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Characterization of the Binding of [^{3}H]L-365,260: A New Potent and Selective Brain Cholecystokinin (CCK-B) and Gastrin Receptor Antagonist Radioligand

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SUMMARY

[^{3}H]L-365,260, [(3*R*-(+)-2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl)-*N'*-(3-methylphenyl)urea], a new potent and selective nonpeptide brain cholecystokinin (CCK-B) and gastrin receptor antagonist, bound saturably and reversibly to guinea pig brain membranes. Scatchard analysis indicated a single class of high affinity (K_d = 2.3 nM) binding sites. The binding of [^{3}H]L-365,260 was stereospecific, because unlabeled L-365,260 (an *R*-enantiomer) was approximately 100 times more potent than its *S*-enantiomer in displacing binding. The relative potencies of various CCK/gastrin-related peptides and nonpeptide peripheral CCK-A antagonists in displacing [^{3}H]L-365,260 brain binding correlated with their potencies in displacing the binding of ^{125}I-CCK to brain receptors but not their potencies in displacing the peripherally selective CCK-A ligand [^{3}H]L-364,718 from pancreatic receptors. The regional distribution of [^{3}H]L-365,260 binding in various brain areas correlated with ^{125}I-CCK binding. Specific [^{3}H]L-365,260 binding to guinea pig brain membranes was reduced by omission of NaCl but was not affected by omission of $MgCl_2$ or addition of guanosine 5'-(β-γ-imido)triphosphate or various pharmacological agents known to interact with other common peptide and nonpeptide receptor systems. [^{3}H]L-365,260 also bound in a specific manner to guinea pig gastric glands but only negligibly to guinea pig or rat pancreas. The binding of [^{3}H]L-365,260 to gastric glands was inhibited by CCK/gastrin antagonists with potencies similar to those for inhibition of ^{125}I-gastrin binding in this tissue. Collectively, the data indicates that [^{3}H]L-365,260 represents a new potent nonpeptide antagonist radioligand suitable for the study of brain CCK-B and gastrin receptors.

CCK is a recognized peptide hormone and proposed neurotransmitter that is found in the gut and central nervous system (1). Distinct CCK receptors in peripheral (CCK-A) and brain (CCK-B) tissues (2) have been characterized using radiolabeled CCK analogues (3–7). The development of the potent peripherally selective antagonist L-364,718 (8, 9) has made possible the characterization of the binding of a radiolabeled nonpeptide antagonist ligand to membrane-bound and detergent-solubilized peripheral CCK-A receptors (10, 11). However, neither nonpeptide radioligands, antagonist radioligands, nor selective radioligands are available for study of brain CCK-B or gastrin receptor interactions.

Recently, L-365,260 [(3*R*-(+)-2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl)-*N'*-(3-methylphenyl)-urea],[1] a potent and selective antagonist for brain CCK-B and gastrin receptors, was developed in our laboratories (12, 13). In the present studies, the binding of [^{3}H]-L-365,260 (Fig. 1) to guinea pig brain CCK-B receptors is characterized. The specific binding of [^{3}H]L-365,260 to gastrin receptors in guinea pig gastric glands and its relative inability to bind to CCK-A receptors in guinea pig or rat pancreas are also reported.

[1] L-365,260 is used in the text to designate the 3*R*-enantiomer unless otherwise indicated.

Materials and Methods

Radioligands. ^{125}I-CCK-8 (2200 Ci/mmol) and ^{125}I-gastrin (2200 Ci/mmol) were purchased from New England Nuclear (Boston, MA). [^{3}H]L-365,260 was prepared, as outlined in the scheme, according to the following three-step procedure (Fig. 1).

Preparation of [^{3}H]-*m*-toluidine (2). 2,4,6-Tribromo-3-methylaniline (1) (34 mg; 0.1 mmol), in 2 ml of ethyl acetate, was treated with triethylamine (0.1 ml) and 5% Pd/C catalyst (30 mg) and was stirred under a tritium atmosphere (1 atm) for 45 min (14). The mixture was filtered and acidified with trifluoroacetic acid (0.3 ml). Solvent and unreacted tritium were removed by concentrating the reaction mixture and redissolving the residue in ethanol. This cycle was repeated twice more. The product was stored in an 80% ethanol/water mixture (25

ABBREVIATIONS: CCK, cholecystokinin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis-(β-aminoethylether)-*N,N,N',N'*-tetracetic acid; Gpp(NH)p, guanosine 5'-(β-γ-imido)triphosphate; CCK-ds, cholecystokinin octapeptide desulfate.

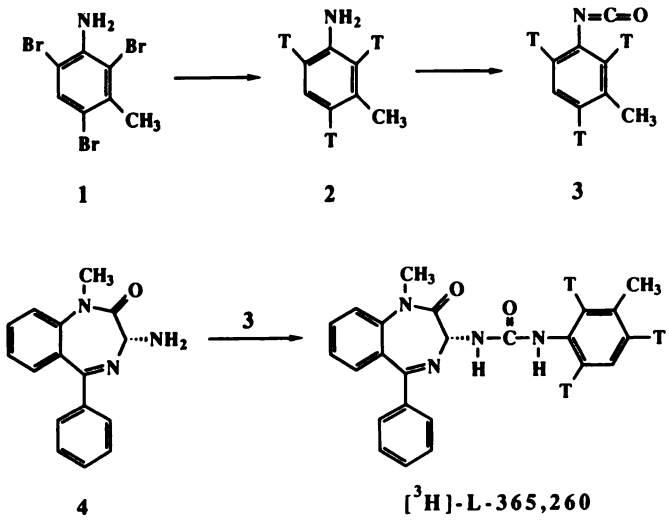

Fig. 1. Synthesis of [^{3}H]L-365,260. *T*, tritium.

ml) that contained sodium bisulfite (50 mg). Based on the total radioactivity (7.2 Ci), the nominal specific activity of the *m*-toluidine thus prepared was 72 Ci/mmol.

Preparation of [^{3}H]-*m*-tolylisocyanate (3). A solution of the [^{3}H]*m*-toluidine trifluoroacetate salt (see above) (3.5 ml; 1.0 Ci; approximately 14 μmol) was evaporated to dryness and the resulting residue was treated with 2 N sodium hydroxide solution (0.3 ml). Extraction with benzene yielded 700 mCi (approximately 10 μmol) of the free base. The solution was evaporated and the residue was dissolved in dry toluene (0.13 ml) to which was added, in succession, a solution of phosgene in toluene (approximately 12% by weight; 30 μl) and 30 μl of tetrahydrofuran. The resulting mixture was stirred at room temperature overnight and then treated with triethylamine (6 μl; 5.7 μmol). After 1 hr, the supernatant was separated from the deposited triethylamine hydrochloride with a fine-tipped pipette and was used directly in the next step.

Preparation of [^{3}H]L-365,260. To the solution of [^{3}H]*m*-tolylisocyanate obtained above was added a solution of tetrahydrofuran (1 ml) that contained (3*R*)-amino-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one (4) (3.0 mg; 7.6 μmol). The reaction mixture was stirred for 40 min and then applied directly to a preabsorbent Analtech silica gel plate (20 × 20 cm × 5 mm; 15% ethyl ether in methylene chloride elution). The product band was eluted with ethyl acetate to yield 60 mCi of crude [^{3}H]L-365,260. A 40 mCi portion was further purified by high performance liquid chromatography (Zorbax ODS semipreparative column; 6 ml/min; 210 nm detection; solvents: A, acetonitrile; B, 0.1% phosphoric acid in water; gradient, 35–80% A over 15 min, employing multiple injections, to afford 20 mCi of greater than 99% pure [^{3}H]L-365,260 with a specific activity of 76.7 Ci/mmol. The free base of [^{3}H]L-365,260 was prepared by adding a slight excess of ammonium hydroxide to the acetonitrile solution and extracting with ethyl acetate. The ethyl acetate was replaced with methanol and the product was stored at −60°.

Radioligand binding assays. Membranes from male guinea pig (Hartley) cerebral cortex or other brain areas (in regional distribution studies) and rat (Sprague Dawley) or guinea pig pancreas were prepared, as described previously (10), by homogenization in 50–100 volumes of Tris·HCl (pH 7.4 at 37°) using a Polytron (Brinkman PT 10, setting 4 for 10 sec). Homogenates were centrifuged at 50,000 × *g* for 10 min and the pellets were resuspended in the same buffer and centrifuged as above. The resulting pellets from brain were routinely resuspended in 160 and 320 volumes (unless stated otherwise) of binding assay buffer (10 mM HEPES, 5 mM $MgCl_2$, 1 mM EGTA, 130 mM NaCl, and 0.25 mg/ml bacitracin, pH 6.5) for [^{3}H]L-365,260 and ^{125}I-CCK-8, respectively. The membrane pellets from guinea pig or rat pancreas were resuspended in 50–400 volumes of the buffer. ^{125}I-CCK-8 binding in brain was performed as described previously (8). To measure [^{3}H]L-365,260 binding in brain or pancreatic membranes, 0.5 ml of tissue was added to triplicate tubes that contained 10 μl of either buffer (for total binding), unlabeled L-365,260 (1 μM final concentration, for nonspecific binding), or displacers (at the desired final concentrations) and 10 μl of [^{3}H]L-365,260 (1 nM final concentration, unless indicated otherwise). After incubation at 25° for 90 min (various time intervals were used in association rate studies), the incubation mixtures were filtered through glass-fiber GF/C filters and washed immediately four times with 4 ml of ice-cold binding assay buffer (without bacitracin). The radioactivity trapped on the filters was counted by liquid scintillation. Specific binding was defined as the radioactivity bound, after subtracting nonspecific binding determined in the presence of 1 μM L-365,260.

[^{3}H]L-365,260 binding to dispersed guinea pig gastric glands (prepared as described in Refs. 15 and 16) was determined similarly as for brain tissue, with modifications noted below. The prepared glands were resuspended in 200 volumes of the same binding buffer used for ^{125}I-gastrin binding (130 mM NaCl, 12 mM $NaHCO_3$, 3 mM NaH_2PO_4, 3 mM Na_2HPO_4, 3 mM K_2HPO_4, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM glucose, 4 mM glutamine, 50 mM HEPES, and 0.25 mg/ml bacitracin). The [^{3}H]L-365,260 binding reaction was performed in quadruplicate at 25° for 30 min. [^{125}I]-Gastrin binding to guinea pig gastric glands was performed as described previously (15, 16).

(±)-[^{3}H]L-365,718 binding to guinea pig pancreas was performed according to our previously published method (10).

Results

Tissue concentration linearity. Specific [^{3}H]L-365,260 binding increased linearly with the concentration of cerebral cortex, up to 6.25 mg/ml (data not shown). A tissue concentration of 6.25 mg/ml was subsequently used for routine binding assays.

Saturation analysis of [^{3}H]L-365,260 binding. The binding of [^{3}H]L-365,260 to guinea pig cerebral cortex was saturable (Fig. 2A). The ratio of total [^{3}H]L-365,260 binding to nonspecific binding was about 3 at a [^{3}H]L-365,260 concentration of 1 nM, which was used for routine binding assays. Scatchard analysis (17) of specific [^{3}H]L-365,260 binding at various concentrations (0.125–8 nM) of [^{3}H]L-365,260 indicated a single class of binding sites with a dissociation constant of 2.3 ± 0.26 nM (Fig. 2B). The maximal number of binding sites for specific [^{3}H]L-365,260 binding was 5.5 ± 0.44 pmol/g of tissue. The maximal number of binding sites for [^{3}H]L-365,260 was not significantly different ($p > 0.05$) from the maximal number of binding sites determined using ^{125}I-CCK-8 (13.7 ± 4.2 pmol/g of tissue; data not shown). A Hill plot (18) of the [^{3}H]L-365,260 binding data gave a Hill coefficient of 0.98 ± 0.03, indicating a single class of binding sites and the absence of positive or negative cooperative interaction (Fig. 2C).

Kinetics of [^{3}H]L-365,260 binding. The specific binding of [^{3}H]L-365,260 to guinea pig cerebral cortical membranes was time dependent, reaching steady state in approximately 5 min (Fig. 3). The calculated association rate constant (k_1) was 1.3 ± 0.045 min^{-1} nM^{-1}. The rate of dissociation was examined by incubating membranes with [^{3}H]L-365,260 to equilibrium and then adding 1 μM unlabeled L-365,260 to prevent rebinding of dissociated [^{3}H]L-365,260. The remaining bound [^{3}H]L-365,260 was measured at different time intervals (Fig. 4A). When plotted on a semilogarithmic scale, the dissociation was linear, indicating a first-order process (Fig. 4B). The dissociation rate constant (k_{-1}) was calculated to be 0.58 ± 0.03 min^{-1}. The dissociation constant determined from the ratio k_{-1}/k_1 was

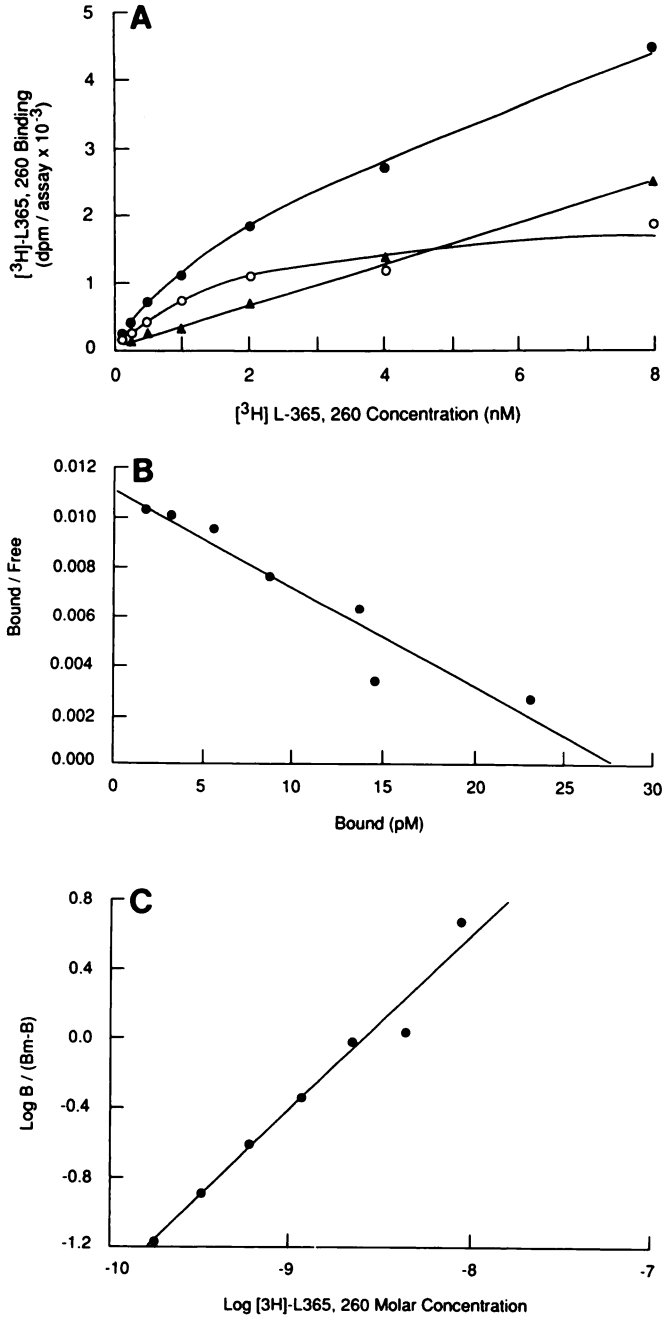

Fig. 2. [^{3}H]L-365,260 binding as a function of increasing concentrations of [^{3}H]L-365,260. The binding assay was performed as described in Materials and Methods, using various concentrations of [^{3}H]L-365,260 (0.125–8 nM). The *points* shown are means of triplicate determinations, which varied less than 25% in each experiment. The experiments were replicated four times with similar results. A, ●, Total binding; ▲, nonspecific binding; ○, specific binding. Nonspecific binding was defined using 1 μM unlabeled L-365,260. Specific binding is the difference between total and nonspecific binding. B, Scatchard plot. The mean (± standard error) K_d value and maximal number of binding sites from the four experiments are given in the text. C, Hill plot. In each of the four experiments, the slope of the Hill plot approximated unity; the mean ± standard error was 0.98 ± 0.03. B, binding; Bm, maximal binding.

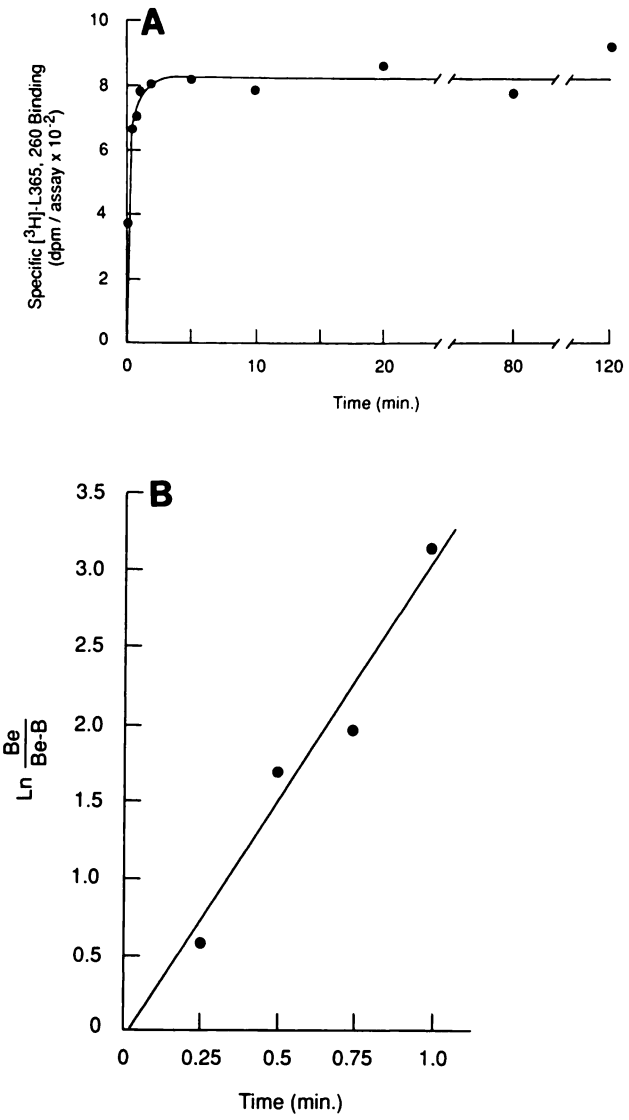

Fig. 3. Time course of association of [^{3}H]L-365,260 binding. The association of [^{3}H]L-365,260 binding to cerebral cortical membranes (6.25 mg/ml original tissue wet weight) was determined at various time intervals, as described in Materials and Methods. Specific binding was defined as the difference between binding obtained in the presence and absence of 1 μM unlabeled L-365,260. The *points* shown are those obtained in a single experiment, performed in triplicate. The experiments were replicated four times with similar results. A, Specific [^{3}H]L-365,260 binding as a function of time. B, Pseudo-first-order kinetic plots of initial specific [^{3}H]L-365,260 binding. On the ordinate, B is the amount of specific [^{3}H]L-365,260 binding at various times and B_e is the amount of specific binding at equilibrium. THe slope of the plot is the observed rate constant (k_{ob}) for the pseudo-first-order reaction. The second-order association rate constant k_1, calculated from $k_1 = (k_{ob} - k_{-1})/[[^3H]L\text{-}365,260]$, was 1.3 ± 0.42 min^{-1} nM^{-1}. k_{-1} is the first-order rate constant for dissociation (from Fig. 4) and [[^{3}H]L-365,260] is the concentration of the radioligand (1 nM).

0.5 nM, slightly lower than the dissociation constant determined in equilibrium studies.

Regional brain distribution of specific [^{3}H]L-365,260 binding: comparison with ^{125}I-CCK-8 binding. Previous studies (3) have indicated variations in the regional distribution of ^{125}I-CCK-33 binding in guinea pig brain. We have confirmed these findings using ^{125}I-CCK-8 and compared the results with data obtained using [^{3}H]L-365,260 (Table 1). The rank orders of distribution of specific [^{3}H]L-365,260 binding and ^{125}I-CCK-8 binding in guinea pig brain regions were similar (olfactory bulb > cerebral cortex > cerebellum > striatum > hippocampus > midbrain > hypothalamus = pons-medulla oblongata) (Table 1).

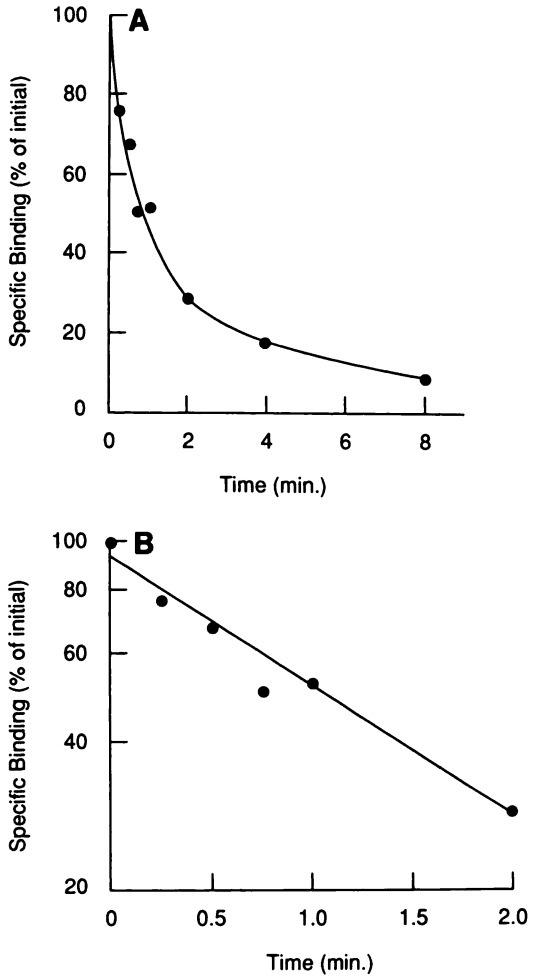

Fig. 4. Dissociation of specific [^{3}H]L-365,260 binding to guinea pig cerebral cortical membranes. Specific [^{3}H]L-365,260 binding assays were performed as described in Materials and Methods. The *points* shown are obtained in a single experiment, performed in triplicate. The experiments were replicated four times with similar results. For dissociation studies, [^{3}H]L-365,260 was first allowed to associate for 90 min at 25°, whereupon 1 μM unlabeled L-365,260 was added to prevent rebinding of dissociated [^{3}H]L-365,260. The dissociation reaction was measured at various time intervals after the addition of unlabeled L-365,260 by rapid filtration, as described in Materials and Methods. A linear plot (A) and a semilogarithmic plot (B) of B/B_e versus t, where B_e and B are binding at equilibrium and time t and t is the time after the addition of excess unlabeled L-365,260, are shown. The dissociation rate constant calculated from the formula $k_{-1} = 2.3 \times$ slope was 0.58 ± 0.03 min^{-1}.

Effect of CCK agonists and antagonists on specific [^{3}H]L-365,260 binding in guinea pig brain. Specific [^{3}H]L-365,260 binding to guinea pig brain membranes was inhibited by both L-365,260 (*R*-enantiomer) and its *S*-enantiomer (Table 2). However, the affinity of L-365,260 was approximately 100 times greater than that of *S*-enantiomer, thus demonstrating the stereoselectivity of bound radioligand. The relative potencies of nonpeptide peripheral CCK-A antagonists [asperlicin (7), L-364,718 (8), and CR-1409 (8, 19)] in displacing [^{3}H]L-365,260 brain binding correlated with their potencies in displacing the binding of ^{125}I-CCK to brain receptors but not their potencies in displacing the peripherally selective CCK-A ligand [^{3}H]L-364,718 (10) from pancreatic receptors (Table 2). The K_i values of all CCK antagonists for inhibiting [^{3}H]L-365,260 binding were similar to the K_i values for inhibiting ^{125}I-CCK

TABLE 1

Regional brain distribution of specific [^{3}H]L-365,260 and ^{125}I-CCK-8 binding

Specific binding was measured using 1 and 0.012 nM concentrations of [^{3}H]L-365,260 and ^{125}I-CCK-8, respectively, and 3.1 and 1.55 mg (original wet weight) of tissue. The data are expressed as per cent binding relative to the olfactory bulb, which bound the highest concentration of the ligands. The values represent the means ± standard errors of three separate experiments performed in triplicate. The total binding and nonspecific binding in the olfactory bulb were 2211 ± 104 and 709 ± 49 dpm for [^{3}H]L-365,260 and 5014 ± 179 and 219 ± 11 cpm for ^{125}I-CCK-8, respectively.

Brain Region	Binding	
	[^{3}H]L-365,260	^{125}I-CCK-8
	%	
Olfactory bulb	100 ± 10	100 ± 3.8
Cerebral cortex	80 ± 8.9	64 ± 6.1
Cerebellum	59 ± 3.4	54 ± 11
Striatum	47 ± 3.6	38 ± 3.9
Hippocampus	43 ± 2.2	32 ± 3.7
Midbrain	35 ± 3.1	20 ± 3.7
Hypothalamus	24 ± 4.4	16 ± 1.4
Pons-medulla	24 ± 3.9	16 ± 0.98

binding in guinea pig brain membranes. The K_i value of L-365,260 (1.4 nM) was also in good agreement with the K_d (2.3 nM) determined above for [^{3}H]L-365,260.

The CCK receptor agonists, including CCK-8, CCK-8-ds, gastrin, and CCK-4, were also effective in inhibiting specific [^{3}H]L-365,260 binding to guinea pig brain membranes, with relative potencies that correlate with their ability to displace brain CCK-B but not pancreatic CCK-A receptor binding (Table 2). The K_i values for CCK-8, CCK-8-ds, gastrin, and CCK-4 for inhibiting specific [^{3}H]L-365,260 brain binding were approximately 3–15-fold higher than their values for inhibiting ^{125}I-CCK-8 brain binding.

The Hill coefficients (n_H) for most of the CCK/gastrin agonists in displacing ^{125}I-CCK-8 brain binding and the Hill coefficients of most of the CCK gastrin antagonists in displacing both ^{125}I-CCK-8 and [^{3}H]L-365,260 brain binding approximated unity. In contrast, the Hill coefficients for CCK/gastrin agonists in displacing [^{3}H]L-365,260 binding appeared to deviate from unity and were significantly less than values obtained using ^{125}I-CCK-8 as a ligand (Table 2).

Differential effect of guanyl nucleotides and ions on specific ^{125}I-CCK-8 and [^{3}H]L-365,260 brain binding. Addition of Gpp(NH)p, a nonhydrolyzable guanyl nucleotide (0.3–100 μM) or omission of $MgCl_2$ from the assay buffer caused a significant reduction in specific ^{125}I-CCK-8 brain binding. However, under the same conditions, no significant effect on specific [^{3}H]L-365,260 binding was observed (Table 3). Omission of NaCl significantly reduced ^{125}I-CCK-8 and [^{3}H]L-365,260 binding in guinea pig brain membranes, by 18 and 48%, respectively. Removal of NaCl and $MgCl_2$ decreased ^{125}I-CCK-8 and [^{3}H]L-365,260 binding by 92 and 59%, respectively (Table 3).

[^{3}H]L-365,260 binding in guinea pig gastric glands and rat and guinea pig pancreas. Specific [^{3}H]L-365,260 binding was also observed in guinea pig gastric glands, another target tissue for gastrin/CCK. However, the ratio of total binding to nonspecific binding (1.54 ± 0.04) was lower than in brain tissue. The low specific binding in gastric glands precluded detailed characterization of the binding as was performed in brain tissue. However, specific [^{3}H]L-365,260 binding in guinea pig gastric glands was inhibited by L-365,260 and

TABLE 2

Displacement of specific [^{3}H]L-365,260 and ^{125}I-CCK guinea pig brain binding and [^{3}H]L-364,718 guinea pig pancreatic binding by various CCK agonists and antagonists

Values are means ± standard errors from three or four experiments performed in triplicate. K_i values were calculated according to the formula $K_i = IC_{50}/(1 + [L]/K_d)$, where $[L]$ is the radioligand concentration and K_d the dissociation constant of radioligand.

Displacers	Brain [^{3}H]L-365,260 Binding		Brain ^{125}I-CCK-8 Binding		Pancreas (±)-[^{3}H]L-364,718 Binding, K_i
	K_i	n_H	K_i	n_H	
	nM		nM		nM
Antagonists					
(*R*)-L-365,260	1.4 ± 0.28	0.71 ± 0.10	1.8 ± 0.27	0.78 ± 0.05	1,700 ± 300
(*S*)-L-365,260	150 ± 44	0.86 ± 0.11	130 ± 11	0.89 ± 0.02	2.4 ± 0.42
L-364,718	56 ± 4.4	0.72 ± 0.03	220 ± 86	0.87 ± 0.05	0.12 ± 0.04
CR-1409	720 ± 200	0.85 ± 0.03	2,000 ± 800	1.0 ± 0.05	47 ± 19
Asperlicin	>35,000		>90,000		480 ± 180
Agonists					
CCK-8	1.2 ± 0.27	0.51 ± 0.02	0.36 ± 0.05	0.91 ± 0.15	61 ± 15
CCK-ds	78 ± 22	0.50 ± 0.04	26 ± 23	0.64 ± 0.07	>600
Gastrin	31 ± 13	0.45 ± 0.06	3.4 ± 1.1	0.75 ± 0.13	>600
CCK-4	670 ± 29	0.41 ± 0.08	48 ± 11	0.83 ± 0.05	

TABLE 3

Effect of guanyl nucleotide and ions on specific [^{3}H]L-365,260 and ^{125}I-CCK-8 binding in guinea pig brain

The data are expressed as percentage of control binding in routine buffer, which contained 5 mM $MgCl_2$, 130 mM NaCl, and no Gpp(NH)p. Values are means ± standard errors from three to five experiments.

Buffer Composition			^{125}I-CCK Binding	[^{3}H]L-365,260 Binding
$MgCl_2$	NaCl	Gpp(NH)p		
mM			% of control	
5	130	0	100	100
5	130	0.0003	70 ± 4*	104 ± 5.6
5	130	0.003	64 ± 4*	110 ± 4.8
5	130	0.03	63 ± 3.8*	120 ± 12
5	130	0.1	62 ± 2.6*	126 ± 16
5	0	0	82 ± 7.0*	52 ± 1.9*
0	130	0	45 ± 0.30*	96 ± 2.5
0	0	0	7.6 ± 1.6*	41 ± 6.3*

* Significantly different than control, $p < 0.05$ (Student's t test).

TABLE 4

Displacement of specific [^{3}H]L-365,260 and ^{125}I-gastrin binding in guinea pig gastric glands by CCK/gastrin receptor agonists and antagonists

Values are means ± standard errors from at least three experiments performed in triplicate.

Displacers	IC_{50}	
	[^{3}H]L-365,260	^{125}I-Gastrin
	nM	
Antagonists		
L-365,260	2.3 ± 0.6	1.1 ± 0.4
(*S*)-L-365,260	203 ± 81	130 ± 45
L-364,718	124 ± 79	300 ± 72
CR1409	1,200 ± 510	1,900 ± 500
Agonists		
CCK-8	330 ± 230	0.9 ± 0.2
CCK-ds	12,000 ± 6,350	110 ± 45
Gastrin	300 ± 184	3 ± 1

(*S*)-L-365,260 in a stereospecific manner, with IC_{50} values of 2.3 and 203 nM, respectively (Table 4). The potencies of L-365,260, its *S*-enantiomer, L-364,718, and CR-1409 in inhibiting specific [^{3}H]L-365,260 binding were comparable to their potencies as inhibitors of specific ^{125}I-gastrin binding in this tissue (Table 4). CCK/gastrin receptor agonists, including CCK-8, CCK-8-ds, and gastrin, also inhibited specific [^{3}H]L-365,260 binding (Table 4). The IC_{50} values of CCK-8, CCK-8-ds, and gastrin for inhibition of specific [^{3}H]L-365,260 binding were 100–300-fold greater than their IC_{50} values for inhibition of ^{125}I-gastrin binding.

Only negligible amounts of specific [^{3}H]L-365,260 binding (≤25% of total binding) were detected in rat or guinea pig pancreatic membranes under conditions in which specific ^{125}I-CCK-8 binding is readily observed in our laboratories (7).

Effect of various pharmacological agents on [^{3}H]L-365,260 binding in guinea pig brain. Although the chemical structure of L-365,260 contains a benzodiazepine moiety (12), specific (±)-[^{3}H]L-365,260 binding in guinea pig brain was not affected by the central or peripheral benzodiazepine receptor ligands flunitrazepam and RO 5-4864, respectively, at concentrations up to 1 μM. Other pharmacological agents, including α- or β-adrenergic, serotonergic, histaminergic, dopaminergic, and cholinergic agonists and/or antagonists or the endogenous peptides substance P, Leu-enkephalin, bradykinin, neurotensin, arginine-vasopressin, vasointestinal polypeptide, neuropeptide Y, and angiotensin II, also had no effect on the specific [^{3}H]L-365,260 binding at concentrations generally considered to be pharmacologically effective (1 μM).

Discussion

The binding of the brain CCK-B and gastrin receptor antagonist [^{3}H]L-365,260 to guinea pig brain membranes was rapid, time and tissue concentration dependent, reversible, and saturable. Scatchard and Hill plot analyses indicated that [^{3}H]L-365,260 binds with high affinity (K_d = 2.3 nM) and recognizes a single class of binding sites. The stereoselectivity of [^{3}H]L-365,260 binding in brain membranes was demonstrated by the much higher potency of unlabeled L-365,260 (an *R*-enantiomer), compared with its *S*-enantiomer, as a displacing agent. In fact, the *S*-enantiomer exhibited selectivity for pancreatic CCK-A receptors, compared with brain CCK-B and gastrin receptors. These data may suggest that, in general, the *S*-configuration may favor the peripheral CCK-A receptor, inasmuch as other selective antagonists such as L-364,718 are also *S*-enantiomers. However, the *R*-enantiomer of L-364,718 does not exhibit selectivity for the brain CCK-B or gastrin receptor.

The rank order of potencies of CCK/gastrin receptor agonists (CCK-8, CCK-8-ds, gastrin, and CCK-4) and antagonists [L-365,260, (*S*)-L-365,260, L-364,718, CR1409, and asperlicin] in

inhibiting specific [^{3}H]L-365,260 binding correlated with their potencies in displacing ^{125}I-CCK-8 binding in brain tissue but not their potencies in displacing the peripherally selective CCK-A ligand [^{3}H]L-364,718 from pancreatic receptors. Moreover, the relative distribution of [^{3}H]L-365,260 binding in various brain regions paralleled the distribution of ^{125}I-CCK binding. Collectively, these data indicate that [^{3}H]L-365,260 represents a new nonpeptide antagonist radioligand suitable for the study of brain CCK-B receptor interactions.

Although the rank order of potencies of CCK agonists were similar (CCK-8 > gastrin > CCK-8-ds > CCK-4) when either [^{3}H]L-365,260 or ^{125}I-CCK-8 was used as a radioligand in brain tissue, the absolute potencies of some agonists, notably gastrin and CCK-4, in displacing [^{3}H]L-365,260 binding were 10–15-fold less than their potencies for inhibition of ^{125}I-CCK-8 binding. In contrast, the absolute potencies of CCK antagonists in displacing ^{125}I-CCK-8 and [^{3}H]L-365,260 were similar (Table 2). A reduced potency for agonists, but not antagonists, in competing for radiolabeled antagonist ligand has also been reported previously for the muscarinic cholinergic receptor (20, 21) and peripheral CCK-A receptor (10) and has been proposed to indicate either two classes of binding sites or two conformational states that have different affinities for agonists but not antagonists.

It is well known that guanyl nucleotides and ions differentially affect agonist and antagonist binding in several neurotransmitter receptor systems (10, 21–24). The removal of NaCl from the incubation buffer significantly reduced specific ^{125}I-CCK-8 and [^{3}H]L-365,260 binding in guinea pig brain membranes. In contrast, removal of $MgCl_2$ or addition of guanyl nucleotide [Gpp(NH)p] resulted in selective reduction of ^{125}I-CCK-8 binding without an affect on [^{3}H]L-365,260 binding. These studies demonstrate a differential effect of guanyl nucleotide and $MgCl_2$ on agonist and antagonist radioligand binding to brain CCK-B receptors. Conceivably, these differences could provide a method for the determination of agonist and antagonist interaction with brain CCK-B receptors, using a binding assay.

L-365,260 has also been previously characterized as a highly potent and selective antagonist for gastrin receptors, compared with peripheral CCK-A receptors (12, 13). In agreement with these results, the present data demonstrate that [^{3}H]L-365,260 binds in a specific manner to guinea pig gastric glands but not rat pancreatic tissue. Specific [^{3}H]L-365,260 binding in gastric glands was inhibited by CCK/gastrin receptor antagonists, with potencies similar to those for inhibition of ^{125}I-gastrin binding in the same tissue. The results indicate that [^{3}H]L-365,260 may label the same receptors as ^{125}I-gastrin in guinea pig gastric glands. However, the finding that CCK/gastrin agonists had a higher potency for ^{125}I-gastrin binding than for [^{3}H]L-365,260 binding may also suggest that agonists and antagonists have two distinct binding sites.

Specific [^{3}H]L-365,260 binding in guinea pig brain was not affected by several pharmacological agents known to interact with other peptide or nonpeptide receptor systems. These results are in agreement with the reported high selectivity of L-365,260 as a brain CCK-B/gastrin receptor antagonist (12) and further demonstrate the utility of [^{3}H]L-365,260 as a selective ligand for brain CCK-B and gastrin receptors. However, the relatively low ratio of total binding to nonspecific binding in guinea pig gastric glands may limit its utility in this tissue.

In summary, [^{3}H]L-365,260 represents a new potent nonpeptide antagonist radioligand selective for brain CCK-B and gastrin receptors. The availability of this ligand and the peripheral CCK-A-selective ligand [^{3}H]L-364,718 (10) should provide valuable tools for the identification of CCK receptor subtypes in various tissues.

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