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Type-A Cholecystokinin Binding Sites in Cow Brain: Characterization using (−)-[^{3}H]L364718 Membrane Binding Assays

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SUMMARY

(−)-[^{3}H]L364718 membrane binding assays were employed to localize and characterize cholecystokinin (CCK)-A binding sites in rat and cow brain. Specific binding was detected in all brain areas tested, but in all areas of rat brain and most areas of cow brain the level was too low to allow characterization of the ligand binding specificity of these sites. Membranes prepared from cow nucleus accumbens and striatum contained higher levels of (−)-[^{3}H]L364718 specific binding which represented 55–70% of total binding. Characterization of the ligand binding properties of (−)-[^{3}H]L364718 binding sites in cow nucleus accumbens revealed that these sites are similar to CCK-A sites found in pancreatic membranes. Binding of (−)-[^{3}H]L364718 was saturable and had high affinity (K_d = 45 pM). Sites labeled by (−)-[^{3}H]L364718 displayed stereospecificity for the stereoisomers of CR1409. The competition curve for CCK8 was shallow and was steepened and shifted to the right by the presence of the stable GTP analog guanosine 5′-(β,δ-imido)triphosphate. The potency of CCK8, but not (−)-L36478, was also affected by the buffer in which the assay was conducted. Future use of (−)-[^{3}H]L364718 membrane binding assays using cow nucleus accumbens and/or striatum will help explore the possibility of differences in ligand recognition among CCK-A sites found in brain and peripheral tissues.

Radioligand binding studies have indicated that CCK binding sites can be divided into two classes (1–3). CCK-B sites are the predominate type found in brain. CCK-A sites are found in peripheral tissues such as pancreas and gallbladder. More recent evidence indicates that CCK-A sites are also present within the central nervous system, albeit at a much lower density than CCK-B sites. CCK-A sites in rodent brain seem to be confined to specific areas of brain and have been documented and localized primarily by autoradiographic techniques (4–9).

(−)-L364718 has been reported to be a high affinity, selective antagonist for CCK-A receptors (10). (±)-[^{3}H]L364718 has been used to characterize CCK-A sites in membranes from pancreas and gallbladder (11). This radioligand has also been an important tool for localizing CCK-A binding sites in brain by autoradiography (4, 6). An important question is whether these sites have ligand binding specificity that is identical to that of CCK-A sites in peripheral tissues. Because CCK-A sites in rodent brain are found in low abundance in discrete brain areas, previous autoradiography studies have been limited in the detail with which ligand binding specificity can be defined. Such studies require at least several different compounds to be tested for their affinity for these sites so that comparisons with affinities for CCK-A sites in other tissues such as pancreas can be made. In addition, competition and saturation experiments with many compound concentrations over a wide concentration range should be performed in order to examine the possibility of binding site heterogeneity. Brain membrane binding studies with higher densities of sites and/or a less limited source of binding sites would have advantages for more comprehensive analysis of ligand binding properties of CCK-A sites found in brain.

We now report that certain areas of cow brain contain a higher density of CCK-A sites than corresponding areas of rat brain. This finding has allowed us to characterize the ligand binding properties of these sites using (−)-[^{3}H]L364718 membrane binding assays.

Experimental Procedures

Materials. CCK8 was purchased from Cambridge Research Biologicals (Valley Stream, NY). (−)-L364718 was obtained from Merck (Rahway, NJ). Boc-CCK4 was synthesized by standard peptide-coupling techniques. The *R* and *S* enantiomers of CR1409 were synthesized

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ABBREVIATIONS: CCK, cholecystokinin; BH-CCK8, Bolton-Hunter cholecystokinin(26–33)amide; Gpp(NH)p, guanosine 5′-(β,δ-imido)triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Boc-CCK4, *t*-butyloxycarbonyl-cholecystokinin(30–33)amide; G protein, guanine nucleotide-binding protein.

by Dr. J. Kerwin (Abbott Laboratories) as described (12). (−)-[^{3}H]L364718 (87 Ci/mmol) was prepared from (±)-[^{3}H]L364718 (New England Nuclear, Boston, MA) by Dr. J. Denisson (Abbott Laboratories) as described (13). ^{125}I-BH-CCK8 was purchased from New England Nuclear. Unless noted, all other reagents were from Sigma Chemical Co. (St. Louis, MO).

Brain dissection and membrane preparation. Cow brains were obtained from a local slaughterhouse and were kept on ice until completion of the dissection (approximately 2 hr after sacrifice). Six areas of brain were dissected, immediately frozen on dry ice, and stored at −80°. Whole cerebellum was removed from the rest of the brain by transection of the cerebellar peduncles. The midbrain and brain stem were removed and placed on a dissecting tray with the posterior surface up. A knife cut was made at the base of the inferior colliculi and another cut was made 1 cm below the obex. The area between these cuts was considered the brain stem. In the forebrain section, the corpus callosum was cut and the lateral surfaces of the lateral ventricles were exposed. The nucleus accumbens was located at the head of the caudate and slightly medial to the bottom of the lateral ventricle. An approximately 0.5-cm thick section of striatum was dissected from the lateral wall of the ventricle. The hippocampus was removed following exposure of the surface of the lateral ventricles. The final brain section was a portion of frontal cortex that was free of underlaying white matter.

Male Sprague-Dawley rats (200–300 g) were decapitated and the brain was removed from the skull. A section of brain containing the nucleus accumbens was dissected as previously described (14). Striatum, hippocampus, cortex, and cerebellum were dissected from the caudal section of brain, which remained after dissection of the nucleus accumbens. A section of brain that was designated brain stem was dissected in a manner similar to that described for cow brain. All brain sections were frozen on dry ice and stored at −80°.

Individual frozen sections of cow brain or pooled sections of rat brain (10–12 rats/group) were weighed and homogenized (Brinkman Polytron, setting 7, 30 sec) in 40 volumes (ml/g) of ice-cold 50 mM Tris·HCl buffer (pH 7.4 at 24°). Pancreases from male guinea pig (250–325 g) were treated in a similar manner except that the buffer contained 0.2 mg/ml soybean trypsin inhibitor. The homogenate was centrifuged at 1,000 × *g* for 10 min and the supernatant was centrifuged at 48,000 × *g* for 20 min. The pellet was then resuspended with a glass/Teflon homogenizer and centrifuged at 48,000 *g* for 20 min. The pellet was resuspended in 10 volumes of Buffer A (20 mM HEPES, 1 mM EGTA, 0.13 M NaCl, 5 mM $MgCl_2$, 5 mM KCl, pH 7.4). A sample was taken for protein determination (Bio-Rad kit, Richmond, CA) and the homogenate was further diluted to 75 volumes for cow brain, 50 volumes for rat brain, or 1000 volumes for guinea pig pancreas with HES buffer (Buffer A containing 100 μM bestatin, 3 μM phosphoramindon, and 0.1% bovine serum albumin). Where noted, Buffer B (50 mM Tris, 5 mM $MgCl_2$, 5 mM dithiothreitol, pH 7.4) and TMD buffer (Buffer B containing 100 μM bestatin, 3 μM phosphoramindon, and 0.1% bovine serum albumin) were substituted for Buffer A and HES buffer, respectively.

The (−)-[^{3}H]L364718 binding assay consisted of 25 μl of HES buffer containing (−)-[^{3}H]L364718, 25 μl of HES buffer with or without competing ligand, and 750 μl of membrane homogenate. The incubation was initiated by addition of the membranes and was allowed to proceed at 37° for 30 min. Reverse phase high pressure liquid chromatographic analysis indicated that >95% of the radioligand was intact after incubation with cow nucleus accumbens membranes at 37° for 30 min (data not shown). Membrane-bound radioligand was collected by rapid filtration on glass fiber filters (No. 32; Schleicher & Schuell, Keene, NH) using a Skatron cell harvester. The filters were washed with approximately 15 ml of ice-cold phosphate-buffered saline and filter-bound radioactivity was measured by liquid scintillation counting. The counting efficiency was 45–48%. Specific binding was defined as the difference in bound radioligand in the presence and absence of 100 nM (−)-L364718.

The ^{125}I-BH-CCK8 binding assay consisted of 50 μl of HES buffer containing ^{125}I-BK-CCK8, 50 μl of HES buffer with or without competing ligand, and 200 μl of membrane homogenate. The assay was initiated and terminated as described above and filter-bound radioactivity was measured by γ counting. The counting efficiency was 75%. Specific binding was defined as the difference in bound radioligand measured in the presence and absence of 1 μM CCK8.

Results

Specific binding of (−)-[^{3}H]L364718 in rat and cow brain membranes. Shown in Table 1 is a comparison of the amount of specific binding of (−)-[^{3}H]L364718 in membranes from different areas of rat and cow brain. In rat brain, specific binding [defined by a difference in the amount bound in the presence and absence of 100 nM (−)-L364718] was detected in all brain regions. Levels of total dpm bound and nonspecific dpm bound from a representative experiment are also shown in Table 1. The percentage of specific binding ranged from 22% in hippocampus to 45% in nucleus accumbens. Because of the low number of specifically bound dpm and the low percentage of specific binding in most brain areas, the characteristics of the binding of (−)-[^{3}H]L364718 were not examined further. Therefore, the results must be viewed with caution. In membranes from rat nucleus accumbens and brain stem, we were able to show that the specific binding of (−)-[^{3}H]L364718 was fully inhibited by 10 μM CR1409 and 10 μM CCK8 (data not shown). We, therefore, tentatively conclude that CCK-A sites are found in membranes from these selected areas of rat brain.

Binding studies in rat brain areas, particularly nucleus accumbens, were limited not only by the low density of sites but also the limited amount of tissue per rat. For example, in the rat nucleus accumbens experiments whose results are given in Table 1, one rat provided only enough membranes for one assay tube. In the hope of circumventing the problem of limited tissue, we tested membranes from areas of cow brain for specific

TABLE 1

Binding of (−)-[^{3}H]L364718 (0.4 nM) to membranes from rat and cow brain

Specific binding was defined as the difference in the total binding of (−) [^{3}H]L364718 and the nonspecific binding determined in the presence of 100 nM L364718. Membrane protein was determined using the Bio-Rad protein assay. Values for the specific binding of (−)-[^{3}H]L364718 which are expressed as fmol/mg protein are the mean and standard error of three experiments each with six determinations of both total and nonspecific binding. DPM values for total and nonspecific binding are from one representative experiment.

Brain region	Specific binding	Binding		
		Total	Nonspecific	Specific
	fmol/mg of protein		*dpm*	
Rat				
Nucleus accumbens	73 ± 19	2306 ± 40	1261 ± 48	1045
Striatum	37 ± 2	1198 ± 15	727 ± 19	471
Cortex	12 ± 1	783 ± 16	591 ± 12	192
Cerebellum	21 ± 1	750 ± 30	561 ± 22	189
Hippocampus	16 ± 1	1071 ± 26	826 ± 34	245
Brainstem	35 ± 1	1355 ± 15	796 ± 19	559
Cow				
Nucleus accumbens	300 ± 20	4373 ± 11	1928 ± 34	2443
Striatum	480 ± 48	5499 ± 41	1766 ± 45	3733
Cortex	58 ± 18	1783 ± 44	1326 ± 42	457
Cerebellum	56 ± 11	1455 ± 30	1213 ± 39	242
Hippocampus	89 ± 23	975 ± 10	818 ± 42	157
Brainstem	81 ± 5	1177 ± 48	860 ± 51	317

binding of (−)-[^{3}H]L364718. The mean results of these studies are shown in Table 1, as well as the dpm values from a representative experiment. Specific binding was detected in all brain areas. Membranes from cortex, cerebellum, hippocampus, and brain stem gave low levels of specific binding and poor ratios of specific to nonspecific binding. However, higher levels of specific binding were detected in membranes from nucleus accumbens and striatum. The levels of specific binding/mg of membrane protein in cow nucleus accumbens and striatum were 4- and 13-fold higher than in the corresponding areas of rat brain. The combination of a high density of specific binding, a higher ratio of specific to nonspecific binding, and the large supply of tissue afforded by using cow brain made more detailed examination of the ligand binding properties of (−)-[^{3}H]L364718 binding sites in brain feasible.

Characterization of (−)-[^{3}H]L364718 binding in cow nucleus accumbens membranes. Additional (−)-[^{3}H]L364718 binding studies were conducted with cow nucleus accumbens membranes. Shown in Fig. 1 are typical competition curves for (−)-L364718 and the stereoisomers of CR1409. Two important conclusions can be made from these studies. First, the binding of (−)-L364718 is clearly saturable, as indicated by the plateau in the competition curve that occurred at concentrations higher than 100 nM. Second, the binding site labeled by (−)-[^{3}H]L364718 shows stereospecificity for the enantiomers of CR1409. The mean K_i values (n = 3) for (−)-L364718, (*S*)-CR1409, and (*R*)-CR1409 were 0.13 ± 0.02, 120 ± 30, and 1700 ± 200 nM, respectively. These K_i values are 3–5-fold higher than the corresponding K_i values that we have previously reported for guinea pig pancreatic membranes under identical conditions (15). In addition, CCK8 competed for (−)-[^{3}H]L364718 binding to cow nucleus accumbens (see below), but Boc-CCK4 and human gastrin I, peptides that are relatively selective for CCK-B sites (16, 17), did not compete at a concentration of 10^{-5} M (Table 2).

Shown in Fig. 2 is a representative Scatchard plot of (−)-[^{3}H]L364718 binding in cow nucleus accumbens membranes. Three such experiments were analyzed by nonlinear least squares curve-fitting of the binding isotherms (LIGAND program) (18). The mean K_d was 45 ± 4 pM, whereas the mean

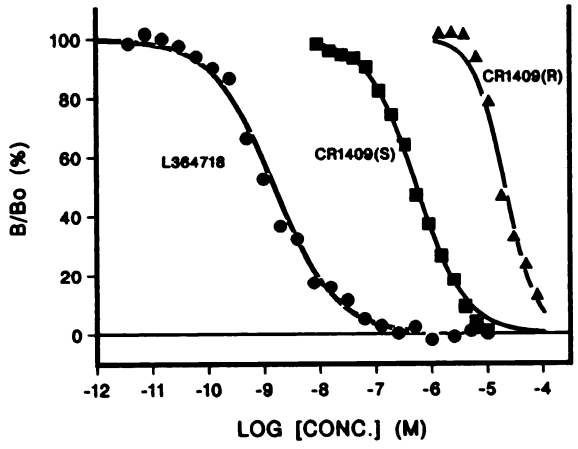

Fig. 1. Competition curves of CCK antagonists versus (−)-[^{3}H]L364718 binding in cow nucleus accumbens membranes. Specific binding of (−)-[^{3}H]L364718 was determined in triplicate in the presence of various concentrations of the antagonists. Results are from a single representative experiment. Mean K_i values from three experiments are given in the text of Results.

B_{max} was 370 ± 50 fmol/mg of protein. In no case did a two-site model provide a statistically significant improvement of the fit of the data. The K_d value in cow nucleus accumbens membranes is slightly higher than the value of 21 ± 1 pM that we have previously determined under identical conditions in guinea pig pancreas membranes (15).

All of the above binding studies were conducted with a buffer in which binding of ^{125}I-BH-CCK8 to CCK-A binding sites in pancreatic membranes is optimal, in terms of specific binding and integrity of the radioligand.[2] However, CCK8 was found to be a weak inhibitor of (−)-[^{3}H]L364718 binding to nucleus accumbens membranes when this buffer was used (Fig. 3, and Table 2). It has previously been demonstrated that NaCl can alter agonist potencies in inhibiting (±)-[^{3}H]L364718 binding to rat pancreatic membranes (11). We, therefore, also determined the potency of CCK8 and (−)-L364718 in a buffer that lacks NaCl and has been previously used to study (±)-[^{3}H]L364718 binding in rat pancreatic membranes (11). For comparison, we conducted parallel experiments with guinea pig pancreatic membranes with both buffers. In both nucleus accumbens and pancreatic membranes, the potency of (−)-L364718 did not differ in the two buffers and in all cases the log-logit slopes of the competition curve were close to unity. However, the potency of CCK8 was dependent on both the tissue and the buffer used. CCK8 was more potent in pancreatic membranes than in nucleus accumbens membranes. In both tissues, CCK8 was more potent in the TMD buffer. In all cases the competition curves were shallow, with log-logit slopes less than 0.6.

In pancreatic membranes (11, 19), rat brain stem membranes (6), and membranes from CHP212 human neuroblastoma cell line (15), CCK-A binding sites are believed to couple to G proteins, as evidence by the fact that guanine nucleotides reduce agonist binding affinity. In cow nucleus accumbens membranes, the stable GTP analog Gpp(NH)p steepened the competition curve of CCK8 and shifted the competition curve to the right (Fig. 3).

It was of interest to estimate the relative densities of CCK-A and CCK-B binding sites in cow nucleus accumbens membranes. ^{125}I-BH-CCK8 binds with high affinity to both CCK-A and CCK-B sites (16, 17), whereas (−)-L364718 is reported to be greater than 1000-fold selective for CCK-A sites (10). Thus, competition studies with these ligands might be expected to reveal binding site heterogeneity if both CCK-A and CCK-B sites were found in near equal densities. In three such experiments, there was no evidence for binding site heterogeneity, i.e., analysis according to a two-site model (LIGAND program) (18) was not statistically better than a single-site model. A representative experiment is shown in Fig. 4. The mean IC_{50} from three experiments was 88 ± 20 nM. Under identical conditions, we have determined the IC_{50} for (−)-L364718 inhibition of ^{125}I-BH-CCK8 binding to guinea pig cortex and guinea pig pancreas membranes to be 120 ± 40 (n = 3) and 0.36 ± 0.10 nM (n = 3), respectively. These data indicate that, within the limits of detection, ^{125}I-BH-CCK8 was bound exclusively to CCK-B sites in cow nucleus accumbens membranes. We conclude that CCK-B sites substantially outnumber CCK-A sites in cow nucleus accumbens membranes.

[2] Unpublished observations.

TABLE 2

IC_{50} values of CCK8 and (−)-L364718 in competing for (−)-[^{3}H]L364718 binding in cow nucleus accumbens membranes

In each experiment, specific binding at each concentration of competing ligand was determined in triplicate. At least 12 concentrations of the competing ligand were tested. The IC_{50} and slope were determined by log-logit analysis of the competition curve. The concentration of (−)-[^{3}H]L364718 was 0.4 nM. Descriptions of the HES and TMD buffers are given in Experimental Procedures. Values are the mean ± standard error from three experiments.

	IC_{50} (Log-logit slope)			
	Cow nucleus accumbens		Guinea pig pancreas	
	HES buffer[a]	TMD buffer	HES buffer	TMD buffer
	nM			
CCK8	2300 ± 500 (0.43 ± 0.01)	360 ± 20 (0.51 ± 0.02)	37 ± 20 (0.45 ± 0.05)	2.2 ± 0.2 (0.54 ± 0.05)
L364718	1.3 ± 0.2 (0.96 ± 0.06)	1.1 ± 0.3 (0.90 ± 0.03)	0.89 ± 0.03 (1.08 ± 0.05)	1.2 ± 0.2 (1.1 ± 0.06)

[a] Under these conditions, Boc-CCK4 and human gastrin I did not significantly inhibit specific binding at 10^{-6} M (two experiments).

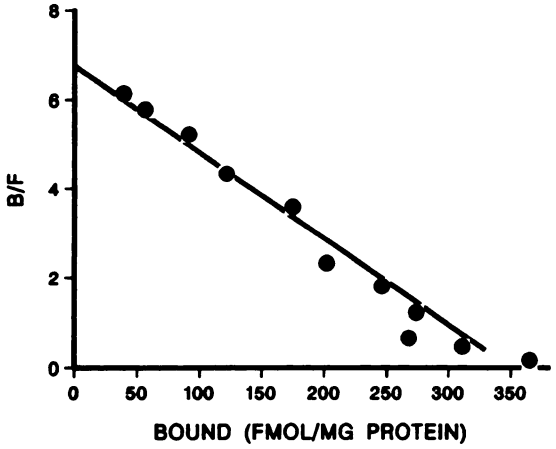

Fig. 2. Scatchard plot of (−)-[^{3}H]L364718 binding to cow nucleus accumbens membranes. Specific binding of various concentrations of (−)-[^{3}H]L364718 was determined in triplicate. The figure is a representative experiment. Mean K_d and B_{max} values from three experiments are given in the text of Results. The units for the *y*-axis are (liter/mg of protein) × 1000.

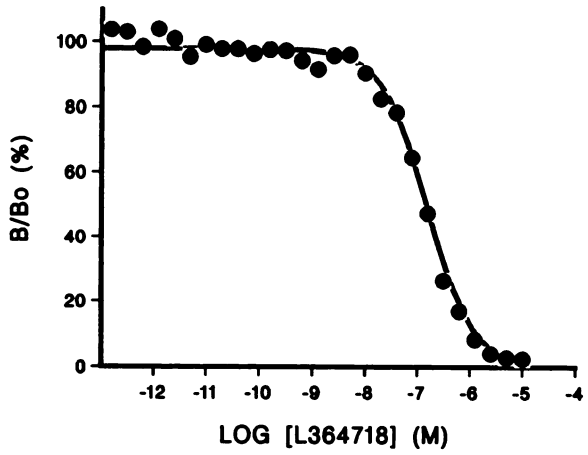

Fig. 4. Competition of (−)-L364718 for ^{125}I-BH-CCK8 binding to cow nucleus accumbens membranes. Specific binding of ^{125}I-BH-CCK8 was determined in triplicate in the presence of various concentrations of (−)-L364718. Results are from a single representative experiment. Mean K_i values from three experiments are given in the text of Results.

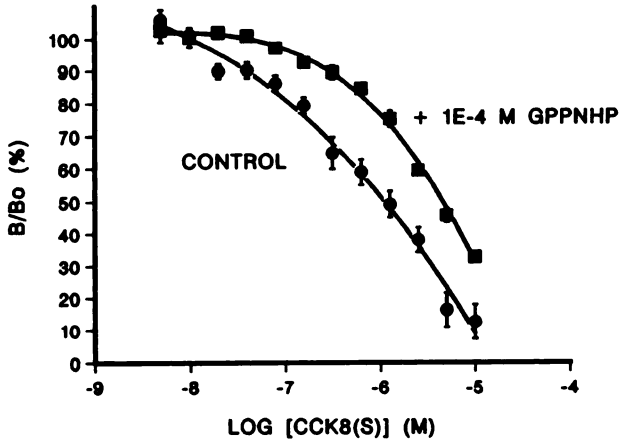

Fig. 3. Effect of Gpp(NH)p on CCK8 competition for (−)-[^{3}H]L364718 binding to cow nucleus accumbens membranes. Cow nucleus accumbens membranes were prepared as described in Experimental Procedures and then divided in half. Gpp(NH)p (100 μM) was added to one half of the membrane preparation. Specific binding of (−)-[^{3}H]L364718 was determined in triplicate in the presence of various concentrations of CCK8. Results are the mean and standard error from three paired experiments.

Discussion

Recent investigations have shown that (−)-L364718, a nonpeptide CCK-A receptor-selective antagonist, is a powerful tool for physiological and biochemical investigations of CCK receptors (10, 11, 20, 21). We now report the use of (−)-[^{3}H]L364718 to detect and characterize CCK-A binding sites in brain membrane preparations. We have demonstrated that various levels of specific binding of (−)-[^{3}H]L364718 are detectable in membranes from specific areas of rat brain, but detailed analysis of the binding characteristics of these sites was not possible due to poor ratios of specific to nonspecific binding and limited amounts of brain tissue. Our novel finding is that the specific binding of (−)-[^{3}H]L364718 to membranes from the nucleus accumbens and striatum of cow brain is appreciably higher than in rat brain and is sufficient to allow characterization of the binding properties of these sites. Our initial investigation of these CCK-A binding sites indicates that they are similar to CCK-A sites in pancreatic membranes.

One factor that aided our investigations of the regional distribution of CCK-A sites and afforded us the ability to study CCK-A sites in membranes from cow nucleus accumbens was the use of (−)-[^{3}H]L364718 rather than the commercially available racemic radioligand (±)-[^{3}H]L364718. CCK-A receptors in pancreas are known to display strong stereospecificity for the enantiomers of L364718. The less active isomer, (+)-L364718, has been shown to have an affinity of 4.5 nM for CCK-A sites in pancreatic membranes, which is approximately 100-fold less than the affinity of (−)-L364718 (10). It, therefore, seems likely that the use of (±)-[^{3}H]L364718 is problematic, particularly when attempting to characterize binding sites found in low abundance. Under our conditions (0.4 nM concentration), half of the racemic radioligand would be expected to contribute to

nonspecific binding but not to appreciably bind to CCK-A sites. Other theoretical problems associated with analysis of binding data when a racemic radioligand is used have been previously presented (22). At the outset of our investigations, we felt it was necessary to utilize (−)-[^{3}H]L364718 rather than the racemic radioligand.

Under our conditions, the amount of specific binding of (−)-[^{3}H]L364718 to membranes prepared from most regions of both rat and cow brain was low and typically represented less than 30% of total binding. The low percentage of specific binding prevented us from experimentally verifying that the specific binding of (−)-[^{3}H]L364718 was actually binding to CCK-A sites. We, therefore, cannot make conclusions about quantitative differences in the density of CCK-A sites in different brain areas. The strongest inference from these data is that, in both rat and cow brain, the nucleus accumbens and striatum displayed higher densities of CCK-A sites than other brain areas.

Given our results with (−)-[^{3}H]L364718 membrane binding assays, it is somewhat surprising that there have been no published reports documentating CCK-A binding sites in rat nucleus accumbens or striatum using autoradiographic techniques. The reason for this is unclear but may be due to poor signal to noise ratios in autoradiographic studies in these brain areas when using ^{125}I-BH-CCK8 with a competing ligand (see below) or when using (±)-[^{3}H]L364718 rather than (−)-[^{3}H]L364718. We detected a relatively high level of (−)-[^{3}H]L364718 specific binding in rat brain stem membranes. This finding is in agreement with a previous report of CCK-A sites on membranes prepared from this brain area (6) and is consistent with autoradiographic studies, which have documented the presence of CCK-A sites in nucleus tractus solitarius, area postrema, and interpeduncular nucleus (4–9).

Nucleus accumbens and striatum are rich in dopaminergic innervation and many investigators have reported that CCK peptides can alter various parameters associated with the function of dopaminergic neurons (13, 23–36). Pharmacological characterization of the receptors (CCK-A or CCK-B) involved in modulating dopaminergic neurons has until recently been difficult, because highly selective agonists and antagonists were not available. The use of type-selective compounds will help discern the physiological role of CCK-A receptors in these dopamine-rich brain areas. For example, it has recently been shown that CCK8 can facilitate potassium-evoked dopamine efflux from rat nucleus accumbens slices and that CCK-A receptors are involved (36). This observation is consistent with our observation of higher levels of (−)-[^{3}H]L364718 binding in this region of rat brain.

We have demonstrated that membranes prepared from both cow nucleus accumbens and striatum possess a high enough density of CCK-A binding sites that specific binding was a major portion of total binding of (−)-[^{3}H]L364718. We chose to conduct further studies with nucleus accumbens membranes. We cannot say with certainty that (−)-[^{3}H]L364718 binding sites in striatal membranes show similar binding characteristics. Our studies indicate that CCK-A sites in nucleus accumbens membranes are very similar to CCK-A sites in pancreatic membranes. Analysis of the binding isotherm of (−)-[^{3}H]L364718 gave no indication of multiple binding sites. Similar to CCK-A sites in guinea pig pancreas (12, 15) and CHP212 neuroblastoma cells (15), CCK-A sites in nucleus accumbens showed stereospecificity for the stereoisomers of CR1409. The affinities of these antagonists were 3–5 times lower than the affinities that we have previously determined in guinea pig pancreatic membranes (15) but the relative ratios of the affinities of these compounds are similar in the two tissues. Another property that is shared by the two sources of CCK-A sites is the shallowness of the competition curve for CCK8 in competing for (−)-[^{3}H]L364718 binding. In both tissues, the potency of the agonist CCK8 is affected by the ionic constituents of the buffer as well as the presence of the guanine nucleotide Gpp(NH)p. The observation that the affinity of CCK8 is reduced by the presence of a guanine nucleotide is consistent with a ternary complex model, which has been proposed for CCK receptors in pancreas (11, 18) as well as other G protein-linked receptors (36–38). Presumably, CCK-A receptors in both nucleus accumbens and pancreatic membranes can exist in two states and the affinity for agonists is dependent on the interaction of the receptor with G protein.

One notable difference between cow nucleus accumbens membranes and guinea pig pancreatic membranes was the potency of CCK8 in competing for (−)-[^{3}H]L364718 binding, regardless of the buffer that was used. CCK8 was 10–30-fold less potent in nucleus accumbens membranes. The reason for this is not readily apparent. We hypothesized that the difference could be attributed to the state of the tissue before membrane preparation. Nucleus accumbens membranes were prepared from frozen tissue whereas guinea pig pancreatic membranes were prepared from fresh pancreases. However, freezing guinea pig pancreases before preparation of the membranes did not affect the potency of CCK8 in competing for (−)-[^{3}H]L364718 binding (data not shown). We have observed similar weak potency of CCK8 in competing for (−)-[^{3}H]L364718 binding in a human neuroblastoma cell line that expresses CCK-A receptors (15). Whether these observations are due to heterogeneity in the CCK-A receptors is not clear at this point. An alternative explanation is that the stoichiometry of CCK-A receptors and G proteins in membranes from a particular tissue or cell is responsible for the differences in potency of CCK8. Computer simulations of the ternary complex model support this hypothesis. This binding model predicts not only agonist competition curves that are shallow under conditions where G protein is stoichiometrically limited, but also agonist potencies that are dependent on the ratio of G protein to receptor (39).

We have attempted to estimate the proportion of CCK-A and CCK-B sites in cow nucleus accumbens membranes using ^{125}I-BH-CCK8 as a nonselective radioligand and (−)-L364718 as a selective competing ligand. The data indicated that, within the detection limits of our procedure, ^{125}I-BH-CCK8 was bound exclusively to CCK-B sites. This observation is consistent with the notion that CCK-B sites outnumber CCK-A sites in nucleus accumbens. Several factors make it impossible to quantitate an exact ratio of the two types of CCK binding sites. We have previously shown that, in two systems that contain a homogeneous population of CCK-A sites, the apparent B_{max} for the agonist ^{125}I-BH-CCK8 is much less than the B_{max} for the antagonist (−)-[^{3}H]L364718. In membranes prepared from guinea pig pancreas and CHP212 neuroblastoma cells, the B_{max} for (−)-[^{3}H]L364718 was higher than the B_{max} for ^{125}I-BH-CCK8 by a factor of 6.5 and 17, respectively (15). Because a CCK-B-selective radiolabeled antagonist is not available, we do not know whether CCK-B sites display this same property.

Therefore, unequivocal quantitation of the amounts of CCK-A and CCK-B receptors in a particular brain area cannot be done at this time.

In summary, we have attempted to localize CCK-A binding sites in both rat and cow brain using (−)-[^{3}H]L364718 membrane binding assays. Although specific binding of (−)-[^{3}H]L364718 could be detected in all brain regions studied, the amount of binding in most brain areas was too low to allow detailed examination of the ligand specificity of these sites. We have discovered that membranes prepared from cow nucleus accumbens and striatum have high enough levels of (−)-[^{3}H]L364718 specific binding to allow investigation of the binding properties of the sites. Our initial characterization of (−)-[^{3}H]L364718 binding sites in cow nucleus accumbens membranes indicates that these sites are similar to CCK-A sites in pancreas. Future studies with structurally diverse agonist and antagonist ligands will explore the possibility of differences in ligand recognition between CCK-A sites located in peripheral tissues such as pancreas and those located within the central nervous system.

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