CHOLECYSTOKININ RECEPTORS: BIOCHEMICAL DEMONSTRATION AND AUTORADIOGRAPHICAL LOCALIZATION IN RAT BRAIN AND PANCREAS USING [³H]CHOLECYSTOKININ₈ AS RADIOLIGAND¹

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Received July 6, 1983; Revised October 11, 1983; Accepted October 19, 1983

Abstract

Since cholecystokinin₈ (CCK₈) seems to be the physiological ligand of CCK receptors in the brain, it would be the most suitable probe for the characterization of CCK receptors in radioligand binding studies. [³H]CCK₈ was synthesized with a specific radioactivity sufficient for the detection of high affinity binding sites. [³H]CCK₈ binds saturably and reversibly to distinct sites in rat brain and pancreas with nanomolar affinity. While the C-terminal tetrapeptide of CCK is the minimal structure required for nanomolar affinity in the brain, the entire octapeptide sequence is required for binding affinity in pancreas. Desulfated CCK₈ and several gastrin-I peptides, which are likewise unsulfated, show virtually no affinity to the binding sites in pancreas but high affinity in cerebral cortex. The ligand specificity of the CCK peptides corresponds to their electrophysiological potency in the brain and their stimulation of secretion in pancreas, respectively. Autoradiographically, high densities of [³H]CCK₈ binding sites were found in cerebral cortex and olfactory bulb, medium levels in nucleus accumbens, hippocampus, dentate gyrus, and striatum with virtually no labeling in cerebellum. This pattern is similar to the distribution of CCK-like immunoreactivity in the brain. In pancreas, equally high levels of [³H]CCK₈ labeling were found in the exocrine and endocrine region.

[³H]CCK₈ binding sites differ from those identified previously with [¹²⁵I]Bolton-Hunter-CCK₃₃ by their sensitivity to guanyl nucleotides in the brain, their ion dependency in the brain and pancreas, and their different autoradiographical localization in some parts of the brain. The distribution of CCK binding sites labeled with [³H]CCK₈ appears to correlate better with the CCK immunoreactivity than those labeled with [¹²⁵I]Bolton-Hunter-CCK₃₃. Thus, [³H]CCK₈ appears to be the radioligand of choice for the investigation of CCK receptors.

The peptide cholecystokinin (CCK) exists in multiple molecular forms both in endocrine cells of the gut and in peripheral and central neurons (Larsson and Rehfeld, 1978, 1979; Rehfeld et al., 1979; Dockray, 1982; Morley, 1982; Emson and Marley, 1983). In the gut, where CCK₈, CCK₃₃, and presumably CCK₂₂ are the predominant fragments (Mutt and Jorpes, 1968; Dockray, 1977; Rehfeld, 1978; Brand and Morgan, 1981; Rehfeld et al., 1982), CCK stimulates gall bladder contraction and the secretion from the exocrine pancreas, acting as a gastrointestinal hormone (Jorpes and Mutt, 1973; Case, 1978).

Pancreatic islet cells, at least in some species, are innervated by CCK neurons containing mainly CCK₄ (Larsson, 1979; Rehfeld et al., 1980). CCK₄ potently stimulates the release of hormones from the endocrine pancreas (Rehfeld et al., 1980).

In the central nervous system, CCK-like immunoreactivity was demonstrated in neuronal perikarya and processes in particular in the cerebral cortex and parts of the limbic system (Straus et al., 1977; Innis et al., 1979; Loren et al., 1979; Schneider et al., 1979; Emson et al., 1980a; Barden et al., 1981; Beinfeld et al., 1981; Beinfeld and Palkovits, 1981; Dockray et al., 1981). The main CCK fragment present in nerve terminal fractions was CCK₈ (Pinget et al., 1978; Emson et al., 1980b). It was released upon K⁺-induced depolarization of synaptosomes and brain slices (Pinget et al., 1979; Dodd et al., 1980; Emson et al., 1980b). Thus, CCK₈, which is also

¹ We wish to thank R. Schubenel, M. Willmann, and W. Lergier for their excellent technical assistance.

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present in human brain (Perry et al., 1981), appears to be the CCK fragment released physiologically from central CCK neurons, suggesting a role for CCK₈ as a neurotransmitter or neuromodulator. Application of CCK₈, CCK₇, or CCK₄ to neurons resulted in a marked excitatory response (Oomura et al., 1978; Dodd and Kelly, 1981; Jeftinija et al., 1981; Skirboll et al., 1981) or in inhibition of neuronal firing (CCK₈; Morin et al., 1983). Pharmacologically, CCK₈ effects include anticonvulsant (Zetler, 1980b), antinociceptive (Zetler, 1980a; Basso et al., 1981; Jurna and Zetler, 1981), and feeding suppressant activity (Della-Fera and Baile, 1979; Stacher et al., 1979; Smith et al., 1981).

CCK receptors in brain and pancreas were investigated previously in biochemical and autoradiographical radioligand binding studies. However, the radioligands used were strongly modified CCK peptides. In most studies (Sankaran et al., 1980; Hays et al., 1980, 1981; Innis and Snyder, 1980a; Saito et al., 1980, 1981; Zarbin et al., 1981, 1983), CCK₃₃ was conjugated with [¹²⁵I]Bolton-Hunter-reagent ([¹²⁵I]BH; Bolton and Hunter, 1973), an N-terminus peptide modification which appeared to be justified since [¹²⁵I]BH-CCK₃₃ retained biological activity in the exocrine pancreas (Sankaran et al., 1979). However, it is unknown whether the compound is active in the endocrine pancreas and in the brain. Furthermore, although the biological activity of CCK resides in the C-terminal part of the peptide, modification at the N-terminus of CCK₃₃ can affect the interaction with CCK receptor sites. Thus, saturable binding sites could only be detected with [¹²⁵I]-monoiodo-labeled BH-CCK₃₃ but not with [¹²⁵I]-diiodo-labeled BH-CCK₃₃ in brain and pancreas (Innis and Snyder, 1980a). In addition, the binding characteristics of [¹²⁵I]BH-CCK₃₃ may not be representative for the receptor interaction of small CCK fragments such as CCK₈; possible CCK receptors with high affinity for CCK₈ but lack of affinity for CCK₃₃ would remain unnoticed with [¹²⁵I]BH-CCK₃₃ as radioligand. Similarly, [¹²⁵I]desaminytyrosyl-CCK₈ (Miller et al., 1981), [³H]pentagastrin (Gaudreau et al., 1983), and [¹²⁵I]imidoester-CCK₈ (Praisman et al., 1983), which were also used as radioligands in binding studies, are greatly modified CCK derivatives.

Since CCK₈ seems to be the physiological ligand of CCK receptors in the brain, the most suitable radioligand for CCK receptor studies in the CNS is the ³H-labeled CCK₈. Although [³H]CCK₈ was prepared earlier, its specific activity was too low for receptor binding studies (Milutinovic et al., 1977; Morgat et al., 1977). Therefore, in the present study, [³H]CCK₈ was prepared with a specific activity sufficient to detect high affinity binding sites. It is the first study to describe the biochemical characteristics and autoradiographical localization of CCK receptor sites in brain and pancreas using [³H]CCK₈ as radioligand. A preliminary communication of our results was published previously (van Dijk et al., 1981).

Materials and Methods

Peptides. All CCK peptides used were sulfated on tyrosine 27 and amidated at the C-terminal end, unless stated otherwise. The following compounds were synthe-

tized according to Gillesen et al. (1979): CCK₈, desulfated CCK₈, CCK₈ acid, the methoxinine-analogues [Mox³]-, [Mox⁶]-, and [Mox^{3,6}]-CCK₈, in which the possible oxidation of the sulfur atom of the methionine residues in position 3 and/or 6 is prevented by replacing methionine by methoxinine (α -amino- γ -methoxybutyric acid), all smaller CCK fragments, the gastrin-I peptides, arginine-vasotocin, lysine-vasopressin, delta sleep-inducing peptide, thyrotropin-releasing hormone, luteinizing hormone-releasing hormone, and secretin. CCK₃₃ was a generous gift from Dr. V. Mutt, Karolinska Institute, Stockholm. Caerulein was kindly provided by Farmitalia Carlo Erba, Milano. Pentagastrin was purchased from Bachem Feinchemikalien AG, Switzerland, motilin and vasoactive intestinal peptide from Peninsula Laboratory, and all remaining peptides from Beckman Bio-products.

Preparation of [³H]CCK₈. Desulfated CCK₈ was labeled with ³H in the phenyl ring of tyrosine and subsequently sulfated with pyridine-SO₃ (D. Gillesen, manuscript in preparation). Purified [³H]CCK₈ (specific activity: 33 Ci/mmol) was stored in liquid nitrogen, and an aliquot was repurified once a week by high pressure liquid chromatography (HPLC) (Spherisorb ODS column 250 × 5 mm; eluent: acetonitril/0.05 M NH₄OAc, pH 4.0 (27:73, v/v)). More than 95% of the label eluted in the same position as nonradioactive CCK₈ as judged by liquid scintillation counting and ultraviolet absorption at 225 nm in two eluent systems (methanol/0.06 M KPO₄ buffer, pH 7.7 (45:55) and the above-mentioned eluent).

Tissue preparation. Brain tissue from male SPF rats (150 gm) was homogenized in 20 vol of ice-cold 0.32 M sucrose, centrifuged at 1000 × g for 10 min, followed by recentrifugation of the supernatant at 140,000 × g for 45 min (P₂ + P₃ pellet). The pellet was resuspended in 20 vol of HEPES buffer (10 mM HEPES, 130 mM NaCl, 5 mM MgCl₂ adjusted with NaOH to pH 7.4), centrifuged for 10 min at 48,000 × g, and used immediately or stored at -20°C. Pancreatic membrane fractions were prepared according to Innis and Snyder (1980a). The tissue was homogenized in 20 vol of ice-cold HEPES buffer and centrifuged at 48,000 × g for 10 min. After resuspension in the same volume and recentrifugation, the pellet was resuspended in 180 vol of HEPES buffer and used immediately.

Binding assays. Frozen brain membranes were used throughout apart from specifically indicated experiments with fresh membranes. Membrane pellets were suspended in 20 vol of HEPES buffer, centrifuged at 48,000 × g for 10 min, and resuspended in 7.5 vol of HEPES buffer. Aliquots containing about 3.5 mg of protein were incubated for 20 min at 22°C in a final volume of 2 ml of HEPES buffer containing bacitracin (0.02% w/v), phenylmethanesulfonylfluoride (PMSF; 1 µg/ml), 130 mM NaCl, and 0.2 nM [³H]CCK₈. Specific binding was defined as the difference between the amount of [³H]CCK₈ bound in the absence (total binding) and in the presence (nonspecific binding) of 1 µM CCK₄. The incubation was terminated by filtration through Whatman GF/B filters, which were washed twice with 3 ml of ice-cold HEPES buffer. After shaking the filters in Aquasol-2 for 1 hr at room temperature, the radioactivity was counted. The

binding assay with pancreatic membranes was the same as that for brain tissue except that 0.2 mg of protein was incubated for 120 min in a final volume of 4 ml. Nonspecific binding of [3 H]CCK $_8$ was determined in the presence of 1 μ M caerulein. There was no displaceable binding of [3 H]CCK $_8$ to the filters.

In the experiments on the nucleotide dependency of [3 H]CCK $_8$ binding, the stability of GTP, ATP, GMP, AMP, ADP, and GDP was tested by applying an aliquot of the supernatant, recovered after completion of the assay, on cellulose thin layer chromatography plates (Merck 5552; solvent system isobutyric acid/1 M ammonia/0.1 M EDTA in H $_2$ O; 50:30:0.8; v/v). Comparison of the UV fluorescent spots with reference compounds indicated no degradation of nucleotides apart from a 50% loss of ATP.

Association and dissociation rate constants were calculated according to Bennett (1978).

Autoradiography. Slide-mounted 10- μ m frontal cryostat sections of fresh brain and pancreas were labeled *in vitro* according to Young and Kuhar (1979) by incubation for 20 min at 22°C with 1 nM [3 H]CCK $_8$ in HEPES buffer containing bacitracin (0.02%) and PMSF (1 μ g/ml), followed by three 15-sec washes in the HEPES buffer and a final dip in distilled water at 4°C. The washing (three $\times$ 15 sec) reduced the amount of specific binding on the sections by about 20% compared to a single dip in water. Alternating sections were incubated with [3 H]CCK $_8$ in the presence of 0.5 μ M caerulein and 0.05 μ M CCK $_4$ for pancreas and brain, respectively. After incubation, the sections were dried at 4°C under a stream of ice-cold nitrogen, apposed to emulsion (Ilford K5)-coated coverslips, and then exposed for 6 to 7 weeks at 4°C. Autoradiograms were evaluated with a Zeiss photomicroscope III using darkfield optics. Semiquantitative analyses of grain densities in various brain regions were performed on projected negatives at a final magnification of $\times$ 640.

Protein determination. Protein was determined according to the method of Lowry et al. (1951).

Results

General characteristics of [3 H]CCK $_8$ binding. The amount of total and nonspecific binding of [3 H]CCK $_8$ to the membrane fractions was 1600 and 400 dpm/assay for cerebral cortex and 12,000 and 200 dpm/assay for pancreas, respectively (Fig. 1, A and B). Specific binding of [3 H]CCK $_8$ showed a linear protein dependency up to 4.0 mg/assay for cerebral cortex and 0.4 mg/assay for pancreas. Incubations were performed at 22°C because specific binding at 0°C was three and 10 times lower in brain and pancreas, respectively. Bovine serum albumin (BSA, 0.2% w/v) was added to the assay medium only when CCK $_{33}$ and caerulein were present in order to prevent their adsorption to the wall of the test tubes. BSA had no influence on the amount of specific binding of [3 H]CCK $_8$ or on the displacing potency of nonradioactive CCK $_8$.

The radioligand did not appear to be metabolized under the assay conditions used. After incubating [3 H]CCK $_8$ (1 nM) with membranes from cerebral cortex or pancreas for 20 and 120 min, respectively, 93 to 95% of

Figure 1. Time dependency of the association and dissociation of [3 H]CCK $_8$ using membranes of cerebral cortex (A) and pancreas (B). For the association experiments membranes were incubated at 22°C with 0.2 nM [3 H]CCK $_8$ in the absence (total) and presence (nonspecific) of 1 μ M CCK $_4$ or caerulein for cortex and pancreas, respectively. Specific binding is the difference between total and nonspecific binding. In the dissociation experiments (insets), an excess of [125 I]-CCK $_8$ (1 μ M) was added after equilibrium was reached between [3 H]CCK $_8$ (0.2 nM) and the membranes. Residual binding was then measured at various time intervals and expressed as percentage of the radioligand bound at equilibrium (% B).

the radioactivity in the supernatant eluted on HPLC in the same position as nonradioactive CCK $_8$.

The binding characteristics in fresh and frozen cerebral cortex were similar as judged by the amount of total and nonspecific binding, the inhibitory potency of all CCK peptides tested, and the regulation by nucleotides and cations (see below).

Kinetics of [3 H]CCK $_8$ binding. Specific binding of [3 H]CCK $_8$ was reversible and time-dependent, a plateau being reached after 20 and 120 min in membranes from cerebral cortex and pancreas, respectively (Fig. 1, A and B). For the determination of the dissociation rate of [3 H]CCK $_8$ from cortical and pancreatic membranes, an excess of [125 I]-CCK $_8$ (1 μ M) was added after equilibrium had been reached, and the residual binding was measured at various intervals (insets Fig. 1, A and B). The dissociation of [3 H]CCK $_8$ from both tissues was biphasic with half-lives of 6 and 19 min for cortical membranes and 9

and 160 min for pancreatic membranes, respectively. The apparent dissociation constants (K_D) calculated from the ratio of the dissociation and association rate constants k_{-1}/k_{+1} were 5.8 and 0.1 nM in the cortex and 0.33 and 0.03 nM in pancreas, respectively.

Saturation of [^3H]CCK $_8$ specific binding sites. Specific binding of [^3H]CCK $_8$ in various brain areas and in pancreas was saturable with increasing concentrations of the radioligand (Fig. 2). The apparent dissociation constants were rather similar throughout the brain, while the maximum number of binding sites (B_{max}) differed markedly in various brain areas (Table I). High values were found in olfactory bulb, striatum, and cerebral cortex, followed by hypothalamus, hippocampus, midbrain, and medulla pons, without detectable specific binding in cerebellum. In pancreas, the K_D value was somewhat lower than in the brain; the maximum number of binding sites, however, exceeded the highest value in the brain by a factor of 40 (Table I). According to the criteria of Scatchard plot linearity, only one population of specific binding sites was apparent in all brain areas tested and in pancreas.

Ligand specificity of [^3H]CCK $_8$ binding sites. Displacement of [^3H]CCK $_8$ from its binding sites was achieved only with peptides structurally related to CCK (Fig. 3, Table II). Caerulein, CCK $_8$, and CCK $_{33}$ revealed highest displacing potencies in both cerebral cortex and pancreas.

The Mox-derivatives of CCK $_8$ displayed marked potencies, with a rank order similar for both tissues. Desulfated CCK $_8$ was devoid of displacing potency in the pancreas, but it was only 16 times less potent than CCK $_8$ in the cortex. Also, the gastrin-I peptides, being unsulfated, showed no displacement in the pancreas but considerable potency in the cortex (Table II).

Of the smaller CCK peptides tested, CCK $_4$ was active in the cortex but not in the pancreas. This differential affinity was also found for the CCK $_4$ derivatives penta-gastrin and [NLeu 2]-CCK $_4$ (Table II).

The presence of the C-terminal amido group in CCK-like peptides appeared to be essential for binding affinity, since the acids of CCK $_4$ and CCK $_8$ were devoid of displacing capacity in both cerebral cortex and pancreas (Table II).

A variety of drugs and peptides chemically unrelated to CCK failed to inhibit [^3H]CCK $_8$ binding in brain and pancreas (see legend to Table II), which underlines the ligand specificity of the [^3H]CCK $_8$ specific binding sites.

Regulation of [^3H]CCK $_8$ binding by ions. The amount

TABLE I
Characteristics of [^3H]CCK $_8$ specific binding in various rat brain areas and in pancreas

Specific [^3H]CCK $_8$ binding was determined as described under "Materials and Methods." The apparent dissociation constant (K_D) and maximum number of binding sites (B_{max}) were calculated from saturation curves (Fig. 2). Results are the means $\pm$ SEM of three separate experiments.

Tissue	K_D nM	B_{max} fmol/mg of protein
Brain		
Olfactory bulb	0.88 $\pm$ 0.25	34.0 $\pm$ 3.0
Striatum	0.99 $\pm$ 0.24	19.4 $\pm$ 0.3
Cerebral cortex	0.86 $\pm$ 0.14	14.0 $\pm$ 1.6
Hypothalamus	1.83 $\pm$ 0.63	8.8 $\pm$ 2.1
Hippocampus	1.03 $\pm$ 0.43	8.5 $\pm$ 2.4
Midbrain	0.99 $\pm$ 0.24	5.6 $\pm$ 0.4
Medulla pons	2.72 $\pm$ 0.68	5.2 $\pm$ 0.3
Cerebellum	ND ^a	ND
Pancreas	0.15 $\pm$ 0.03	1140 $\pm$ 80

^a ND, not detectable.

Figure 2. Saturation of [^3H]CCK $_8$ specific binding using membranes from rat pancreas and cerebral cortex. The points are means $\pm$ SEM of triplicate determinations from one experiment performed as described under "Materials and Methods." The K_D and B_{max} values calculated from three independent experiments are given in Table I.

Figure 3. Inhibition of [^3H]CCK $_8$ specific binding to rat cerebral cortex membranes by CCK-related peptides. Membranes were incubated with 0.2 nM [^3H]CCK $_8$ and varying concentrations of peptides for 20 min at 22°C (Gastrin refers to gastrin-1-(4-17)). The points are means of triplicate determinations differing less than 15%. The IC_{50} values are listed in Table II.

of specifically bound [^3H]CCK $_8$ in pancreas and cerebral cortex was markedly enhanced in the presence of the divalent cations Ca^{2+} or Mg^{2+} . In the presence of 5 mM MgCl_2 , MgSO_4 , or CaCl_2 , the amount of specifically bound [^3H]CCK $_8$ was increased about 14-fold in pancreas and 2- to 7-fold in fresh and frozen tissue from cerebral cortex, respectively (Fig. 4). In contrast, there was no major influence of monovalent cations on the amount of [^3H]CCK $_8$ specific binding. When, in addition to 5 mM MgCl_2 , increasing concentrations of NaCl, LiCl, or KCl were added, a decrease by only 15% was found at very high salt concentration (160 mM NaCl, LiCl, or KCl in pancreas and frozen or fresh cerebral cortex). Thus, the assay buffer for [^3H]CCK $_8$ binding routinely contained 5 mM MgCl_2 and, for osmolarity, 130 mM NaCl.

Regulation of [^3H]CCK $_8$ binding by nucleotides. In pancreas, CCK-stimulated amylase secretion is inhibited by cyclic guanyl but not adenylyl nucleotides (Peikin et al., 1979), suggesting that CCK receptors are linked to an adenylate cyclase. A corresponding influence of nucleotides on [^3H]CCK $_8$ binding in pancreas was found. Specific binding was inhibited by various guanyl nucleotides but not adenylyl nucleotides (Table III). In contrast, in brain tissue (fresh or previously frozen), there was no inhibition of [^3H]CCK $_8$ specific binding by guanyl nucleotides (Table III), indicating that central CCK $_8$ binding sites are not linked to an adenylate cyclase.

Autoradiographical localization of [^3H]CCK $_8$ binding sites. To localize [^3H]CCK $_8$ specific binding sites in tissue sections by light microscopic autoradiography, it had to be demonstrated that the characteristics of [^3H]CCK $_8$ binding in sections were similar to those obtained with membrane fractions. Tissue sections were incubated with

TABLE II

Potencies of various peptides in inhibiting [^3H]CCK $_8$ specific binding in rat cerebral cortex and in pancreas

Membranes from cerebral cortex or pancreas were incubated with [^3H]CCK $_8$ (0.2 nM) and increasing concentrations of various peptides and other drugs as described under "Materials and Methods." The 50% inhibitory concentration (IC_{50}) was calculated by log logit analysis. No displacement of [^3H]CCK $_8$ in either cerebral cortex or pancreas was found for the following compounds up to 10^{-5} M: ACTH 4-10, α -melanocyte-stimulating hormone, β -endorphin, Leu- and Met-enkephalin, arginine-vasotocin, lysine-vasopressin, vasoactive intestinal peptide, delta sleep-inducing peptide, melanocyte-inhibiting factor, luteinizing hormone-releasing hormone, thyrotropin-releasing hormone, substance P, angiotensin, neurotensin, bombesin, physalaemin, bradykinin, motilin, secretin, somatostatin, serotonin, dopamine, γ -aminobutyric acid, noradrenaline, morphine, apomorphine, phenoxybenzamine, and fenfluramine.

Peptide	IC_{50}	
	Cerebral Cortex ^a	Pancreas
	nM	
Caerulein	1.6 ± 0.2	0.31 ± 0.02
CCK $_8$	1.8 ± 0.3	0.7 ± 0.1
CCK $_{33}$	8.8 ± 1.1	3.3 ± 0.3
Desulfated CCK $_8$	28.6 ± 6.8	$>10^3$
CCK $_4$	31.7 ± 4.7	$>10^4$
CCK $_3$	$>10^4$	$>10^4$
CCK $_2$	$>10^4$	$>10^4$
CCK $_8$ acid	$>10^3$	$>10^3$
CCK $_4$ acid	$>10^4$	$>10^4$
CCK-(26-29)-tetrapeptide	$>10^4$	$>10^4$
CCK-(30-32)-tripeptide	$>10^4$	$>10^4$
Pentagastrin ^b	8.2 ± 1.3	748 ± 44
[NLeu 2]-CCK $_4$	51.3 ± 5.9	$>10^4$
[Mox 3]-CCK $_8$ ^c	4.4 ± 0.8	2.0 ± 0.4
[Mox 6]-CCK $_8$ ^c	25.5 ± 3.2	9.2 ± 0.5
[Mox 3,6]-CCK $_8$ ^c	51.7 ± 9.8	23.0 ± 5.1
Gastrin-I-(4-17)	15.9 ± 2.7	$>10^3$
Gastrin-I-(5-17)	21.7 ± 6.1	$>10^3$
Gastrin-I-(1-17)	27.1 ± 3.8	$>10^3$

^a The values were determined in previously frozen tissue. They were similar to those obtained with fresh tissue.

^b BOC- β -Ala-CCK $_4$.

^c [Mox 3]-, [Mox 6]-, [Mox 3,6]-CCK $_8$ contain methoxinin-residues (α -amino- γ -methoxybutyric acid) instead of methionine in position 3 and/or 6 of CCK $_8$.

[^3H]CCK $_8$ in the absence and presence of 0.5 μM CCK $_4$ (brain) and 0.5 μM caerulein (pancreas), washed and dried as described under "Materials and Methods." The amount of radioactivity bound to the tissue was determined by scintillation counting after scraping the tissue off the slide. Specific binding of [^3H]CCK $_8$ to brain and pancreas slices amounted to 70 and 95% of total binding, respectively. Half-maximum inhibition of [^3H]CCK $_8$ specific binding by CCK $_4$ in brain sections occurred at 25 nM. These results are in agreement with those found with membrane fractions (Table II), indicating that the [^3H]CCK $_8$ specific binding sites detected autoradiographically were representative for those characterized in membrane fractions.

The autoradiographic distribution of [^3H]CCK $_8$ binding sites was investigated in brain and pancreas (Figs. 5 to 7, Table IV).

Brain. A high density of [^3H]CCK $_8$ labeling was ob-

Figure 4. Influence of divalent cations on [³H]CCK₈ specific binding in pancreas and cerebral cortex. The [³H]CCK₈ binding assay was performed as described under "Materials and Methods" except that the incubation medium contained either no divalent cations (control) or 5 mM CaCl₂, 5 mM MgSO₄, or 5 mM MgCl₂ as indicated. Open columns represent pancreatic membranes. Horizontally and diagonally striped columns correspond to fresh and frozen membranes, respectively, from cerebral cortex; the absolute control value in frozen tissue was 20% lower than in fresh tissue.

Figure 5. Diagrammatic representation of the distribution of [³H]CCK₈ binding sites in rat brain. The densities of labeling obtained in frontal sections (Table IV, Figs. 6 and 7) have been transposed, for an overview, into the sagittal plane (L 1160 μ m, König and Klippel, 1963) and are represented as stippled regions. a, nucleus accumbens; cbm, cerebellum; CG, substantia grisea centralis; cp, nucleus caudatus putamen; ctx, cortex frontalis; dg, gyrus dentatus; FPT, fibrae pontis transversae; GCC, genu corporis callosi; hi, hippocampus, pars anterior; hl, nucleus lateralis hypothalami; ic, brachium colliculi inferioris; lc, locus ceruleus; nts, nucleus tractus solitarius; ol, bulbus olfactorius; ot, tuberculum olfactorium; pbm, nucleus parabrachialis medialis; pol, nucleus preopticus lateralis; pv, nucleus periventricularis hypothalami; r, nucleus raphé; rpc, nucleus reticularis pontis caudalis; rpo, nucleus reticularis pontis oralis; sc, brachium colliculi superioris; sl, nucleus septi lateralis; sn, substantia nigra; SR, sulcus rhinalis; st, nucleus interstitialis striae terminalis; t, thalamus.

TABLE III
Potencies of various nucleotides in inhibiting [³H]CCK₈ specific binding in rat pancreas and cerebral cortex

Nucleotide	IC ₅₀	
	Pancreas ^a	Cerebral cortex ^{a,b}
	μ M	
Guanylyl imidodiphosphate	0.15	>1000
GTP	0.35	>1000
GDP	0.50	>1000
Dibutyl cGMP	70	>1000
GMP	200	>1000
ATP	>1000	>1000
ADP	>1000	>1000
AMP		>1000

^a There was no degradation of the nucleotides during incubation apart from a 50% loss of ATP.

^b The same results were obtained whether fresh or previously frozen tissue was used, tested either in the presence or absence of NaCl in the incubation medium.

served in the olfactory bulb, sulcus rhinalis, olfactory tubercle, nucleus accumbens, hypothalamus, dentate gyrus, amygdala, and layers III and IV of the cerebral cortex (Fig. 6). Cortical labeling was most pronounced in cingular (Fig. 6A) and occipital (Fig. 7A) regions, but it was also marked in frontal (Fig. 6E), pyriform, and entorhinal cortex. In the olfactory bulb, dense labeling was observed in the external plexiform layer and to a lesser extent in the glomerular layer (Fig. 6C). High density labeling was detected in the olfactory tubercle, but only background levels were present in the olfactory tract (Fig. 6D). Whereas labeling in the nucleus accumbens was higher than in the neostriatum, only background levels were observed in the septum (Fig. 6F). In the hippocampal area only the hilus (polymorphic region) of the dentate gyrus and its caudal ventrolateral extension were markedly labeled (Table IV). Appreciable labeling was found in the locus ceruleus. A rather low amount of labeling was found in the substantia nigra and even less

Figure 6. Autoradiographical localization of [³H]CCK₈ binding sites in 10- μ m cryostat sections of various brain regions in frontal plane with darkfield optics. Calibration bars = 200 μ m. A, Cortex cinguli—total binding. Note the high grain density in layers III and IV (*) and the much lower density in the adjacent genu corporis callosi (GCC). B, Cortex cinguli—nonspecific binding, equivalent to background levels of a section adjacent to that shown in A. C, Bulbus olfactorius—lamina plexiformis externi bulbi olfactorii (LPE) is highly labeled. LG, lamina glomerulosa bulbi olfactorii; LGE, lamina granularis bulbi olfactorii; LCM, lamina cellularum mitralium bulbi olfactorii; LN, lamina nervosa bulbi olfactorii. D, Tuberculum olfactorium (TU) and tractus olfactorius (TO). E, Cortex frontalis—highest densities occurred in layers III and IV (*). F, Nucleus caudatus putamen (cp), nucleus accumbens (a), nucleus septi lateralis (sl), and ventriculus lateralis (VL).

Figure 6

Figure 7. Autoradiographical localization of [^3H]CCK $_8$ binding sites in 10- μm sagittal sections of brain (A and B) and in pancreas (C and D) with darkfield optics. Calibration bars = 200 μm . A, Cortex occipitalis (CO)—note the intense labeling in layers III and IV (*) and the virtual absence of labeling in the adjacent brachium colliculi superioris (SC). B, Cerebellum—this region contains virtually background levels of labeling. gr, lamina granularis; mol, lamina molecularis; my, myelin. C and D, Pancreas—total (C) and nonspecific binding (D) in adjacent sections.

TABLE IV

Autoradiographical distribution pattern of [^3H]CCK $_8$ specific binding sites in rat brain and pancreas

Grain densities were counted in the most intensely labeled parts of the respective region on projected negatives at a final magnification of $\times 640$. Unit area = 62.5 μm^2 .

Tissue	Grain Counts/Unit Area
Cortex occipitalis	54.7 $\pm$ 1.8
Cortex cinguli	43.6 $\pm$ 1.6
Cortex pyriformis	39.5 $\pm$ 1.2
Tuberculum olfactorium	39.3 $\pm$ 2.7
Sulcus rhinalis	39.2 $\pm$ 1.4
Nucleus accumbens	39.0 $\pm$ 1.3
Bulbus olfactorius (lam. plex. ext.)	36.2 $\pm$ 1.5
Nucleus amygdaloideus, pars posterior	36.0 $\pm$ 0.6
Nucleus caudatus putamen	20.3 $\pm$ 0.9
Locus ceruleus	20.5 $\pm$ 2.8
Gyrus dentatus	18.1 $\pm$ 0.8
Nucleus ventromedialis (hypothalami)	16.7 $\pm$ 1.3
Substantia nigra (zona reticulata)	6.8 $\pm$ 1.6
Cerebellum (lamina molecularis)	2.8 $\pm$ 0.5
Pancreas	101.8 $\pm$ 5.0

in the molecular layer of the cerebellum (Fig. 7B, Table IV). Specific accumulation of radioactivity in cell bodies was never observed in the brain regions examined. The distribution of [^3H]CCK $_8$ specific binding sites in the brain areas studied is summarized schematically in Figure 5.

Nonspecific labeling of [^3H]CCK $_8$ in the presence of CCK $_4$ was similar to the background levels in gray as well as in white matter as illustrated for the cingulate cortex (Fig. 6B).

Pancreas. The pancreas was the most intensely labeled tissue compared to all brain areas studied (Fig. 7C, Table IV). The labeling was equally distributed over the endocrine and exocrine pancreas. Caerulein, but not CCK $_4$, was able to prevent this labeling (Fig. 7D).

Discussion

Since CCK $_8$ is the CCK fragment released from central neurons, it is the most suitable probe for the identification of CCK receptors in radioligand binding studies. We have prepared [^3H]CCK $_8$ as a new probe with a specific

radioactivity suitable for high affinity binding studies. The biochemical characteristics and autoradiographical distribution of the [3 H]CCK $_8$ specific binding sites identified in brain and pancreas are in line with the concept that they represent CCK receptors. In the following, the ligand specificity, regional distribution, and autoradiographical localization of the [3 H]CCK $_8$ binding sites in brain and pancreas are discussed, followed by a comparison of the characteristics of the sites identified by [3 H]CCK $_8$ or other CCK radioligands in brain and pancreas.

[3 H]CCK $_8$ specific binding sites in rat brain

Ligand specificity. The saturable specific binding sites for [3 H]CCK $_8$ were highly specific for CCK-like peptides. Caerulein and CCK $_{33}$, which have seven and eight amino acids, respectively, in common with CCK $_8$, were the most potent displacing agents besides CCK $_8$ itself (Fig. 3, Table II). Desulfated CCK $_8$ was 16 times less potent than CCK $_8$. CCK $_4$ and all gastrin-I peptides which share the last five C-terminal amino acids with CCK $_8$ displayed considerable potency, whereas a wide variety of other peptides and nonpeptidergic compounds were completely inactive in inhibiting [3 H]CCK $_8$ binding (Table II). It appears that the four C-terminal amino acid residues of CCK are essential for the expression of affinity to the [3 H]CCK $_8$ binding sites in cerebral cortex. All smaller peptides comprising only part of the amidated C-terminal tetrapeptide were inactive. Accordingly, structural changes in parts other than the tetrapeptide, such as in pentagastrin (BOC- β Ala-CCK $_4$) or [Mox 3]-CCK $_8$, resulted in virtually no loss in binding affinity, whereas changes within this part (NLeu 2 -CCK $_4$; [Mox 6]-CCK $_8$; [Mox 3,6]-CCK $_8$) resulted in a clear decrease of the inhibitory potency compared to CCK $_8$.

This ligand specificity of the [3 H]CCK $_8$ specific binding sites compares well with the electrophysiological activity of CCK peptides. CCK $_8$, CCK $_7$, and CCK $_4$ produce a marked excitatory response of neurons in various brain areas (Oomura et al., 1978; Dodd and Kelly, 1981; Jeftinija et al., 1981; Skirboll et al., 1981). A discrepancy exists only for nonsulfated CCK $_8$; although a potent displacer of [3 H]CCK $_8$ binding in rat cerebral cortex (Table II), it was found to leave neuronal activity unaltered in rat hippocampal slices (Dodd and Kelly, 1981).

Regional distribution. Saturation of the [3 H]CCK $_8$ binding sites revealed rather similar dissociation constants for CCK $_8$ in different brain regions (Table I). Although only one population of binding sites was suggested using the criterion of Scatchard plot linearity in all brain areas tested, the biphasic dissociation of CCK $_8$ in cerebral cortex may indicate a heterogeneity of central CCK binding sites (Fig. 1A).

The density of the [3 H]CCK $_8$ specific binding sites varied considerably in different brain regions (Table I). Their rank order largely corresponded to that of CCK-like immunoreactivity. Notably, both cerebral cortex and olfactory bulb showed high amounts of [3 H]CCK $_8$ specific binding (Table I) and contain CCK-like immunoreactivity (Schneider et al., 1979; Beinfeld et al., 1981; Dockray et al., 1981).

The cerebellum contained no appreciable amount of either [3 H]CCK $_8$ specific binding sites (Table I) or CCK-

like immunoreactivity (Schneider et al., 1979; Beinfeld et al., 1981; Dockray et al., 1981), underlining the physiological significance of the binding sites identified by [3 H]CCK $_8$.

Autoradiographical visualization. [3 H]CCK $_8$ specific binding sites were found widely distributed throughout the CNS with enrichment in cortical and limbic areas. Highest densities of [3 H]CCK $_8$ binding sites were observed throughout the cortex (notably layers III and IV), olfactory tubercle, nucleus accumbens, olfactory bulb, sulcus rhinalis, and amygdala with no appreciable binding in cerebellum (Figs. 5 to 7). This pattern corresponded largely to the distribution of CCK-like immunoreactivity. Immunohistochemically, in rat neocortex, CCK $_8$ terminals are found in all parts of the cortex (Emson and Hunt, 1981; Hendry et al., 1983). In addition, dense fluorescent fibers have been located in the sulcus rhinalis, central nuclei of the amygdala, and dorsomedial and ventromedial hypothalamus but not in the cerebellum (Innis et al., 1979; Larsson and Rehfeld, 1979; Hökfelt et al., 1980a; Vanderhaeghen et al., 1980). Furthermore, thin fibers, but no positively stained cell bodies, were detected in the olfactory tubercle and nucleus accumbens (Vanderhaeghen et al., 1980).

The hippocampus and dentate gyrus contain a moderate number of immunoreactive CCK terminals (Emson et al., 1980b; Handelsmann et al., 1981). Our study revealed consistently higher [3 H]CCK $_8$ binding in the hilus of the dentate gyrus than in the hippocampus. Electrophysiologically, a strong excitatory response was found after application of CCK $_8$ to hippocampal CA $_1$ pyramidal cells (Dodd and Kelly, 1981).

It has been reported that dopamine and CCK coexist in a subpopulation of dopaminergic neurons in the A $_9$ /A $_{10}$ region (Hökfelt et al., 1980b; Studler et al., 1981). Although the immunoreactivity is mainly in cell bodies in this area (Hökfelt et al., 1980a; Vanderhaeghen et al., 1980), there is also evidence for CCK terminals (Williams et al., 1981). Ionophoretic application of CCK $_7$ induced bursting patterns of A $_{10}$ neurons in which CCK-like immunoreactivity was present (Skirboll et al., 1981). We found only a moderate amount of [3 H]CCK $_8$ labeling in the A $_9$ but no labeling in the A $_{10}$ region, indicating a receptor density in the A $_{10}$ region below the detection limit of our assay. In general, however, there was a marked parallelism between the distribution of [3 H]CCK $_8$ labeling and CCK-like immunoreactive material. This evidence, taken together with the ligand specificity of the [3 H]CCK $_8$ specific binding sites, supports their role as CCK receptors.

[3 H]CCK $_8$ specific binding sites in rat pancreas

Saturable high affinity binding sites for [3 H]CCK $_8$ were also found in pancreas. Caerulein, CCK $_8$ and CCK $_{33}$ were the most potent displacing agents, whereas CCK $_4$, in striking contrast to the binding sites in the brain, was virtually inactive in displacing [3 H]CCK $_8$ (Table II). Furthermore, deamination of CCK $_8$ (CCK $_8$ acid) and desulfation (desulfated CCK $_8$ and gastrin-I series) both dramatically reduced the displacing potency in comparison to CCK $_8$ (Table II). Thus, the entire CCK $_8$ sequence appears to be required for a high affinity binding in

pancreas. The ligand specificity of the binding sites in pancreas corresponded to their biological activity. CCK₈, caerulein, and CCK₃₃, but not CCK₄, are potent stimulators of pancreatic exocrine secretion (Rehfeld et al., 1980; Jensen et al., 1981). Furthermore, the displacing potency of the Mox-derivatives in [³H]CCK₈ binding in pancreas correlates with their biological response (Gillissen et al., 1979). Thus, the [³H]CCK₈ specific binding sites may represent the pancreatic target sites for the action of CCK as a gastrointestinal hormone.

CCK₄ (but not CCK₃₃ and CCK₈) is very potent in stimulating hormone secretion from pancreatic islet cells as shown in pig and man (Rehfeld et al., 1980). These cells are innervated in several species (pig, cat, hamster) by CCK neurons containing mainly CCK₄ (Larsson, 1970; Rehfeld et al., 1980). Thus, receptors for CCK₄ would be expected to be present in the endocrine pancreas. However, in the rat, the species used in this study, there is no indication of an interaction of CCK₄ with islet cell function. Likewise, a pancreatic innervation by CCK neurons is unknown. Correspondingly, CCK₄ lacked inhibitory potency in [³H]CCK₈ binding in pancreas (Table II). Autoradiographically, the uniform labelling of [³H]CCK₈ throughout the pancreas could be displaced by caerulein (Fig. 7, C and D) but in no part by CCK₄. Hence, within the detection limit of this assay, our results suggest that the islets of Langerhans in the rat are not under the control of CCK₄.

[³H]CCK₈ specific binding in pancreas, but not in cerebral cortex, was inhibited by several guanyl nucleotides (Table III). The pattern of specificity was similar to that of the other neurotransmitter and hormone receptors that are regulated by guanyl nucleotides and linked to an adenylate cyclase. Thus, CCK receptors in pancreas, but not in brain, may be linked to an adenylate cyclase.

Comparison of binding sites labeled by [³H]CCK₈ or other CCK radioligands

Previously, [¹²⁵I]BH-CCK₃₃ was used in an attempt to characterize CCK receptors in the brain of the rat (Hays et al., 1980; Saito et al., 1980; Zarbin et al., 1983), mouse (Saito et al., 1981), and guinea pig (Innis and Snyder, 1980a; Zarbin et al., 1981, 1983). Furthermore, [³H]pentagastrin (Gaudreau et al., 1981) and [¹²⁵I]imidoester-CCK₈ (Praisman, 1983) were used in rat brain. CCK binding sites in the pancreas were previously characterized by [¹²⁵I]BH-CCK₃₃ binding in the rat (Sankaran et al., 1979; Innis and Snyder, 1980b; Steigerwaldt and Williams, 1981) and guinea pig (Jensen et al., 1980). In addition, [¹²⁵I]desaminotyrosyl-CCK₈ was used with rat pancreas (Miller et al., 1981). In the following, these studies are compared to our results obtained in rat brain and pancreas with [³H]CCK₈ as radioligand.

Brain. The characteristics of [³H]CCK₈ specific binding resemble those obtained with [¹²⁵I]BH-CCK₃₃ with regard to the *K_D* value, Scatchard plot linearity, and rank order of inhibitory potencies of CCK peptides (Innis and Snyder, 1980a; Saito et al., 1980). However, our findings are at variance with those of Hays et al. (1980), who found no difference in displacing potency between CCK₈-

sulfated CCK₈-unsulfated, CCK₃₃, and caerulein in [¹²⁵I]BH-CCK₃₃ binding in rat brain. A further difference in displacing potency exists for sulfated and nonsulfated CCK₈. These peptides can be differentiated by their displacing potencies in binding assays with [³H]CCK₈ (Table II), [¹²⁵I]BH-CCK₃₃ (Innis and Snyder, 1980a; Saito et al., 1980; Saito et al., 1981), or [¹²⁵I]imidoester-CCK₈ (Praisman et al., 1983), but not with [³H]pentagastrin, as radioligand (rat brain; Gaudreau et al., 1983).

The binding sites labeled by [³H]CCK₈ or [¹²⁵I]BH-CCK₃₃ differed in their sensitivity to guanyl nucleotides and monovalent ions. While guanylyl-imidodiphosphate and GTP inhibited [¹²⁵I]BH-CCK₃₃ binding in guinea pig brain considerably (*IC*₅₀ = 35 and 560 μM respectively) (Innis and Snyder, 1980a), there was no inhibition of [³H]CCK₈ binding in either fresh or frozen rat cerebral cortex by guanylyl imidodiphosphate, GTP, GDP, or GMP (Table III). Furthermore, while both [³H]CCK₈ and [¹²⁵I]BH-CCK₃₃ binding were enhanced by divalent cations to a similar degree (Fig. 4; Innis and Snyder, 1980a; Saito et al., 1981), they differed considerably in their sensitivity to monovalent cations. In the presence of NaCl, LiCl, or KCl (60 mM), [³H]CCK₈ specific binding remained unaltered in either frozen or fresh rat cerebral cortex, whereas [¹²⁵I]BH-CCK₃₃ binding in guinea pig brain (Innis and Snyder, 1980a) and mouse brain (Saito et al., 1981) was reduced by 50%. Although a species difference between rat (this study), guinea pig (Innis and Snyder, 1980a), and mouse brain (Saito et al., 1981) cannot be excluded, it is conceivable that [¹²⁵I]BH-CCK₃₃ interacts with a larger domain of the CCK receptor protein than [³H]CCK₈, including sites sensitive to guanyl nucleotides and monovalent cations. However, these nucleotide and cation recognition sites are probably not relevant for the regulation of the ligand binding reaction physiologically inasmuch as the binding of the physiological ligand CCK₈ is not regulated by guanyl nucleotides or monovalent cations. Alternatively, it is also feasible that the two radioligands label, at least in part, subtypes of CCK sites with different regulatory characteristics.

Some indications for a possible heterogeneity of CCK binding sites are given by the dissociation rates of the radioligands. A biphasic dissociation curve was found for [³H]CCK₈ in rat brain (Fig. 1A) and for [¹²⁵I]BH-CCK₃₃ in mouse brain (Saito et al., 1980). However, a monophasic dissociation was observed for [¹²⁵I]BH-CCK₃₃ in rat and guinea pig brain (Hays et al., 1980; Innis and Snyder, 1980a).

Autoradiographically, the labeling pattern for [³H]CCK₈ (rat brain) was similar to that of [¹²⁵I]BH-CCK₃₃ (guinea pig brain; Zarbin et al., 1981, 1983), notably for the laminar distribution in cerebral cortex. However, the cerebellum, interpeduncular nucleus, olfactory tract, optic tract, parts of the hippocampus, and layer VI of cerebral cortex were strongly labeled by [¹²⁵I]BH-CCK₃₃ (Zarbin et al., 1981, 1983) but not by [³H]CCK₈ (Figs. 5 and 6). Thus, [¹²⁵I]BH-CCK₃₃ may, at least in part, label sites unrelated to CCK receptors. This appears to be especially pertinent in guinea pig cerebellum, which contains virtually no CCK immunoreactivity but was very strongly labeled with [¹²⁵I]BH-CCK₃₃ (Zarbin et al.,

1983). In rat cerebellum, which likewise contains virtually no CCK immunoreactivity, only marginal labeling was found with [^3H]CCK $_8$ (Fig. 7B). Possible species differences in the labeling pattern of [^3H]CCK $_8$ and [^{125}I]BH-CCK $_{33}$ remain to be investigated.

Among other CCK radioligands there is only a preliminary autoradiographical study with [^3H]pentagastrin (Gaudreau et al., 1983), which, however, does not permit a detailed comparison with our results. Furthermore, as mentioned above, [^3H]pentagastrin binding differs from other CCK radioligands in ligand specificity. Thus, [^3H]CCK $_8$ appears to be the ligand of choice for studying central CCK receptors.

Pancreas. The characteristics of [^3H]CCK $_8$ binding sites in rat pancreatic membranes were very similar to those identified in the same species with [^{125}I]BH-CCK $_{33}$ with regard to their ligand specificity, biphasic dissociation of the radioligand, Scatchard plot linearity, inhibition of binding by guanyl nucleotides, and K_D values (Innis and Snyder, 1980a, b; Steigerwaldt and Williams, 1981). However, the binding sites differed in their sensitivity to cations. Monovalent cations (Li^+ , Na^+ , K^+ , 120 mM) reduced [^{125}I]BH-CCK $_{33}$ binding by 50%, but there was only a 15% reduction in [^3H]CCK $_8$ specific binding. In contrast, the enhancement by divalent cations (5 mM Mg^{2+} or Ca^{2+}) was much more pronounced for [^3H]CCK $_8$ binding (11- to 15-fold increase, Fig. 4) than for [^{125}I]BH-CCK $_{33}$ binding (2- to 4-fold increase; Innis and Snyder, 1980a; Steigerwaldt and Williams, 1981). Thus, similar to the binding sites in brain, [^{125}I]BH-CCK $_{33}$ may interact with a larger receptor domain than [^3H]CCK $_8$, which is reflected in the differential sensitivity to cations. Alternatively, the two radioligands may interact with different subpopulations of pancreatic CCK receptors.

Whether the sites labeled by [^{125}I]desaminotyrosyl-CCK $_8$ in pancreatic acini (Miller et al., 1981) resemble those labeled by [^3H]CCK $_8$ is difficult to assess since the displacing potency of only four CCK peptides was tested. [^3H]-Pentagastrin cannot be used for CCK binding studies in pancreas due to lack of sufficient affinity (Table II).

Whether [^3H]CCK $_8$ and other CCK radioligands label the same maximum number of sites or, at least in part, subpopulations of CCK receptors in pancreas or brain can only be determined in comparative studies under the same experimental conditions. However, our biochemical and autoradiographical results demonstrate that [^3H]CCK $_8$ is a radioligand superior to those used previously for the study of CCK receptors.

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