58	−24 (0.99)
25	82	dec. 202	F: 0.24; H: 0.49	−22 (0.79)	**50**	71	dec. 130	F: 0.19; P: 0.44	−23 (0.98)
26	69	108–110	E: 0.35; F: 0.51	−8 (0.92)	**51**	83	dec. 145	F: 0.33; P: 0.62	−20 (0.89)
27	80	178–180	E: 0.30; F: 0.44	−18 (0.86)					
28	78	dec. 160	E: 0.26; F: 0.41	−19 (0.98)					
29	78	dec. 151	R: 0.49; M: 0.76	−21 (0.82)					

cally reduced the size of the cyclic structure, resulted in a severe loss of both CCK-B and CCK-A receptor affinity, and concomitantly in a severe decrease of the potency of the analogue on the secretory response. Substitution of the lysine residues in position 28 and 31 by two ornithine residues and introduction of a glutaryl or an adipyl replacing the succinyl moiety of compound JMV320 produced compounds **55** and **57**. The affinity of these compounds for CCK-B receptors dramatically decreased. In contrast, their affinity for CCK-A receptors significantly increased (IC_{50} = 1700 ± 500 nM for compound **55**, and 800 ± 200 nM for compound **57**). Interestingly, the analogue **55** exhibited only a partial agonist activity on amylase secretion, its maximal efficacy being approximately 40% of the maximal response induced by CCK-8 at 10 μM. The analogue **57**, up to a 10 μM concentration, was devoid of agonist activity on CCK-A receptors and behaved as a weak CCK-A receptor antagonist on CCK-8-induced amylase secretion. These results indicate that depending on the nature and the size of the cyclic structure in analogues of CCK cyclized between residues 28 and 31, it is possible to obtain highly potent and selective CCK-B receptor ligands, partial CCK-A agonists or CCK-A receptor antagonists.

This study showed that:

(1) replacement of the succinyl moiety of JMV320 by a malonyl, glutaryl or adipyl moiety produced CCK analogues with high affinity for CCK-B receptors (the malonyl group yielding the most potent compound) and retaining significant affinity for CCK-A receptors;

(2) replacement of the two lysines by ornithine residues and cyclization with glutaryl or adipyl moieties

Table II. Physical data of cyclic CCK analogues.

Compound	Mp	R_f	$(\alpha)_D^{20}$ (*c*, DMF)	t_R (min) [(A)/(B)[a]]	FAB + MS (*m/e*)
52	dec. 160	H: 0.71	–3 (0.99, DMSO)	39.12 [55/45]	1038
53	dec. 180	I: 0.52	–18 (0.95, DMSO)	19.54 [55/45]	1010
54	dec. 168	K: 0.54	–19 (0.8)	22.10 [50/50]	1080
55	dec. 208	M: 0.44	–20 (0.59)	41.56 [52/48]	1052
56	dec. > 220	G: 0.65	–10 (1.05, DMSO)	20.34 [50/50]	1094
57	dec. 211	K: 0.21	–16 (1.08)	29.78 [55/45]	1066
58	dec. 153	P: 0.53	–20 (1.04)	21.36 [52/48]	1052
59	dec. 154	K: 0.55	–11 (0.86)	19.92 [52/48]	1052

[a](A) ammonium acetate 0.05 M, pH 6.5; (B) methanol.

produced compounds of very low affinity for CCK-B receptors but exhibiting significant affinity for CCK-A receptors. In fact, as compared with compound JMV320, these cyclic analogues started to show CCK-A selectivity. In addition, according to the nature of the linker (glutaric or adipic acid), they were either partial agonists on amylase secretion in rat pancreatic acini or without intrinsic activity. These constrained cyclic analogues may be useful in the study of molecular interactions of CCK ligands with CCK-A and CCK-B receptors. Conformational studies have been undertaken in order to further understand these pharmacological results.

Table III. Receptor binding assay and stimulation of amylase secretion by cyclic CCK analogues.

Ac-Tyr-X1-Gly-Trp-X2-Asp-Phe-NH_2 $CO-(CH_2)_x-CO$				Rat pancreatic acini		Guinea pig brain membranes	
X1	X2	*x*	Compound	Amylase secretion EC_{50} (nM)	Binding IC_{50} (nM)	Binding IC_{50} (nM)	Selectivity factor (sf)[a]
Lys	Lys	1	**58**	3 ± 1	300 ± 10	1.4 ± 0.1	214
Lys	Lys	3	**54**	50 ± 10	4000 ± 500	240 ± 30	16.7
Lys	Lys	4	**56**	55 ± 8	800 ± 200	37 ± 7	21.6
Orn	Orn	2	**52**	5000 ± 700	25 000 ± 2000	3200 ± 700	7.8
Orn	Orn	3	**55**	40% max (10 000)	1700 ± 500	4000 ± 1000	0.43
Orn	Orn	4	**57**	no agonist (10 000)	800 ± 200	> 10 000	
Orn	Lys	2	**59**	100 ± 30	13 000 ± 1500	180 ± 20	72.2
A_2Bu	A_2Bu	2	**53**	100 000 ± 1000	92 500 ± 5000	8500 ± 2000	10.9
Boc-[Nle28,31]-CCK-7				0.05 ± 0.01	2.32 ± 0.48	0.29 ± 0.05	8
Boc-Trp-Leu-Asp-Phe-NH_2				–	4000 ± 851	2.22 ± 0.29	1802
JMV320				70 ± 30	21 000 ± 2000	2.03 ± 0.26	10 345

[a]sf: selectivity factor, ratio of the IC_{50} value of binding to rat pancreatic acini over the IC_{50} value of the binding to guinea pig brain membranes.

4. Experimental protocols

4.1. Chemistry

Melting points were taken on a Bücchi apparatus in open capillary tubes. Optical rotations were determined with a Perkin-Elmer 141 polarimeter. ^{1}H NMR spectra were recorded on a Bruker 360 instrument in DMSO-d_6 at 293 K. Ascending TLC was performed on precoated plates of silica gel 60 F_{254} (Merck) using the following solvent systems (by volume).
A: AcOEt;
B: AcOEt/hexane, 7:3;
C: AcOEt/hexane, 3:7;
D: chloroform/methanol/acetic acid, 120:10:5;
E: chloroform/methanol/acetic acid, 85:10:5;
F: chloroform/methanol/acetic acid, 60:10:5;
G: AcOEt/pyridine/acetic acid/water, 80:20:3:3;
H: AcOEt/pyridine/acetic acid/water, 80:20:5:10;
I: AcOEt/pyridine/acetic acid/water, 60:20:5:10;
K: AcOEt/pyridine/acetic acid/water, 50:20:5:10;
M: *n*BuOH/acetic acid/water, 3:1:1;
O: chloroform/methanol/acetic acid, 70:10:5;
P: chloroform/methanol/acetic acid, 40:10:5;
R: chloroform/methanol/acetic acid, 50:10:5.

Peptide derivatives were located with UV light (254 nm), charring reagent or ninhydrin. Column chromatographies were performed with silica gel 60, 60–229 mesh, ASTM (Merck). HPLC purifications were run on a Merck/Hitachi instrument on a Beckman Ultrasphere® ODS (10 μm) 150 x 22.5 mm column, with an UV detection at 279 nm, at a flow rate of 7 mL/min of a mixture of A: ammonium acetate 0.05 M, pH 6.5, and B: methanol. Mass spectra were recorded on a Jeol JMS-DX-300 apparatus. L-amino acids and derivatives were purchased from Bachem (Switzerland). All reagents and solvents were of analytical grade. BOP was recrystallized from acetone and ether. The following abbreviation was used: OBg, *N*-benzhydrylglycolamide. Other abbreviations used were those recommended by the IUPAC-IUB Commission [Eur. J. Biochem. 138 (1984) 9–37].

4.1.1. Boc-Orn(Z)-Gly-OBg **1**

To a cold (–20 °C) solution of Boc-Orn(Z)-OH (3 g, 8.2 mmol) in DMF (30 mL), were successively added NMM (N-methylmorpholine) (0.92 mL, 8.2 mmol) and IBCF (isobutyl chloroformate) (1.11 mL, 8.2 mmol). After 5 min stirring, TFA, H-Gly-OBg (3.54 g, 8.6 mmol) and NMM (1.2 mL) were added and the mixture was stirred at room temperature for 1 h. The reaction mixture was diluted with ethyl acetate (200 mL), washed with a 1 N aqueous potassium bisulfate solution, water, a saturated aqueous sodium bicarbonate solution, brine, dried over magnesium sulfate, and concentrated under reduced pressure to afford a residue that crystallized upon trituration in ether/hexane.

4.1.2. Boc-Tyr-Orn(Z)-Gly-OBg **2**

Boc-Orn(Z)-Gly-OBg (5 g, 7.7 mmol) was partially deprotected with TFA. The partially deprotected material was added to a solution of Boc-Tyr-OSu (2.8 g, 7.35 mmol) in DMF (10 mL) followed by DIEA (1.5 mL) and the mixture was stirred for 1 h at room temperature. The expected compound precipitated upon addition of a 1 N aqueous potassium bisulfate solution. It was collected, washed with water, a 2% aqueous sodium bicarbonate solution, water, hexane and dried in vacuo.

4.1.3. Boc-Tyr-Orn(CO-$(CH_2)_2$-COOH)-Gly-OBg **3**

This compound was prepared as previously described for the synthesis of Boc-Tyr-Lys(CO-$(CH_2)_2$-COOH)-Gly-OBg [11].

4.1.4. Z-Orn(Fmoc)-OH **4**

This compound was prepared as previously described for the synthesis of Z-Lys(Fmoc)-OH [11].

4.1.5. Z-Orn(Fmoc)-Asp(OtBu)-Phe-NH_2 **5**

This compound was prepared as previously described for the synthesis of Z-Lys(Fmoc)-Asp(OBut)-Phe-NH_2 [11].

4.1.6. Boc-Tyr-Lys[CO-$(CH_2)_3$-COOH]-Gly-OBg **21**

Boc-Tyr-Lys(Z)-Gly-OBg (3.5 g, 4.2 mmol) was hydrogenated overnight at room temperature in EtOH (100 mL) containing concentrated hydrochloric acid in the presence of a 10% Pd/C catalyst. The catalyst was removed by filtration and the filtrate concentrated in vacuo to leave a residue that solidified upon trituration in ether. It was collected, washed with ether, dried in vacuo over KOH pellets. To a solution of this partially deprotected peptide in DMF (15 mL) was added glutaric anhydride (0.47 g, 4.2 mmol) and DIEA (1 mL). The reaction mixture was stirred at room temperature for 5 h. The reaction mixture was diluted with ethyl acetate (200 mL), washed with a 1 N aqueous potassium bisulfate solution, water, brine, dried over magnesium sulfate, and concentrated under reduced pressure to afford the title compound that crystallized upon trituration in ether. Compound Boc-Tyr-Orn[CO-$(CH_2)_3$-COOH]-Gly-OBg **26** was prepared according to the same synthetic pathway.

4.1.7. Boc-Tyr-Lys[CO-$(CH_2)_4$-COOBzl]-Gly-OBg **31**

To a solution of the partially deprotected peptide Boc-Tyr-Lys-Gly-OBg (6.3 g, 8.7 mmol) in DMF (20 mL) was added adipic acid mono benzyl ester (2 g, 8.65 mmol), BOP (3.82 g, 8.65 mmol) and NMM (2 mL, 18 mmol). The reaction mixture was stirred at room temperature overnight. The reaction mixture was diluted with ethyl acetate (200 mL), washed with a 1 N aqueous potassium bisulfate solution, water, a saturated aqueous sodium bicarbonate solution, brine, dried over magnesium sulfate, and concentrated under reduced pressure to afford the title compound that crystallized upon trituration in ether. Compound Boc-Tyr-Orn[CO-$(CH_2)_4$-COOBzl]-Gly-OBg **37** was synthesized according to the same procedure.

4.1.8. Boc-Tyr-Lys[CO-$(CH_2)_4$-COOH]-Gly-OBg **32**

Compound **31** (6.5 g, 7.16 mmol) was hydrogenated overnight at room temperature in DMF (50 mL) in the presence of a 10% Pd/C catalyst. The catalyst was removed by filtration and the filtrate concentrated in vacuo to afford the title compound that crystallized upon trituration in ether. Compound Boc-Tyr-Orn[CO-$(CH_2)_4$-COOH]-Gly-OBg **38** was prepared according to the same synthetic pathway from compound **37**.

4.1.9. Z-Lys(CO-CH_2-COOBzl)-Asp(OBut)-Phe-NH_2 **47**

To a solution of Z-Lys-Asp(OBut)-Phe-NH_2 [11] (2.6 g, 4.4 mmol) in DMF (10 mL) were added malonic acid mono benzyl ester (0.9 g, 4.6 mmol), BOP (2.05 g, 4.6 mmol) and NMM (0.6 mL, 5 mmol). The reaction mixture was stirred overnight at room temperature. The reaction mixture was diluted with ethyl acetate (200 mL), washed with a 1 N aqueous potassium bisulfate solution, water, a saturated aqueous sodium bicarbonate solution, brine, dried over magnesium sulfate, and concentrated under reduced pressure to afford the title compound that crystallized upon trituration in ether. Compound Z-Lys[CO-$(CH_2)_4$-COOBzl]-Asp(OBut)-Phe-NH_2 **74** was synthesized according to the same procedure.

4.1.10. ZTrp-Lys-Asp(OtBu)-Phe-NH_2 ***48***

$CO\text{-}CH_2\text{-}COOH$

Z-Lys($CO\text{-}CH_2\text{-}COOBzl$)-Asp(OBut)-Phe-$NH_2$ **47** (2.2 g, 2.84 mmol) was hydrogenated overnight at room temperature in DMF (50 mL) in the presence of a 10% Pd/C catalyst. The catalyst was removed by filtration and the filtrate concentrated in vacuo. To a solution of the partially deprotected peptide H-Lys-($CO\text{-}CH_2\text{-}COOH$)-Asp(OBut)-Phe-$NH_2$ [11] (1 mmol) in DMF (20 mL) were successively added Z-Trp-OSu (1.24 g, 2.84 mmol) followed by DIEA (0.5 mL) and the mixture was stirred for 1 h at room temperature. The expected compound precipitated upon addition of a 1 N aqueous potassium bisulfate solution. It was collected, washed with water, hexane/ether and dried in vacuo.

4.1.11. Boc-Gln-Gly-OBg ***10***

This compound was prepared according to the procedure described for the synthesis of Boc-Orn(Z)-Gly-OBg **1**.

4.1.12. Boc-A_2Bu-($CO\text{-}CH_2\text{-}CH_2\text{-}COOH$)-Gly-OBg ***11***

To a solution of compound **10** (5 g, 9.5 mmol) in a mixture of acetonitrile and water (100 mL, 1:1) was added BTIB (6.12 g, 14.24 mmol). After 48 h at room temperature, BTIB (1.3 g) was added. After 5 days at room temperature, the reaction mixture was concentrated in vacuo to leave an oil. It was dissolved in DMF (20 mL), containing succinic anhydride (1 g, 9.98 mmol) and DIEA (3.27 mL, 19 mmol). After 2 h at room temperature, the reaction mixture was diluted with ethyl acetate (200 mL), washed with a 1 N aqueous potassium bisulfate solution (3 x 100 mL), water, brine, dried over magnesium sulfate, and concentrated under reduced pressure to afford a residue that crystallized upon trituration in ether.

4.1.13. Boc-Tyr-A_2Bu-($CO\text{-}CH_2\text{-}CH_2\text{-}COOH$)-Gly-OBg ***15***

Compound **11** (3.4 g, 5.68 mmol) was partially deprotected with TFA. The partially deprotected material (3.22 g, 5.26 mmol) was added to a solution of Boc-Tyr-OSu (1.89 g, 4.99 mmol) in DMF (15 mL) followed by DIEA (0.90 mL, 5.26 mmol) and the mixture was stirred for 1 h at room temperature. The reaction mixture was then diluted with ethyl acetate (200 mL), washed with a 1 N aqueous potassium bisulfate solution (3 x 100 mL), water, brine, dried over magnesium sulfate, and concentrated under reduced pressure. Evaporation of the solvent and trituration in ether afforded the expected material as a white solid.

4.1.14. Z-A_2Bu(Boc)-OMe ***12***

To a solution of Z-Gln-OMe (6.5 g, 22.1 mmol) in a mixture of acetonitrile and water (100 mL, 1:1) was added BTIB (14.2 g, 39.1 mmol). After 24 h at room temperature, 4.3 g of BTIB were added. After 5 days at room temperature, the reaction mixture was concentrated in vacuo to leave an oil. It was dissolved in dioxane (30 mL) containing (di-*tert*-butyl)-dicarbonate (Boc_2O) (5.8 g, 26.5 mmol) and DIEA (3.8 mL, 22.1 mmol). After 3 h at room temperature, the reaction mixture was concentrated to dryness and the residue dissolved in ethyl acetate (200 mL), washed with a 1 N aqueous potassium bisulfate solution (3 x 100 mL), water, a saturated aqueous sodium bicarbonate solution (3 x 100 mL), brine, dried over magnesium sulfate, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography, with ethyl acetate–hexane (3:7) as solvent. Pure fractions were pooled, concentrated under reduced pressure to afford the title compound as an oil. Yield 4.77 g (59 %).

4.1.15. Z-A_2Bu(Boc)-OH ***13***

Compound **12** (4.54 g, 12.39 mmol) was dissolved in methanol (20 mL), and treated with 1 N sodium hydroxide (13.7 mL, 13.6 mmol). After 2 h at room temperature, the reaction mixture was diluted with water (100 mL) and extracted with ether (3 x 50 mL). The aqueous phase was acidified with 1 M potassium bisulfate and extracted with ethyl acetate (3 x 50 mL). The organic extracts were washed with 1 M potassium bisulfate, brine, dried over magnesium sulfate, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography, with chloroform–methanol–acetic acid (120:10:5) as solvent. Pure fractions were pooled, concentrated in vacuo to leave an oil. Yield 3.43 g (78 %).

4.1.16. Z-A_2Bu(Fmoc)-OH ***14***

Compound **13** (3.93 g, 11.15 mmol) was partially deprotected with TFA. The partially deprotected material (2.82 g, 7.7 mmol) was dissolved in a 1 M aqueous sodium carbonate solution (25 mL), and the mixture was cooled down to 4 °C. 1,4-dioxane (15 mL) was added, followed by Fmoc-Cl (2.09 g, 8.1 mmol). After 2 h stirring, the reaction mixture was diluted with water (200 mL) and extracted with ether (3 x 100 mL). The aqueous phase was acidified with concentrated hydrochloric acid and extracted with ethyl acetate (3 x 100 mL). The combined organic fractions were washed with 1 M potassium bisulfate, water, brine, dried over magnesium sulfate, and concentrated under reduced pressure to leave the title compound as an oil. Yield 3.34 g (92 %).

4.1.17. Z-A_2Bu(Fmoc)-Asp(OtBu)-Phe-NH_2 ***16***

This compound was prepared as previously described for the synthesis of Z-Lys(Fmoc)-Asp(OBut)-Phe-NH_2 [11].

4.1.18. Boc-Tyr-Lys-Gly-OBg Z-Trp-Lys-Asp(OBut)-Phe-NH_2 ***49***

$CO\text{-}CH_2\text{-}CO$

Boc-Tyr-Lys-Gly-OBg.HCl (1.09 g, 1.49 mmol) [11] was added to a solution of compound **48** [Z-Trp-Lys($COCH_2CO_2H$)-Asp(OBut)-Phe-NH_2] (1.3 g, 1.49 mmol) and BOP (0.66 g, 1.49 mmol) in DMF (20 mL), followed by NMM (0.33 mL, 2.98 mmol) and the mixture was stirred for 2 h at room temperature. The expected compound precipitated upon addition of a 2% aqueous sodium bicarbonate solution (300 mL). It was collected, washed with water, a 1 N aqueous potassium bisulfate solution, water. The residue was purified by silica gel column chromatography, with chloroform–methanol–acetic acid (60:10:5) as eluant. Pure fractions were pooled, concentrated in vacuo to afford a residue that crystallized upon trituration in ether.

4.1.19. Compounds ***6–9, 17–20, 22–25, 27–30, 33–36, 39–46, 50–59***

All the above-mentioned compounds were prepared according to the synthetic pathway described for the preparation of compound JMV320 and its intermediates [11] and are listed in *table IV*. Physical and analytical characteristics of the compounds are reported in *table I*. ^1H NMR data of compounds **52** to **59** are available as supplementary material upon request directly from the authors.

4.2. Pharmacology

Male guinea pigs (280–300 g) were obtained from le Centre d'Elevage d'Animaux de Laboratoire (Ardenay, France); male Wistar rats (180–200 g) were from Effa-Credo (Saint Germain l'Arbresle, France). Hepes was from Boehringer–Mannheim;

Table IV. Compounds prepared according to the synthetic pathway described for the preparation of compounds JMV 320 and its intermediates [11].

Compound	Structure	Compound	Structure
6	Boc-Tyr-Orn-Gly-OBg Z-Orn-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_2-CO$	**41**	Boc-Tyr-Orn-Gly-OH Z-Trp-Orn-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_4-CO$
17	Boc-Tyr-A_2Bu-Gly-OBg Z-A_2Bu(OBut)-Asp-Phe-NH_2 $CO-(CH_2)_2-CO$	**45**	Boc-Tyr-Orn-Gly-OH Z-Trp-Lys-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_2-CO$
22	Boc-Tyr-Lys-Gly-OBg Z-Lys-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_3-CO$	**50**	Boc-Tyr-Lys-Gly-OH Z-Trp-Lys-Asp(OBut)-Phe-NH_2 $CO-CH_2-CO$
27	Boc-Tyr-Orn-Gly-OBg Z-Orn-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_3-CO$	**9**	Boc-Tyr-Orn-Gly-Trp-Orn-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_2-CO$
33	Boc-Tyr-Lys-Gly-OBg Z-Lys-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_4-CO$	**20**	Boc-Tyr-A_2Bu-Gly-Trp-A_2Bu(OBut)-Asp-Phe-NH_2 $CO-(CH_2)_2-CO$
39	Boc-Tyr-Orn-Gly-OBg Z-Orn-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_4-CO$	**25**	Boc-Tyr-Lys-Gly-Trp-Lys-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_3-CO$
43	Boc-Tyr-Orn-Gly-OBg Z-Lys-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_2-CO$	**30**	Boc-Tyr-Orn-Gly-Trp-Orn-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_3-CO$
7	Boc-Tyr-Orn-Gly-OBg Z-Trp-Orn-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_2-CO$	**36**	Boc-Tyr-Lys-Gly-Trp-Lys-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_4-CO$
18	Boc-Tyr-A_2Bu-Gly-OBg Z-Trp-A_2Bu(OBut)-Asp-Phe-NH_2 $CO-(CH)_2)_2-CO$	**42**	Boc-Tyr-Orn-Gly-Trp-Orn-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_4-CO$
23	Boc-Tyr-Lys-Gly-OBg Z-Trp-Lys-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_3-CO$	**46**	Boc-Tyr-Orn-Gly-Trp-Lys-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_2-CO$
28	Boc-Tyr-Orn-Gly-OBg Z-Trp-Orn-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_3-CO$	**51**	Boc-Tyr-Lys-Gly-Trp-Lys-Asp(OBut)-Phe-NH_2 $CO-CH_2-CO$
34	Boc-Tyr-Lys-Gly-OBg Z-Trp-Lys-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_4-CO$	**52**	Ac-Tyr-Orn-Gly-Trp-Orn-Asp-Phe-NH_2 $CO-(CH_2)_2-CO$
40	Boc-Tyr-Orn-Gly-OBg Z-Trp-Orn-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_4-CO$	**53**	Ac-Tyr-A_2Bu-Gly-Trp-A_2Bu-Asp-Phe-NH_2 $CO-(CH_2)_2-CO$
44	Boc-Tyr-Orn-Gly-OBg Z-Trp-Lys-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_2-CO$	**54**	Ac-Tyr-Lys-Gly-Trp-Lys-Asp-Phe-NH_2 $CO-(CH_2)_3-CO$
8	Boc-Tyr-Orn-Gly-OH Z-Trp-Orn-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_2-CO$	**55**	Ac-Tyr-Orn-Gly-Trp-Orn-Asp-Phe-NH_2 $CO-(CH_2)_3-CO$
19	Boc-Tyr-A_2Bu-Gly-OH Z-Trp-A_2Bu(OBut)-Asp-Phe-NH_2 $CO-(CH_2)_2-CO$	**56**	Ac-Tyr-Lys-Gly-Trp-Lys-Asp-Phe-NH_2 $CO-(CH_2)_4-CO$
24	Boc-Tyr-Lys-Gly-OH Z-Trp-Lys-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_3-CO$	**57**	Ac-Tyr-Orn-Gly-Trp-Orn-Asp-Phe-NH_2 $CO-(CH_2)_4-CO$
29	Boc-Tyr-Orn-Gly-OH Z-Trp-Orn-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_3-CO$	**58**	Ac-Tyr-Lys-Gly-Trp-Lys-Asp-Phe-NH_2 $CO-CH_2-CO$
35	Boc-Tyr-Lys-Gly-OH Z-Trp-Lys-Asp(OBut)-Phe-NH_2 $CO-(CH_2)_4-CO$	**59**	Ac-Tyr-Orn-Gly-Trp-Lys-Asp-Phe-NH_2 $CO-(CH_2)_2-CO$

purified collagenase was from Serva (Garden City Park, NY); soybean trypsin inhibitor from Sigma (St Louis, MO); Eagle's basal amino acid medium (100 times concentrated) was from GIBCO (Grand Island, NY); essential vitamine mixture (100 times concentrated) was from Microbiological Associates (Bethesda, MD); Bovine Plasma Albumin (fraction V) was from Miles Laboratories Inc. (Elkhart, IN); Phadebas® amylase test was from Pharmacia Diagnostics (Piscataway, NJ) and ^{125}I-labeled *N*-succinimidyl-3-(4-hydroxyphenyl)propionyl-CCK-8 ([^{125}I]BH-CCK-8) was from Amersham Corp. (Buckinghamshire, UK). Unless otherwise stated, the standard incubation solutions contained 24.5 mM Hepes (pH 7.4), 98 mM NaCl, 6 mM KCl, 2.5 mM NaH_2PO_4, 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM sodium glutamate, 2 mM glutamine, 11.5 mM glucose, 0.5 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5 mg/mL bacitracin, 0.2% (w/v) albumin, 0.03% (w/v) soybean trypsin inhibitor, 1% (v/v) essential amino acid mixture, and 1% (v/v) essential vitamine mixture. The incubation solution was equilibrated with 95% O_2, 5% CO_2 as the gas phase.

Dispersed acini from rat pancreas were prepared according the previously described modifications (22) of the method of Peikin et al. [23]. Guinea pig brain membranes were prepared following the procedures described by Pelaprat et al. [24].

Amylase release was measured using the procedure already described [25]. Briefly, acini were resuspended in the standard incubation solution complemented with 1% bovine serum albumin, 1 mM calcium, and 5 mM theophylline containing about 1 mg protein/mL, and samples (1 mL) were incubated at 37 °C for 30 min. Amylase activity was determined by the method of Ceska et al. [26] using the Phadebas® reagent. Amylase release was measured as the difference of amylase activity at the end of incubation that was released into the extracellular medium, with and without secretagogue and expressed as the percentage of maximal stimulation obtained with Boc-[Nle^{28}, Nle^{31}]-CCK-7 (40 ± 5% of the total amylase contained in the acini) minus the basal amylase secretion (10 ± 2% of the total amylase contained in the acini) obtained without secretagogue.

Binding of [^{125}I]BH-CCK-8 to rat pancreatic acini was performed as previously described [25]. Briefly, samples (0.5 mL containing ~1 mg/mL protein) were incubated with the appropriate peptide concentrations for 30 min at 37 °C in the presence of 10 pM of [^{125}I]BH-CCK-8 plus various concentrations of Boc-[Nle^{28}, Nle^{31}]-CCK-7. After centrifugation at 10 000 *g* for 10 min and washings, the radioactivity associated with the acinar pellet was measured. Values are expressed as the percentage of the value obtained with labelled CCK-8 alone. The specific activities of the ligand used in our experiments were 2000 Ci/mmol. Acini from three rat pancreata were suspended in 100 mL of standard incubation solution. Specific binding in the absence of any unlabelled CCK-peptide was 13 ± 3% of the total radioactivity present in the sample. Non-specific binding was determined in the presence of 1 μM Boc-[Nle^{28}, Nle^{31}]-CCK-7 and was always less than 15% of the total binding. Results are the means of at least four independent experiments in duplicate.

Binding of ^{125}I-CCK-8 to guinea pig membranes was performed according to Pelaprat et al. [24]. The buffer used was 50 mM (Tris)•HCl, 5 mM $MgCl_2$, 0.1 mg/mL bacitracin, pH 7.4 (Tris-$MgCl_2$-bacitracin buffer). Briefly, displacement experiments were performed by incubation of 1 mL of brain membranes (approximately 0.5 mg protein) in the presence of 20 pM [^{125}I]BH-CCK-8 for 60 min at 25 °C in the presence of various concentrations of Boc-[Nle^{28}, Nle^{31}]-CCK-7 or peptide analog in a total volume of 1 mL. Non-specific binding was determined in the presence of 1 μM Boc-[Nle^{28}, Nle^{31}]-CCK-7 and was always less than 25% of the total binding. Total binding was about 10% of the total radioactivity contained in the sample. Results are the means of at least four independent experiments in duplicate.

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