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Distinct cholecystokinin receptors in brain and pancreas

(receptor binding/gastrin/guanyl regulation)

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ABSTRACT **^{125}I-Labeled (Bolton–Hunter) cholecystokinin triacontatriapeptide (CCK-33) binds saturably and reversibly to distinct receptors in brain and pancreatic membranes. The peptide specificity of pancreatic CCK binding is the same as that for pancreatic amylase release. In brain, gastrin and pentagastrin display nanomolar affinity for binding sites, whereas in pancreas these two peptides are virtually inactive. Though these differences indicate that brain and pancreas possess distinct CCK receptors, the two tissues show some similarities. Both pancreas and brain receptors show greater sensitivity to sulfated than to desulfated COOH-terminal octapeptide of CCK and display dissociation constants of 0.3–0.5 nM. The pancreas possesses about 300 times more binding sites than does brain. CCK binding in both brain and pancreas is enhanced by divalent cations and reduced by monovalent cations. Receptor binding in both tissues is regulated in a selective fashion by guanyl nucleotides.**

Cholecystokinin (CCK) was first isolated as a 33-amino acid intestinal peptide hormone that releases pancreatic amylase and other enzymes (1). CCK also occurs in neurons in the brain (2–8). In both intestine and brain, CCK exists in multiple molecular forms with the COOH-terminal octapeptide (CCK-8) representing the predominant molecular species (4, 9). Rehfeld *et al.* (10) demonstrated that pancreatic islet neurons contain the COOH-terminal tetrapeptide of CCK (CCK-4). CCK-4 potently releases insulin and other islet hormones but is virtually inactive in releasing amylase, whereas CCK-8 is almost inactive in releasing insulin but is potent as a releaser of amylase (10); this suggests that CCK-4 and CCK-8 act at chemically distinct receptors located on the islet and acinar cells, respectively. Because CCK-4 occurs in brain (6), distinct forms of CCK receptors might also be expected to exist in brain.

By utilizing ^{125}I-labeled (Bolton–Hunter) CCK triacontatriapeptide (^{125}I-CCK-33), CCK receptors have been labeled in pancreas (11–13) and brain (13, 14). In these initial studies, the peptide specificity of CCK receptors in the brain differed from that in the pancreas. CCK-4 and pentagastrin (which shares an identical COOH-terminal tetrapeptide sequence with gastrin and CCK) were virtually inactive in the pancreas but rather potent in the brain (13, 14). Here we describe detailed properties of the apparently distinct CCK receptors in brain and pancreas, including differential regulation by guanyl nucleotides.

MATERIALS AND METHODS

^{125}I-CCK-33 was prepared as described (15). Because ^{125}I-CCK-33 is as potent as CCK-33 in stimulating amylase release (15), the specific activity of the radioligand was determined from a standard curve of amylase release by native CCK-33 (16). Amylase was determined colorimetrically with assay kit 700 (Sigma). The specific activity of ^{125}I-CCK-33 made with freshly prepared Bolton–Hunter ^{125}I reagent (New England Nuclear) was 800–1000 Ci/mmol (1 Ci = 3.7×10^{10} becquerels).

Rat pancreas or guinea pig brain was homogenized in 20 vol of 50 mM Tris·HCl buffer (pH 7.7) at 25°C with a Brinkmann Polytron PT 10. The homogenates were centrifuged twice at $50,000 \times g$ for 10 min with an intermediate rehomogenization in fresh buffer. The final pellets were resuspended in 20 vol (brain) or 250 vol (pancreas) of incubation buffer [50 mM Tris/HCl, pH 7.7 (at 25°C)/0.2% bovine serum albumin (fraction V, Sigma)/5 mM $MgCl_2$/5 mM dithiothreitol/0.1 mM bacitracin]. To triplicate polypropylene incubation tubes were added, in a total volume of 0.5 ml, 400 μl of freshly resuspended tissue, 45,000 dpm of ^{125}I-CCK-33 (final concentration, 50–100 pM), and displacing agents. After incubation for 30 min at 37°C, 1.0 ml of ice-cold incubation buffer was added, and the tubes were centrifuged for 4 min at $10,500 \times g$ in a Brinkmann Microfuge. The supernatant was aspirated and the radioactivity in the pellet was measured. Specific binding was defined as the excess binding over that in blanks containing 0.5 μM CCK-8.

The kinetic parameters of association and dissociation were calculated as follows: the dissociation rate constant (k_{-1}) was determined as $0.693/t_{1/2}$, in which $t_{1/2}$ equals the time required for 50% of the specifically bound ligand to be dissociated in the presence of excess CCK-8 (0.5 μM). The association rate constant (k_1) was calculated as $k_1 = (k_{obs} - k_{-1})/[L]$. The observed association rate constant (k_{obs}) was taken as the slope of $\ln (B_{eq}/(B_{eq} - B_t))$ vs. time, in which B_{eq} is the concentration of radioligand bound at equilibrium and B_t is the concentration bound at various times t. The concentration of radiologand $[L]$ was calculated from the specific activity of ^{125}I-CCK-33.

CCK-related peptides were gifts from the following sources: CCK-33 (Victor Mutt, Karolinska Institute, Sweden), CCK-4 (Lars-Inge Larsson, University of Aahrus, Denmark), CCK-8 and desulfated CCK-8 (Squibb), caerulein (Adria Laboratories, Columbus, OH), and pentagastrin (Ayerst Laboratory, New York). [Leu^{15}]Gastrin-17-I was obtained from Beckman (Palo Alto, CA).

RESULTS

General Properties of ^{125}I-CCK-33 Binding to Brain and Pancreatic Membranes. In both rat pancreas and guinea pig brain ^{125}I-CCK-33 binding was saturable. At 50 pM ^{125}I-CCK-33, used in routine experiments, total binding was 7000 and 5000 cpm in rat pancreas and guinea pig brain, respectively; nonspecific binding in the presence of 0.5 μM CCK-8 was about 1500 cpm in rat pancreas and 2500 cpm in guinea

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Abbreviations: CCK, cholecystokinin; CCK-33, CCK triacontatriapeptide; ^{125}I-CCK-33, ^{125}I-labeled (Bolton–Hunter) CCK-33; CCK-8, CCK COOH-terminal octapeptide; CCK-4, CCK COOH-terminal tetrapeptide; p[NH]ppG, 5′-guanylylimidodiphosphate.

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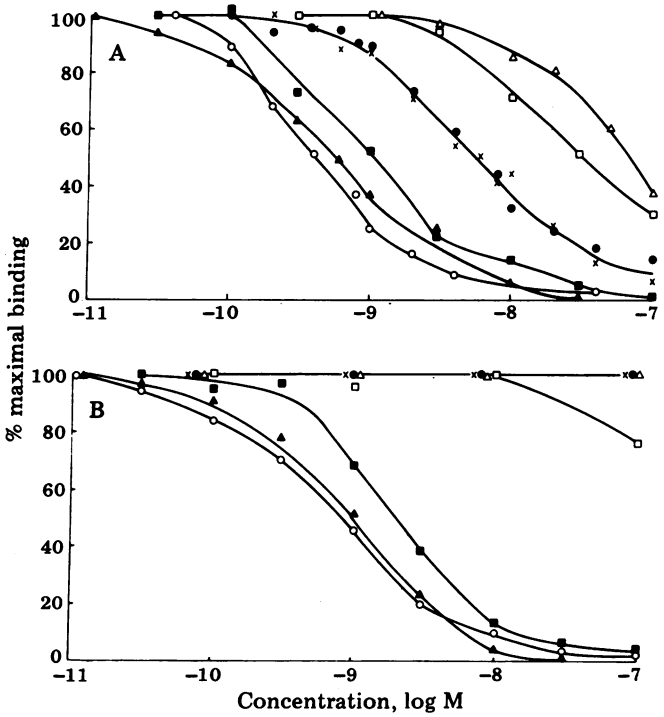

FIG. 1. Displacement of ^{125}I-CCK-33 from guinea pig brain (*A*) and rat pancreatic membranes (*B*) by CCK-related peptides. Residual binding of ^{125}I-CCK-33 to membranes was measured at several peptide concentrations after incubation for 30 min at 37°C. ○, CCK-33; ▲, CCK-8; ■, caerulein; □, disulfated CCK-8; ×, pentagastrin; ●, [Leu^{15}]gastrin-17-I; △, CCK-4. Data are expressed as the percentage of maximal binding measured in the absence of unlabeled peptide. Results are the average of triplicate determinations from a representative experiment that was repeated at least four times with similar results.

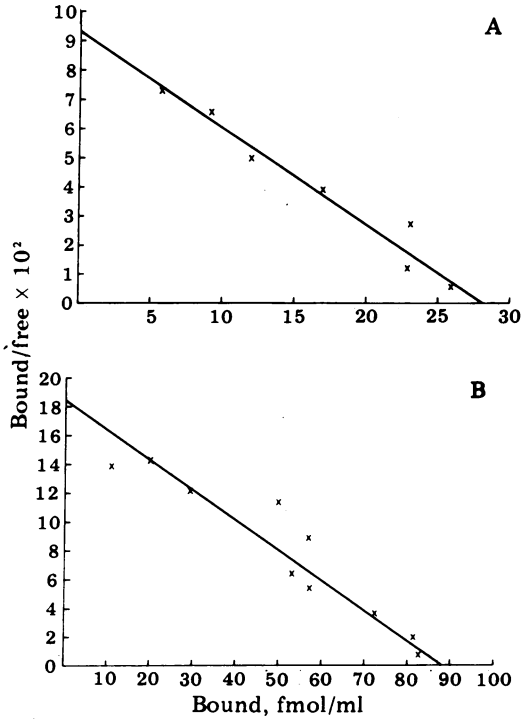

FIG. 2. Scatchard analysis of the displacement of ^{125}I-CCK-33 by CCK-33 from guinea pig brain (*A*) and rat pancreatic membranes (*B*). Each point represents the average of triplicate determinations, which were replicated at least four times. K_d, 0.3 nM (guinea pig brain) and 0.5 nM (rat pancreas); B_{max}, 0.7 pmol/g (guinea pig brain) and 220 pmol/g (rat pancreas).

pig brain. Saturable binding of ^{125}I-CCK-33 was demonstrable in rat brain membranes, but the ratio of total to nonspecific binding was less than that in guinea pig brain, consequently guinea pig brain membranes were used for routine experiments. Potencies of CCK-4, pentagastrin, CCK-33, CCK-8, and desulfated CCK-8 were the same in rat and guinea pig brain membranes. Thus, the recognition site of the receptor in brain membranes of the two species appeared to be identical. Differences in the regional distribution of receptors in guinea pig and rat brain are discussed below.

In initial experiments we attempted to evaluate receptor binding with ^{125}I-labeled CCK-8 (iodinated with chloramine-T) but could detect no saturable binding, perhaps because the iodine is located adjacent to the critical sulfate on the tyrosine of CCK-8. Saturable binding also could not be detected with [^{125}I]diiodo-labeled (Bolton–Hunter) CCK-33 so that in all experiments only monoiodinated Bolton–Hunter reagent was employed.

Optimal incubation conditions for receptor binding appeared to be the same in guinea pig brain and rat pancreas. Routine binding conditions included 0.2% bovine serum albumin, which decreased adsorption of the ^{125}I-CCK-33 to polypropylene test tube surfaces. Bacitracin (0.1 mM) enhanced binding by about 50%, presumably by inhibiting CCK degradation. Dithiothreitol (5 mM) and $MgCl_2$ (5 mM) were included, as they enhanced binding 20% and 200%, respectively. Incubations were conducted at 37°C, as lower temperatures increased adsorption of ^{125}I-CCK-33 to tube surfaces.

^{125}I-CCK-33 appeared not to be metabolized during incubation with brain and pancreatic membranes. After incubation of ^{125}I-CCK-33 with brain or pancreatic membranes for 30 min and centrifugation, the supernatant fractions eluted from a Sephadex G-25 (fine) column with elution profiles identical to those of authentic ^{125}I-CCK-33.

Specific ^{125}I-CCK-33 binding was linear with up to 5 mg and 25 mg (original wet weight) of rat pancreas and guinea pig brain, respectively. All assays were conducted within the linear range.

Saturation Analysis of ^{125}I-CCK-33 Binding. We progressively diluted ^{125}I-CCK-33 with increasing concentrations of CCK-33 (Fig. 1). Binding in brain and pancreas was saturable, showing linear Scatchard plots with dissociation constants (K_d) of 0.3 and 0.5 nM for membranes from guinea pig brain and rat pancreas, respectively (Fig. 2). Binding in intact acini of rat (12) and guinea pig (11) pancreas is described by biphasic Scatchard plots. Though brain and pancreatic receptors had similar affinities for CCK, the pancreas (B_{max} = 220 pmol/g) possessed about 300 times more binding sites than guinea pig brain had (0.7 pmol/g).

Peptide Specificity of CCK-Receptors. Displacement of ^{125}I-CCK-33 binding to guinea pig brain and rat pancreas membranes by CCK derivatives gave parallel curves for all peptides evaluated (Fig. 1). Hill coefficients for displacement by all peptides were about 1.0, indicating the absence of positive or negative cooperative interactions.

Although CCK-8 and CCK-33 showed similar affinities in guinea pig brain and rat pancreas, there were pronounced differences for some of the other peptides. Whereas desulfation of CCK-8 reduced potency 300 to 400-fold in rat pancreas, it lowered receptor affinity for guinea pig brain membranes only 50-fold. Even greater discrepancies occurred with gastrin, pentagastrin, and CCK-4. [Leu^{15}]Gastrin-17-I, pentagastrin, and CCK-4 were 500–2000 times more potent in the brain than in the pancreas. Because the peptide potencies were the same

in rat as in guinea pig brain membranes, these differences cannot be attributed to species variations.

The specificity of the binding sites for the CCK group of peptides was indicated by the inactivity of other gut and brain and related peptides, including physalaemin, substance P, insulin, glucagon, bradykinin, neurotensin, [Met]- and [Leu]-enkephalin, bombesin, eledoisin, prolylleucylglycinamide, and prolylleucylphenylalanylamide.

The binding specificity obtained here resembles earlier results in brain and pancreas obtained by ourselves (13) and others (14). The peptide selectivity of pancreatic binding sites is the same as in stimulating amylase secretion, with CCK-8 highly potent and CCK-4 virtually inactive. The peptide specificity of brain binding sites resembles the influences on pancreatic insulin release in the considerable potency of CCK-4 but differs in that CCK-33 is potent at brain receptors but does not release pancreatic insulin (10).

Kinetics of CCK-Receptor Binding in Pancreas and Brain. Specific binding of ^{125}I-CCK-33 in both guinea pig brain and rat pancreas membranes was reversible and time dependent. Association was more rapid in pancreas than in brain. Pancreatic binding was half maximal by 3 min at 37°C and reached equilibrium values by 15 min (Fig. 3). By contrast, in guinea pig brain membranes half-maximal binding occurred at 6 min and plateaued by about 30 min.

To measure dissociation, brain and pancreatic membranes were incubated for 30 min at 37°C with ^{125}I-CCK-33; then 0.5 μM CCK-8 was added and residual binding was measured at various intervals (Fig. 3). Dissociation from pancreatic membranes was biphasic, with the two components displaying half-lives of 3 and 27 min. By contrast, dissociation under the same conditions in guinea pig brain membranes was monophasic with a half-life of 12 min. With intact pancreatic acini, ^{125}I-CCK-33 showed a monophasic dissociation with a half-life of 30 min (12). As expected, the dissociation of ^{125}I-CCK-33 from rat brain membranes at 4°C (14) was substantially slower than that observed here at 37°C.

The dissociation rate constants were 0.23 and 0.026 min^{-1} for the rapid and slow components in rat pancreas and 0.058 min^{-1} for guinea pig brain membranes. The association rate constants, with the same observed association rate constant (k_{obs}) for both pancreatic components, were 7.9×10^8 and 4.5×10^9 min^{-1} M^{-1} for the pancreas and 5.4×10^8 min^{-1} M^{-1} for brain. The K_d calculated from the ratio k_{-1}/k_1 in guinea pig brain was 0.1 nM—somewhat lower than the value obtained in equilibrium experiments. The two K_d values calculated by this ratio in the rat pancreas were 0.06 and 0.2 nM.

Regulation of CCK-Receptor Binding by Nucleotides and Ions. We have described the selective reduction of ^{125}I-CCK-33 binding to rat pancreatic membranes by certain guanine nucleotides (13). In this study we compared in some detail influences of guanine nucleotides on brain and pancreatic receptor binding (Fig. 4). In confirmation of our earlier finding, GTP and 5′-guanylylimidodiphosphate (p[NH]ppG) were the most potent nucleotides, decreasing binding about 50% in the low micromolar range, with GDP being slightly less active. By contrast, GMP and the adenine nucleotides ATP, ADP, and AMP were virtually inactive in reducing binding. The relative and absolute potencies of p[NH]ppG and GTP differed in guinea pig brain and rat pancreas. The lesser potency of p[NH]ppG in brain (IC_{50} = 35 μM) than pancreas (IC_{50} = 0.7 μM) may reflect endogenous GDP bound to the guanyl nucleotide regulatory site in brain (17). (IC_{50} is the concentration that inhibits specific binding by 50%.) In pancreas, GTP was half as potent as p[NH]ppG in decreasing ^{125}I-CCK-33 receptor binding, whereas GTP was only 1/16th as potent as p[NH]ppG in brain. The decreased relative potency of GTP compared with p[NH]ppG may reflect GTP metabolism in brain membranes or real differences in the nucleotide recognition sites.

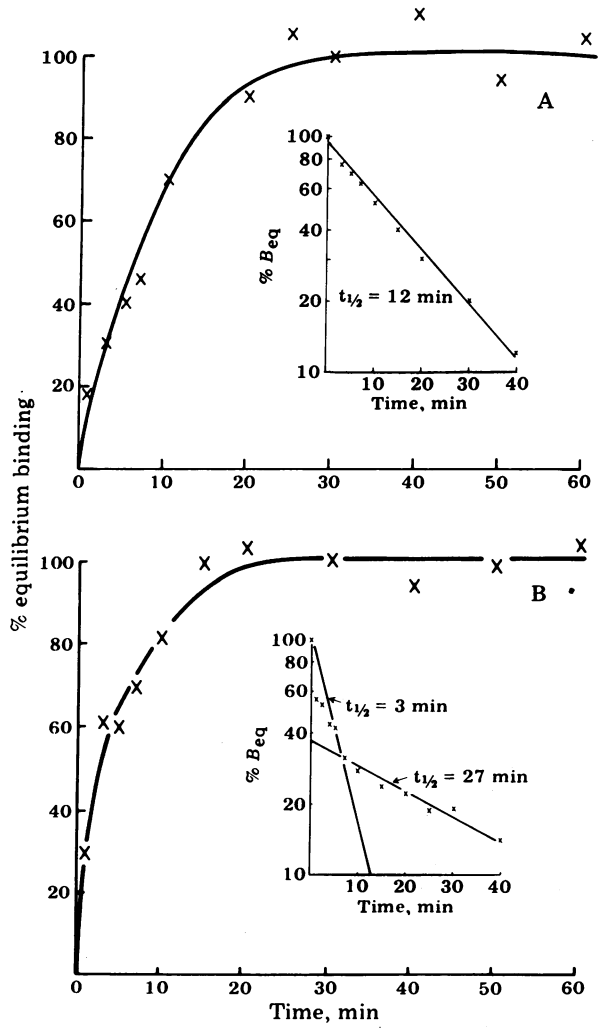

FIG. 3. Association and dissociation of ^{125}I-CCK-33 binding to guinea pig brain (*A*) and rat pancreatic membranes (*B*). For association, 50–100 pM ^{125}I-CCK-33 was added to brain and pancreatic membranes and incubated at 37°C. The reaction was stopped at various time intervals by addition of 1.0 ml of ice-cold incubation buffer and rapid centrifugation. Specific binding is plotted as percent of equilibrium binding (taken at 30 min). For dissociation, ^{125}I-CCK-33 was first allowed to associate for 30 min at 37°C, whereupon 0.5 μM CCK-8 was added and residual binding was measured at various time intervals. Data are expressed as the percent of equilibrium binding present at the end of the association for 30 min at 37°C. Results are averages of triplicate determinations performed three times. B_{eq}, concentration of radioligand bound at equilibrium.

Opiate (18), α-adrenergic (19), and histamine H_1 (20) receptors are regulated by monovalent cations. Sodium and lithium, but not other monovalent cations, decrease affinities of agonists but not antagonists for binding sites. Monovalent cations reduced ^{125}I-CCK-33 binding to pancreatic and brain membranes. However, potencies were similar for sodium, potassium, lithium, rubidium, and cesium, with 50% reduction at about 60 mM for brain membranes and at 120 mM for pancreatic membranes. This is not a simple effect of ionic strength, since divalent cations enhanced binding (Fig. 5). Calcium and magnesium were roughly equipotent, doubling binding at 5 mM concentration, whereas manganese at the same concentration increased specific binding 2.5- to 2-fold. This effect differs from that in opiate (21), α-adrenergic (22), and hista-

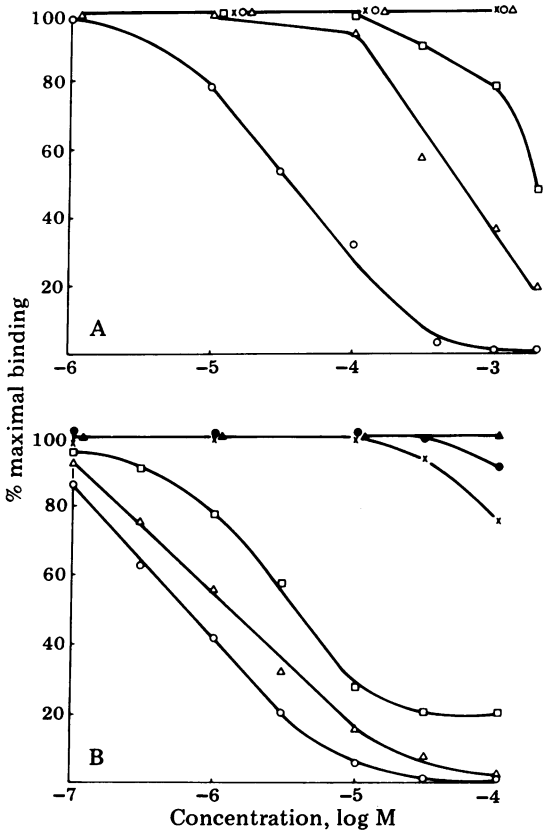

FIG. 4. Guanyl nucleotide inhibition of ^{125}I-CCK-33 binding to guinea pig brain (*A*) and rat pancreatic membranes (*B*). ^{125}I-CCK-33 (50–100 pM) was incubated for 30 min at 37°C with various concentrations of nucleotides. ○, p[NH]ppG; △, GTP; □, GDP; ×, ADP; ●, GMP; ▲, ATP. Data are a measure of the residual specific binding and are the average of triplicate determinations performed at least three times. Results are expressed as the percent of maximal binding determined in the absence of exogenous nucleotide.

mine H_1 (20) receptors, where the effect of calcium is much weaker than that of magnesium and manganese. The inhibiting effects of monovalent cations and guanine nucleotides on ^{125}I-CCK-33 binding were additive in both brain and pancreas. Thus, when concentrations of sodium and GTP that separately reduce binding by 25% were combined, a 50% reduction in binding occurred.

Regional Distribution of CCK-Receptor Binding in Brain. Immunohistochemical (5–8) and chemical (23) studies of endogenous CCK have indicated considerable regional variations in rat and guinea pig brain, with highest CCK content in the cerebral cortex and lowest levels in the cerebellum. Saito *et al.* (14) reported that regional variations of ^{125}I-CCK-33 binding in rat brain resemble the distribution of endogenous CCK. We found similar regional variations of ^{125}I-CCK-33 binding in rat brain (Table 1). However, guinea pig brain had a markedly different pattern of regional variations. Whereas the cerebellum had the lowest levels of binding in the rat, it displayed the second highest receptor binding in guinea pig brain (Table 1). As in the rat, cerebral cortical binding levels were fairly high, whereas spinal cord and brain stem levels were lowest, and intermediate levels of binding occurred in hypothalamus, hippocampus, and caudate. Whether the regional variations in CCK receptor binding in guinea pig brain correlate with the distribution of endogenous CCK is unclear, because the relative concentrations of CCK-8 and CCK-4 in all brain regions have not been determined (6). The receptor in guinea pig cerebral cortex and that in cerebellum appear to be identical, as the

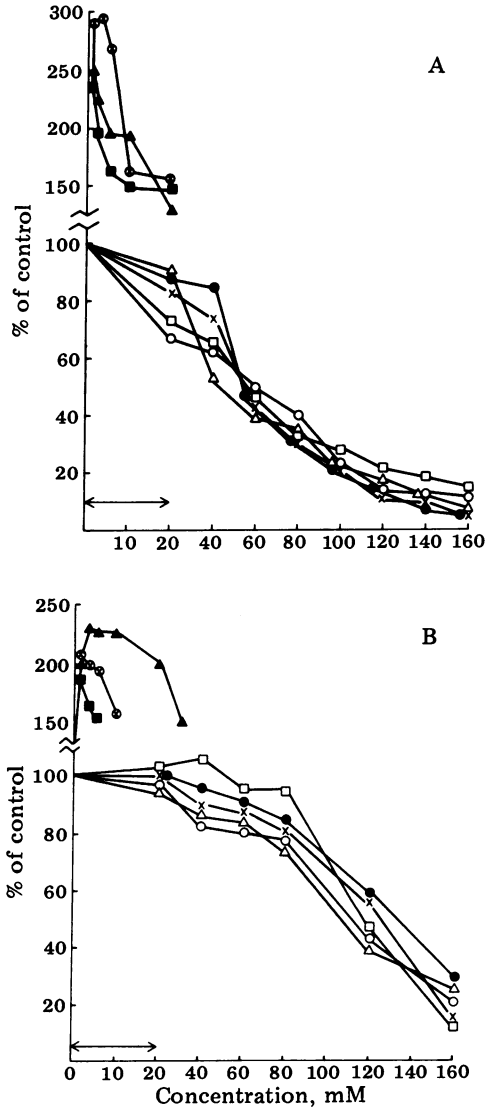

FIG. 5. Monovalent cation inhibition and divalent cation enhancement of ^{125}I-CCK-33 binding to guinea pig brain (*A*) and rat pancreatic membranes (*B*). Various concentrations of monovalent cations were incubated with membranes and 50–100 pM ^{125}I-CCK-33 for 30 min at 37°C in incubation buffer. △, CsCl; ●, KCl; □, RbCl; ×, NaCl; ○, LiCl. Data represent the residual specific binding in the presence of increasing concentrations of monovalent cation. Experiments with the divalent cations were performed identically, except the incubation buffer did not contain $MgCl_2$ and the maximal binding was determined in the absence of any exogenous divalent cation. ■, $CaCl_2$; ⊗, $MnCl_2$; ▲, $MgCl_2$. All points are the average of triplicate determinations performed at least four times. The arrow in the lower left of each graph designates the area of the expanded scale on the abscissa.

potencies of CCK-33, CCK-8, desulfated CCK-8, [Leu^{15}]gastrin-17-I, and pentagastrin were indistinguishable in these two regions.

DISCUSSION

The present study shows that pancreatic and brain CCK receptors differ markedly. The pancreatic binding sites correspond in peptide specificity to the amylase stimulating actions of CCK. We did not find evidence for binding sites with a specificity characteristic of the selective releasing actions of CCK-4 for insulin and other hormones. Presumably CCK-33, which is very weak in releasing insulin, lacks adequate affinity to label the putative CCK-4 receptors (10).

Though CCK-4 is potent at the brain binding sites, the con-

Table 1. Regional distribution of ^{125}I-CCK-33 binding in rat and guinea pig brain

Region	Rat*	Guinea pig*
Cerebral cortex	100	40
Olfactory bulb	87	100
Hypothalamus	85	18
Pons/medulla oblongata	70	15
Caudate/putamen	66	23
Hippocampus	40	24
Spinal cord (cervical/thoracic)	25	11
Cerebellum	10	54

The specific binding was measured in the presence of 50–100 pM ^{125}I-CCK-33 (90,000 dpm/ml) and 20 mg (original wet weight) of tissue. The numbers represent the average of three separate determinations each done in triplicate, which differed by less than 15%. The total binding and nonspecific binding were respectively 4500 and 3300 cpm in the rat cerebral cortex and 6200 and 2300 cpm in guinea pig olfactory bulb.

* Percentage of region with highest binding.

siderable activities of CCK-8, CCK-33, and [Leu^{15}]gastrin-17-I indicate that the pancreatic receptor binding described here differs from the pancreatic islet sites associated with enhancement of insulin release (10). Though [Leu^{15}]gastrin-17-I is potent at brain sites, these receptors probably do not represent primarily gastrin receptors because the peptide specificity of displacement of ^{125}I-labeled [Leu^{15}]gastrin-17-I binding to rat stomach (24) differs from that described here. For example, CCK-33 is less potent than [Leu^{15}]gastrin-17-I in displacing ^{125}I-labeled [Leu^{15}]gastrin-17-I bound to rat stomach, whereas the opposite is the case for ^{125}I-CCK-33 bound to guinea pig brain. Perhaps a single type of receptor in the brain interacts with multiple forms of CCK and gastrin released from various populations of neurons. Alternatively, the binding to brain membranes may represent multiple forms of a cholecystokinin/gastrin receptor, each specific for CCK-4, CCK-8, CCK-33, or gastrin, which we cannot distinguish using ^{125}I-CCK-33 as the radiolabeled probe.

We know of only one instance of multiple subtypes of peptide hormone receptors, the mu and delta opiate receptors (25–27). Recently we have observed that the differential localization of mu and delta receptors in brain areas determined by light microscopic autoradiography (28) corresponds to the different localizations of [Met]- and [Leu]enkephalin-containing neurons (29). Thus, the mu and delta receptors appear to be distinct opiate receptors for [Met]enkephalin and [Leu]enkephalin, respectively.

There is conflicting evidence as to whether CCK actions in the pancreas involve cyclic AMP (30). Accordingly, it is not clear whether the regulation of CCK-receptor binding by guanyl nucleotides relfects an association with adenylate cyclase. Receptors for peptide hormones such as angiotensin II (with no known adenylate cyclase linkage) also manifest specific regulation by guanine nucleotides (31). CCK enhancement of amylase secretion in the pancreas is associated with calcium outflux and cyclic GMP accumulation (32, 33). Conceivably the GTP effect is associated with calcium regulatory sites.

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