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ACCELERATED COMMUNICATION

Type-A Cholecystokinin Receptors in CHP212 Neuroblastoma Cells: Evidence for Association with G Protein and Activation of Phosphoinositide Hydrolysis

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SUMMARY

^{125}I-Bolton Hunter-cholecystokinin octapeptide (BH-CCK8) and (−)-[^{3}H]L-364718 membrane binding assays were used to identify and characterize cholecystokinin (CCK) receptors in CHP212 human neuroblastoma cells. The ligand binding properties of CCK receptors in these cells are similar to those found in pancreas (CCK-A sites) and differ from the predominant type of CCK binding site found in brain (CCK-B sites). The specific binding of ^{125}I-BH-CCK8 but not (−)-[^{3}H]L-364718 was reduced by the metabolically stable GTP analog guanosine 5′-(β-δ-imido)trisphosphate. A substantial difference in the B_{max} for the radiolabeled agonist (^{125}I-BH-CCK8) and antagonist [(−)-[^{3}H]L-364718] was noted. These observations are consistent with CCK receptors existing in guanine nucleotide-binding protein-coupled and -uncoupled states. Similar to its action in pancreatic acinar cells, CCK8(S) stimulated the accumulation of [^{3}H]inositol phosphates in cells prelabeled with [^{3}H]myo-inositol (EC_{50} = 3.2 ± 0.4 nM; maximum response = 4.5 ± 0.4 × basal). The intrinsic activity of CCK analogues in stimulating phosphoinositide hydrolysis was substantially less than their reported intrinsic activity in stimulating phosphoinositide hydrolysis in pancreatic acinar cells. The CHP212 neuroblastoma cell may serve as a useful model for the recently reported CCK-A binding site found in the central nervous system.

Various molecular forms of CCK peptides exist in mammalian blood, gut, and brain (1). CCK peptides are believed to play a role in the regulation of gall bladder contraction, pancreatic enzyme secretion, and smooth muscle contraction of the gut. Relative to other neuropeptides, high concentrations of CCK peptides are found in specific areas of the brain (2) and CCK meets many of the criteria for consideration as a neurotransmitter (3). There is evidence to suggest that CCK peptides are involved in the regulation of food intake, locomotion, seizures, and pain perception (4–7). However, a definition of its mechanism of action at the neuronal level has been elusive.

Radioligand binding studies have demonstrated the presence of CCK binding sites in brain. The ligand binding specificity of these sites differs from CCK binding sites in the pancreas and gall bladder (8–10). This observation has led to the conclusion that at least two types of CCK binding sites exist. Using the nomenclature originally suggested by Moran *et al.* (11), CCK-A binding sites are found in peripheral tissues such as pancreas and in certain areas of the brain. CCK-B binding sites are the predominant type of CCK binding site found in brain. Signal transduction mechanisms associated with activation of CCK-A receptors in the pancreatic acinar cells have been well characterized (12–14). However, little is known about the second messengers or signalling systems associated with CCK-B binding sites in the brain. In the hope of finding a cell line in which CCK-B binding site activation could be studied, we tested a number of human neuroblastoma cell lines for the presence of CCK binding sites. We now report the characterization of CCK receptors in CHP212 neuroblastoma cells. To our surprise, these receptors are of the CCK-A subclass. They may, therefore, represent a model system for investigations of recently described CCK-A binding sites in brain (11, 15–18).

Experimental Procedures

Materials. CCK8 and CCK8(DS) were purchased from Cambridge Research Biochemicals (Valley Stream, NY). CCK7 and CCK7(DS) were purchased from Research Plus (Bayonne, NJ). All other peptides were synthesized using standard peptide-coupling techniques. All pep-

ABBREVIATIONS: CCK, cholecystokinin; BH-CCK8, Bolton Hunter cholecystokinin(26–33)amide; CCK8(DS), cholecystokinin(26–33)amide, nonsulfated; CCK7, cholecystokinin(27–33)amide; CCK7(DS), cholecystokinin(27–33)amide, nonsulfated; BOC-CCK6, *t*-butyloxycarbonyl-cholecystokinin(28–33)amide; BOC-CCK5, *t*-butyloxycarbonyl-cholecystokinin(29–33)amide; BOC-CCK4, *t*-butyloxycarbonyl-cholecystokinin(30–33)amide; Gpp(NH)p, guanosine 5′-(β-δ-imido)triphosphate; PBS, phosphate buffered saline, EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N*^{1},*N*^{1}-tetraacetate; HEPES, 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; G protein, guanine nucleotide-binding protein.

tides were greater than 95% pure as assessed by reverse phase HPLC and their structures confirmed by fast atom bombardment mass spectrometry. Racemic CR-1409 was purchased from Peninsula Laboratories (Belmount, CA). The *R*- and *S*-enantiomers of CR-1409 were synthesized by Dr. J. Kerwin, Abbott Laboratories. (−)-L-364718 was obtained from Merck, Inc. (Rahway, NJ). Cell culture and other reagents were from Sigma Chemical Co. (St. Louis, MO). [^{3}H]myo-Inositol and ^{125}I-BH-CCK8 were from New England Nuclear (Boston, MA). The preparation of (−)-[^{3}H]L-364718 (Dr. J. Denisson, Abbott Laboratories) by chiral HPLC from (±)-[^{3}H]L-364718 (New England Nuclear) will be described elsewhere (19).

Cell culture. The human neuroblastoma cell line CHP212 was obtained from Dr. J. Beidler (Memorial Sloan-Kettering Cancer Center). CHP212 cells were grown at 37° in 5% carbon dioxide in 150-cm^2 flasks in a 1:1 mixture of Eagle's minimum essential medium and Ham's F-12 medium with 15% heat-inactivated fetal calf serum, nonessential amino acids, 100 IU penicillin, and 100 μg/ml streptomycin. Once a week, cells were detached from the flask with PBS containing 0.05% trypsin and 0.02% EDTA, centrifuged at 750 × *g* for 5 min, and split at a ratio of 1:3 into new flasks containing fresh medium. Medium was exchanged with fresh medium 4 days after passage. Initial experiments were with passages 20–25; however, the cells have been passaged continuously for 8 months with little change in CCK radioligand binding or CCK-induced phosphoinositide hydrolysis.

Radioligand binding assays. Cells were detached from flasks with 15 ml of PBS containing 0.02% EDTA, triturated with a pasteur pipette, and counted. After centrifugation at 750 × *g* for 5 min, the cell pellet was weighed and then homogenized using a Teflon/glass homogenizer in 30 ml of ice-cold 50 mM Tris (pH 7.4 at 23°). After centrifugation for 20 min at 40,000 × *g*, the pellet was rehomogenized in the same buffer and centrifuged again. The final pellet was homogenized in 4 ml of Buffer A (20 mM HEPES, 1 mM EGTA, 0.13 M NaCl, 5 mM $MgCl_2$, and 5 mM KCl, pH 7.4) and an aliquot was removed for protein determination (Bio-Rad Laboratories, Richmond, CA). The homogenate was further diluted [30 ml/g of cells for ^{125}I-BH-CCK8 binding; 300 ml/g of cells for (−)-[^{3}H]L-364718 binding] with Buffer B (Buffer A containing 100 μM bestatin, 3 μM phosphoramidon, and 0.1% bovine serum albumin). The pancreases from male guinea pigs (250–325 g) were homogenized in 50 mM Tris buffer containing 0.2 mg/ml soybean trypsin inhibitor followed by centrifugation for 10 min at 1000 × *g*. The supernatant was then treated as described for the CHP212 cell homogenate and was used at a dilution of 30 ml/g of wet weight for ^{125}I-BH-CCK8 assays and 1000 ml/g of wet weight for (−)-[^{3}H]L-364718 assays.

Radioligands and competing ligands were diluted in Buffer B. The (−)-[^{3}H]L-364718 binding assay consisted of 25 μl of buffer containing (−)-[^{3}H]L-364718, 25 μl of buffer with or without competing ligands, and 750 μl of the membrane homogenate. The ^{125}I-BH-CCK8 binding assay consisted of 50 μl of buffer containing ^{125}I-BH-CCK8, 50 μl of buffer with or without competing ligand, and 200 μl of membrane homogenate. The incubation was initiated by addition of the membranes and incubated at 37° for 30 min. Membrane-bound radioligand was collected by rapid filtration on glass fiber filters (No. 32; Schleicher & Schuell, Inc, Keene NH) using a Skatron cell harvester. Membranes were washed with approximately 15 ml of PBS containing 0.1% bovine serum albumin and filter-bound radioactivity was measured by liquid scintillation or γ counting. Specific binding of ^{125}I-BH-CCK8 and (−)-[^{3}H]L-364718 was defined as the difference in radioligand binding in the presence and absence of 1 μM CCK8(S) and 100 nM (−)-L-364718, respectively. At a concentration of 50 pM (used in competition experiments), nonspecific binding of ^{125}I-BH-CCK8 was approximately 25% of total binding for CHP212 membranes and approximately 5% of total binding for pancreatic membranes. At a concentration of 0.4 nM (used in competition experiments), nonspecific binding of (−)-[^{3}H]L-364718 was approximately 10% of total binding for CHP212 membranes and approximately 5% of total binding for pancreatic membranes.

Competition experiments using a wide range of concentrations indicated that 1 μM CCK8 and 100 nM (−)-L-364718 were appropriate for defining saturable and nonsaturable binding. Time course experiments indicated that the specific binding of both radioligands reached a steady state by 20 min and was constant up to 40 min. The integrity of the radioligands, as monitored by reverse phase HPLC, was greater than 90% after incubation with membranes for 30 min at 37°.

Screening of human neuroblastoma cell lines for ^{125}I-BH-CCK8 binding. Membranes from frozen cell pellets (200–400 mg of packed cells) were prepared in a manner similar to that described above for CHP212 cells and were assayed for specific binding of ^{125}I-BH-CCK8 (50 pM). Each assay tube contained the equivalent of approximately 5 mg of wet weight of cells. The presence of specific binding of ^{125}I-BH-CCK8 was determined by statistical comparison (*t* test; $p < 0.05$) of triplicate determinations of total and nonspecific binding.

Phosphoinositide hydrolysis assay. Subconfluent CHP212 cells (3–4 days after passage) were incubated at 37° with fresh culture medium containing [^{3}H]myo-inositol (2 μCi/ml) for 18 hr. Cells were detached with PBS/EDTA and washed once with Earle's balanced salt sodium (GIBCO, Grand Island, NY) containing 10 mM LiCl, 0.025% bacitracin, 0.1% bovine serum albumin, and 10 μg/ml insulin. After incubation in the same buffer for 20 min at 37°, cells were pipetted into tubes (200 μl/tube; approximately 2 × 10^5 cells/tube) containing 100 μl of buffer with test compounds. The reaction was terminated after incubation for 30 min at 37° by vortexing of the tube after addition of 1 ml of chloroform/methanol (2:1) and 0.8 ml of water. After centrifugation, 1 ml of the aqueous phase was diluted with 2 ml of water and applied to a 1-ml AG1-X8 (formate form; 100–200 mesh; Bio-Rad Laboratories) anion-exchange column. The columns were washed with 20 ml of 5 mM myo-inositol, followed by 6 ml of 60 mM sodium formate/5 mM sodium tetraborate. [^{3}H]Inositol phosphates were eluted with 5 ml of 1 M ammonium formate/0.1 M formic acid and counted by liquid scintillation counting. Preliminary experiments with [^{14}C]inositol monophosphate added to cells before addition of the chloroform/methanol gave a consistent recovery of 66–70%.

Results

Screening of human neuroblastoma cell lines. The nonselective radioligand ^{125}I-BH-CCK8 was used to test for the presence of CCK binding sites (CCK-A or CCK-B) in membranes prepared from 13 human neuroblastoma cell lines. The following cell lines lacked detectable specific binding of ^{125}I-BH-CCK8: SKN-BEZ, SKN-MC, SKN-BE1, SMS-SAN, SMS-KAN, SMS-KANR, SMS-KCN, SMS-MSN, LAN-N1, LAN-2, CHP234, SH-SY5Y, and IMR-32. These data do not preclude the possibility that these cell lines express low densities of CCK receptors, which were undetected by our procedure. Specific binding of ^{125}I-BH-CCK8 was detected in membranes from the CHP212 cell line.

Radioligand binding studies. In order to characterize the ligand specificity of CCK binding sites present in CHP212 cells, CCK-related compounds that are known to discriminate between CCK-A and CCK-B binding sites were tested for their potency to inhibit the specific binding of ^{125}I-BH-CCK8 (Table 1). For comparison, the potency of these compounds in inhibiting ^{125}I-BH-CCK8 binding to guinea pig pancreatic membranes under identical conditions was determined. In general, the K_{i50} values of compounds are greater in CHP212 membranes than in pancreatic membranes. However, the quantitative rank order of potency of the compounds is very similar in the two types of membranes and clearly differs from the relative potencies of these compounds in inhibiting ^{125}I-BH-CCK8 binding in brain membranes (8, 9, 20, 21). These results indicate that CCK binding sites in the CHP212 cells can be classified as CCK-A binding sites.

TABLE 1

Binding affinities of CCK-related compounds

Specific binding in the presence of 12 concentrations at quarter log intervals was determined in triplicate. The IC_{50} was determined by log-logit analysis and the K_i was computed using the equation:

$$K_i = \frac{IC_{50}}{1 + ([L]/K_d)}$$

Mean K_d values given in Table 2 were used in the calculation. The concentration of ^{125}I-BH-CCK8 was 50 pM and the concentration of (−)-[^{3}H]L-364718 was 400 pM. Except where noted, the computed log-logit slopes ranged from 0.90 to 1.1. For all compounds, the maximal inhibition of specific binding was greater than 90%. Values are the mean ± standard error from three experiments.

Compound	IC_{50}	
	CHP212 Cells	Guinea pig pancreas
	nM	
^{125}I-BH-CCK8 binding		
CCK8	1.9 ± 0.8	0.21 ± 0.03
CCK8(DS)	600 ± 20	27 ± 5
BOC-CCK4	11000 ± 3900	5600 ± 2600
(−)-L-364718	0.62 ± 0.20	0.27 ± 0.08
(−) -[^{3}H]L-364718 binding		
(−)-L-364718	0.053 ± 0.001	0.044 ± 0.002
CR-1409(*S*)	65 ± 19	21 ± 4
CR-1409(*R*)	1600 ± 100	480 ± 20
CCK8	58 ± 11[a]	1.9 ± 0.9[a]

[a] Log-logit slope was significantly less than 1 (CHP212 cells = 0.48 ± 0.08; guinea pig pancreas = 0.45 ± 0.05).

The racemic radioligand (±)-[^{3}H]L-364718 has been reported to selectively bind to CCK-A binding sites in pancreas and gall bladder (22) and in specific areas of the central nervous system (15, 16). (+)-L-364718 has been reported to have greater than 100-fold less affinity for CCK-A binding sites than (−)-L-364718 (22). However, (+)-L-364718 still has relatively high affinity (4–8 nM) for CCK-A sites. The problems associated with the analysis of binding data using racemic radioligands and stereoselective binding sites have been previously discussed (23). We, therefore, have utilized the higher affinity isomer, (−)-[^{3}H]L-364718, for studies of CCK-A binding sites in CHP212 cells and pancreas.

Competition studies with unlabeled (−)-L-364718 and (−)-[^{3}H]L-364718 demonstrated that the binding of the radioligand to membranes from both CHP212 cells and guinea pig pancreas was saturable and of high affinity (Table 1). The binding site labeled by (−)-[^{3}H]L-364718 in membranes from CHP212 cells and guinea pig pancreas displayed similar stereoselectivity for the enantiomers of the CCK antagonist CR-1409 (Table 1). We have confirmed the observation (22) that the competition curve for CCK8 versus radiolabeled L-364718 in pancreatic membranes is shallow and demonstrated that this is also true in CHP212 cell membranes (Table 1).

It has been previously reported that the total number of binding sites labeled by (−)-[^{3}H]L-364718 is substantially greater than the number of sites for ^{125}I-BH-CCK8 (22). We have confirmed this observation in pancreatic membranes with the pure enantiomer (−)-[^{3}H]L-364718. The B_{max} for (−)-[^{3}H]L-364718 was 6.6-fold greater than the B_{max} for ^{125}I-BH-CCK8 (Table 2). For both radioligands, there was no indication of heterogeneity of binding sites because analysis according to a two-site model using the LIGAND program (23) did not significantly reduce the sum of squares of the residuals (n = 3). Representative Scatchard plots of these data are shown in Fig. 1.

On the basis of membrane protein, CHP212 cells have a lower density of CCK binding sites than guinea pig pancreatic membranes (Table 2). Similar to pancreatic membranes, the B_{max} for (−)-[^{3}H]L-364718 was substantially greater (17-fold) than the B_{max} for ^{125}I-BH-CCK8. A density for (−)-[^{3}H]L-364718 binding sites was calculated to be 29400 ± 2500 receptors/cell whereas the density of ^{125}I-BH-CCK8 binding sites was 1900 ± 370 receptors/cell. Computerized analysis of the binding isotherms indicated that there was no evidence for binding site heterogeneity for either radioligand (n = 3). Representative Scatchard plots are shown in Fig. 1. The K_d of ^{125}I-BH-CCK8 was significantly higher in CHP212 membranes, compared with pancreatic membranes (t test; $p < 0.05$), whereas the K_d for (−)-[^{3}H]L-364718 was not different between the two types of membranes (Table 2).

TABLE 2

Binding parameters of ^{125}I-BH-CCK8 and (−)-[^{3}H]L-364718 to membranes from CHP212 cells and guinea pig pancreas

Specific binding was determined at various concentrations of the radioligands and the data were analyzed by nonlinear least squares curve fitting according to the law of mass action [LIGAND program (24)]. Values are the mean ± standard error of three experiments. Representative Scatchard plots are shown in Fig. 1.

	^{125}I-BH-CCK8	(−)-[^{3}H]L-364718
CHP212 cells		
K_d (pM)	540 ± 100	28 ± 7
B_{max} (fmol/mg of protein)	110 ± 40	1900 ± 370
Guinea pig pancreas		
K_d (pM)	150 ± 20	21 ± 1
B_{max} (fmol/mg of protein)	540 ± 130	3550 ± 60

The specific binding of ^{125}I-BH-CCK8, but not (−)-[^{3}H]L-364718, was reduced by the stable guanine nucleotide Gpp(NH)p (Fig. 2). The maximal inhibition of ^{125}I-BH-CCK8 binding was >90%. Similar results have been previously reported in guinea pancreatic membranes (22).

Phosphoinositide hydrolysis studies. Activation of CCK-A receptors in pancreatic acinar cells leads to the stimulation of phosphoinositide hydrolysis (12, 13). In CHP212 cells prelabeled with [^{3}H]myo-inositol, CCK8 stimulated the accumulation of [^{3}H]inositol phosphates. The full dose-response curve is shown in Fig. 3A. The EC_{50} was 3.2 ± 0.4 nM and the maximum response was 4.5 ± 0.3-fold above the basal level of [^{3}H]inositol phosphate accumulation (four experiments). The dose response curve was shallow with a log-logit slope of 0.45.

CCK8 stimulation of phosphoinositide hydrolysis was inhibited by the CCK-A receptor-selective antagonists (−)-L-364718 and CR-1409 (Fig. 3B). The IC_{50} values for (−)-L-364718 and CR-1409 were 0.93 ± 0.34 nM and 360 ± 80 nM, respectively. The potency of these antagonists is similar to their reported potency in inhibiting amylase release in pancreatic ancinar cells (25–27).

The ability of several CCK analogues to stimulate phosphoinositide hydrolysis in CHP212 cells was investigated (Fig. 4). In order to ascertain the maximal effects of these analogues, a concentration of 100 μM was used. This concentration is 10 to 1000 times greater than the IC_{50} of these compounds in inhibiting ^{125}I-BH-CCK8 binding to CCK-A binding sites in CHP212 or guinea pig pancreas membranes (13).[1] Furthermore, 100 μM concentrations of these CCK analogues have been reported to elicit full or nearly full agonist effects in phosphoinositide hydrolysis assays in pancreatic acinar cells (13). In the CHP212 cells phosphoinositide hydrolysis assay, CCK7 had approximately the same maximal effect as CCK8. However, removal

[1] Unpublished data.

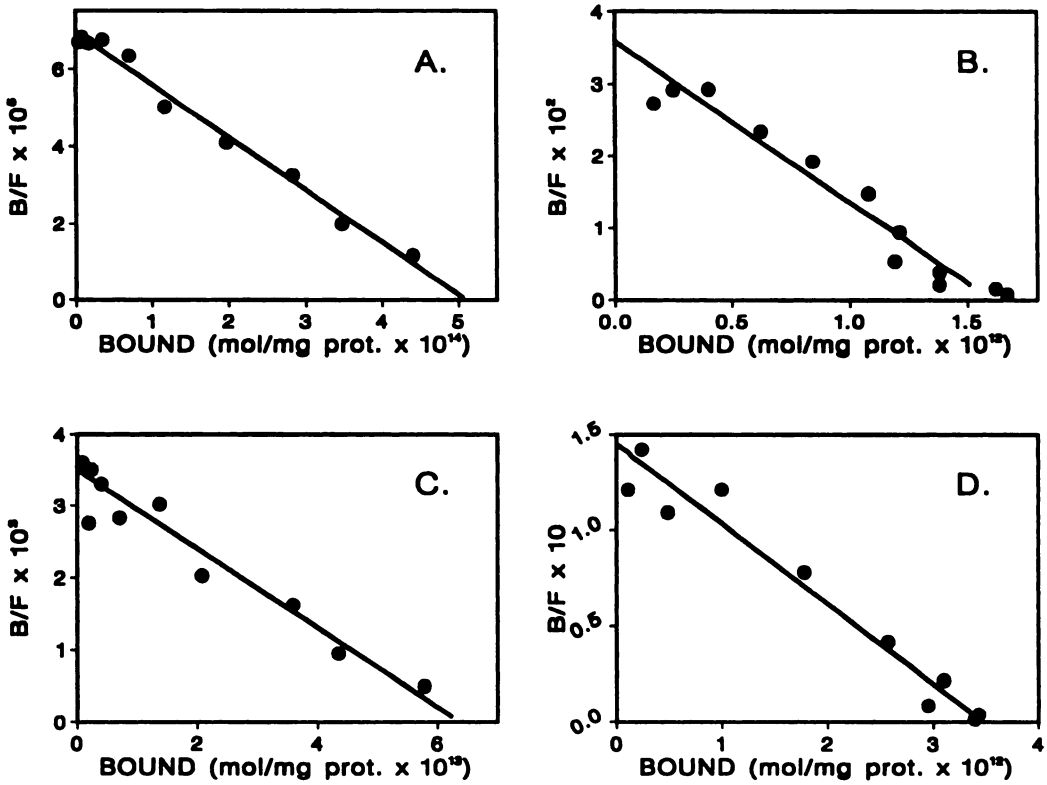

Fig. 1. Representative Scatchard plots of ^{125}I-BH-CCK8 and (−)-[^{3}H]L-364718 binding to membranes from CHP212 cells and guinea pig pancreas. Details of the experiment are given in Table 3. A, ^{125}I-BH-CCK8 binding in CHP212 membranes. B, (−)-[^{3}H]L-364718 binding in CHP212 membranes. C, ^{125}I-BH-CCK8 binding in guinea pig pancreatic membranes. D, (−)-[^{3}H]L-364718 binding in guinea pig pancreatic membranes.

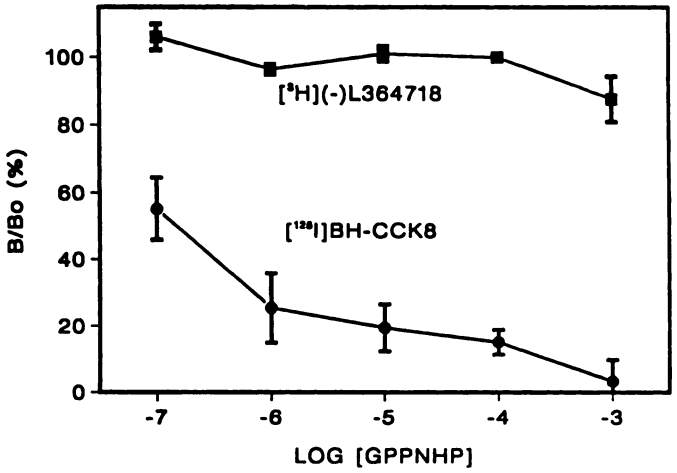

Fig. 2. Effect of Gpp(NH)p on ^{125}I-BH-CCK8 and (−)-[^{3}H]L-364718 binding to membranes from CHP212 cells. The concentration of ^{125}I-BH-CCK8 and (−)-[^{3}H]L-364718 were 50 pM and 400 pM, respectively. Results are expressed as a percent of specific binding in the absence of Gpp(NH)p and are the mean ± standard error of three experiments, each performed in triplicate.

of the sulfate from CCK8 and CCK7 caused a dramatic reduction in the maximal response. BOC-CCK6, BOC-CCK5, and BOC-CCK4 had maximal effects that were less than 10% of the CCK8 response. In order to establish that metabolic instability, absorptive losses, or unexpected low binding affinity in whole cells were not the reasons for the very low response of BOC-CCK4, we tested it as an antagonist of CCK8-stimulated phosphoinositide hydrolysis. Fig. 3B shows that BOC-CCK4 antagonizes the CCK8 response with an IC_{50} of approximately 20 μM. We conclude that BOC-CCK4 has little intrinsic activity in the phosphoinositide hydrolysis assay.

Discussion

Continuous cell lines that express receptors of interest have proven to be extremely useful tools in studying cellular events associated with receptor activation. For this reason, we tested a number of human neuroblastoma cell lines for the presence of CCK binding sites. The CHP212 cell line was the only one in which specific binding of ^{125}I-BH-CCK to membranes was detected. We have shown that CCK binding sites in CHP212 cells are of the CCK-A subclass. We have directly compared the binding of ^{125}I-BH-CCK8, an agonist, and (−)-[^{3}H]L-364718, a CCK antagonist, to membranes prepared from CHP212 cells and guinea pig pancreas. In both cases, the calculated B_{max} for (−)-[^{3}H]L-364718 was substantially greater than the B_{max} for ^{125}I-BH-CCK8. In addition, we showed that, similar to what has been reported in pancreas (6, 22), the binding of the ^{125}I-BH-CCK8, but not (−)-[^{3}H]L-364718, was reduced by the guanine nucleotide Gpp(NH)p. These observations are consistent with the ternary complex model proposed for other G protein-linked receptors where the affinity of the receptor for agonist is dependent on receptor/G protein coupling (28, 29). Finally, we have demonstrated that CCK agonists stimulate phosphoinositide hydrolysis in CHP212 cells and have characterized the requirements for agonism using a series of truncated CCK8 analogs.

Radioligand binding studies have shown that the vast majority of CCK binding sites in membranes prepared from brain

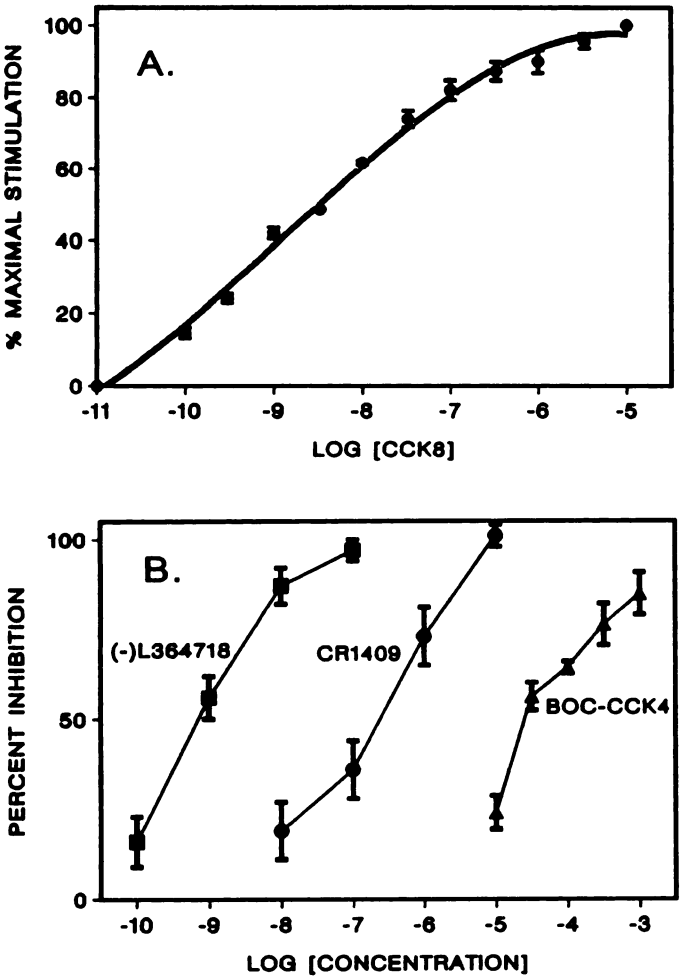

Fig. 3. Characterization of CCK8 stimulation of phosphoinositide hydrolysis in CHP212 cells. A, CCK8 concentration-response curve. For each experiment, results were calculated as the percentage of the stimulation over the basal response that occurred with 10 μM CCK8. Individual experiments were analyzed with the ALLFIT program (36). Results are given in the text. The *points* represent the mean ± standard error of 4 experiments, each done in triplicate. B, Inhibition of CCK8 (10 nM) response with CCK-A receptor-selective antagonists. Each concentration of antagonist was added to CHP212 cells simultaneous to the addition of 10 nM CCK8. The results were calculated as the percentage of inhibition of the response of 10 nM CCK8 alone. Individual experiments were analyzed with the ALLFIT program (36) and results are given in the text. The *points* represent the mean ± standard error of four experiments, each done in triplicate.

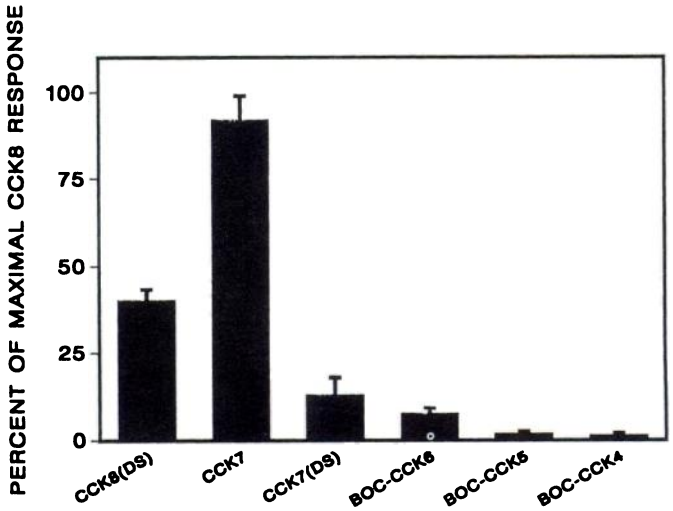

Fig. 4. Stimulation of phosphoinositide hydrolysis in CHP212 cells by C-terminal fragments of CCK8. All CCK8 fragments were tested at a concentration of 100 μM. In individual experiments, results were calculated as the percentage of the response to 10 μM CCK8. The *bars* represent the mean ± standard error for three experiments, each done in triplicate.

(CCK-B sites) differ in their ligand specificity from CCK binding sites in pancreas, gall bladder, and gut smooth muscle (CCK-A sites) (8–10, 21). Neuroblastoma tumors are believed to be derived from cells of neural crest origin and the neuronal phenotype of CHP212 cells is indicated by the presence of catacholaminergic and cholinergic synthetic enzymes and neuronal cell surface antigens in this cell line (29, 30). It, therefore, may be surprising that CHP212 cells express CCK-A sites. However, recently it has become clear that low densities of CCK-A sites are present in neuronal tissue. Autoradiography studies have allowed the detection of CCK-A sites in rat area postrema and intrapenduncular nucleus (11, 15–17), monkey spinal cord (18), and rat vagal nerve (31). Membrane binding studies using radiolabeled L-364718 have also documented the presence of type A sites in discreet brain areas (15).[2] Although it is not definitively known in what cell type (neuronal or glial) the CCK-A sites within the central nervous system are located, it is tempting to speculate that CHP212 cells may be a general model system for investigations of CCK-A receptors on cells with a neuronal phenotype.

CCK stimulation of phosphoinositide hydrolysis has been reported in the human embryonic pituitary cell line FLOW 900 (32) and in gastric mucosal cells (33, 34) but the receptor subtype involved has not been well characterized. CCK-A receptor-mediated stimulation of phosphoinositide hydrolysis has been well characterized in pancreatic acinar cells (12, 13). Thus, it appears that, in at least two types of cells, CCK-A receptors are linked via a G protein to phospholipase C. In acinar cells, stimulation of phosphoinositide hydrolysis is believed to play a critical role in the secretory response to CCK. We have recently observed that CCK elevates the intracellular concentration of calcium in CHP212 cells.[1] It will be interesting to see whether a similar signal transduction scheme is associated with CCK-A sites in brain. Of interest is the recent report that CCK facilitation of stimulated dopamine release from nucleus accumbens is mediated by CCK-A receptors (35). Whether CCK stimulation of phosphoinositide hydrolysis and regulation of intracellular calcium concentration play a role in regulating neurotransmitter release remains to be elucidated.

An important question is whether CCK-A receptors in CHP212 cells are different from CCK-A receptors in peripheral tissues such as pancreas. Using membrane binding studies, we have directly compared the binding affinities of several CCK-related compounds for CCK-A sites in CHP212 cells and guinea pig pancreas. Some differences in the affinity of compounds for ^{125}I-BH-CCK8 or (−)-[^{3}H]L-364718 binding sites were noted, particularly for the agonist CCK8. However, the rank order of the potency of the tested compounds was similar in CHP212 cells and pancreas and the CCK-A sites from both sources displayed an almost identical stereoselectivity for the stereoiso-

[2] Barrett *et al.*, manuscript in preparation.

mers of CR-1409. Until more compounds are tested, we believe that it is premature to draw conclusions about whether CCK-A sites in CHP212 cells differ from pancreatic CCK-A sites in terms of ligand-binding specificity.

It is worth noting that the dose-response curve for CCK8 was shallower than would be expected if occupancy of a single receptor type was directly proportional to amount of phosphoinositide hydrolysis. One possible explanation for this observation is that CCK8 elicits its response in whole CHP212 cells through interaction with more than one type of receptor that differ in their affinity for CCK8. The fact that CCK-A-selective antagonists L-364718 and CR-1409 fully inhibit the CCK8 response suggests that CCK-B receptors, present in levels that were not detected in membrane binding studies, were not involved in CCK8-stimulated phosphoinositide hydrolysis in CHP212 cells. We attempted to conduct radioligand binding studies using whole CHP212 cells to address the possibility that variant types of CCK-A receptors were expressed by CHP212 cells, but one type was lost during the preparation of membranes. However, we were unable to reliably detect specific ^{125}I-BH-CCK binding in whole cells and detailed binding studies with (−)-[^{3}H]L-364718 were not ideal because of the high amount of nonspecific binding, possibly due to the internalization of this lipophilic ligand (data not shown). Therefore, our data are insufficient for drawing conclusions about multiple types of CCK-A receptors being involved in CCK8 stimulation of phosphoinositide hydrolysis. An alternative hypothesis regarding the nature of the shallow dose-response curve for CCK8 is that it is somehow a manifestation of high and low agonist affinity states, which we have postulated to exist in membranes. Pertinent to this hypothesis is the observation that the competition curve of CCK8 for sites labeled by (−)-[^{3}H]L-364718 in CHP212 membranes also has a shallow slope. Further studies are clearly needed to address the reason for the shallow dose-response curve for CC8-stimulated phosphoinositide hydrolysis.

We have examined the ability of several CCK-related compounds to mimic or antagonize the ability of CCK8 to stimulate phosphoinositide hydrolysis in CHP212 cells. As mentioned above, the potency of (−)-L-364718 and CR-1409 are consistent with the hypothesis that the response to CCK8 is mediated via an interaction with the CCK-A sites characterized in membrane binding studies. Various peptide fragments of CCK8 were also tested to determine the structural requirements for activity in the phosphoinositide hydrolysis assay. It is clear that sulfation of the tyrosine in CCK8 and CCK7 is important for maximal activity. Truncation from the N-terminus beyond the tyrosine leads to a dramatic decrease in activity. We believe that all the peptide fragments were tested at a concentration (100 μM) that fully occupies CCK-A receptors and, thus, the reported activities reflect the intrinsic activity of these compounds in stimulating phosphoinositide hydrolysis. In the case of BOC-CCK4, we showed that this peptide fragment behaved as an antagonist. These data are clearly different from what has been reported for the same compounds in pancreatic acinar cells, where all of the CCK fragments are full agonists (13). The disparity in intrinsic activity of CCK compounds in the two cell types could be a consequence of differences in the receptor reserve, i.e., the intrinsic efficacy of nonsulfated CCK fragments at CCK-A receptors is low but they behave as full agonists in pancreatic acinar cells because of highly efficient receptor coupling and/or a large percentage of spare receptors. The fact that the maximal response to partial agonists is dependent on tissue/cell receptor reserve has been documented in many systems (37). We are currently undertaking experiments designed to examine this possibility. Alternatively, the difference may be attributable to receptor heterogeneity with regard to the structural requirements for agonism. Ultimately, this issue will be resolved by identification of CCK receptor gene(s) and expression and characterization of recombinant receptor(s) in foreign cells.

In summary, CHP212 cells express CCK-A receptors that are associated with a G protein. Activation of these receptors leads to an increase in phosphoinositide hydrolysis. Future investigations with this cell line may prove useful in establishing additional cellular events associated with CCK-A receptor activation in neuronal cells and determining whether these CCK-A receptors are identical to those found in nonneuronal tissues.

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