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CHARACTERIZATION OF [^{3}H](±)L-364,718 BINDING TO SOLUBILIZED CHOLECYSTOKININ (CCK) RECEPTORS OF RAT PANCREAS

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Abstract—The binding of [^{3}H](±)L-364,718 (3*S*(−)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepine-3-yl)-1*H*-indole-2-carboxamide], an extremely potent nonpeptide cholecystokinin (CCK) receptor antagonist, to digitonin-solubilized CCK receptors from rat pancreas was characterized. [^{3}H](±)L-364,718 binding to digitonin-solubilized receptors was assayed using polyethylene glycol precipitation followed by rapid filtration to separate free and bound [^{3}H](±)L-364,718. Specific [^{3}H](±)L-364,718 binding to solubilized receptors was dependent on the digitonin and receptor concentration and, under optimal conditions, represented greater than 90% of the total binding. Scatchard analysis indicated a single class of binding sites with a K_d of 0.53 nM and a B_{max} of 3.1 pmol/mg protein. Specific [^{3}H](±)L-364,718 binding to solubilized CCK receptors was inhibited by both CCK receptor agonists and antagonists in a stereospecific manner. After solubilization, the affinities of various antagonists to displace specific [^{3}H](±)L-364,718 binding were similar to those obtained with membrane-bound receptors; however, the affinities of CCK agonists were reduced 10–100 times. Collectively, the data presented indicate that [^{3}H](±)L-364,718 represents a new antagonist ligand which has apparent advantages over the agonist ligand [^{125}I]CCK in assaying digitonin-solubilized receptors. Gel filtration of the digitonin-solubilized CCK receptors followed by [^{3}H](±)L-364,718 binding determinations revealed an estimated molecular weight of 400,000 daltons.

The membrane-bound receptors for cholecystokinin (CCK†), a hormone and putative neurotransmitter [1], have been characterized using various radiolabeled CCK receptor agonists [2–5] and antagonists [6]. [^{125}I]CCK binding to digitonin-solubilized receptors from mouse pancreatic tissue has also been reported by Szecowka *et al.* [7]. However, although the affinities of CCK antagonists for solubilized CCK receptors are similar to membrane-bound receptors, the affinities of CCK agonists for solubilized receptor are reduced 50–100 times [7]. Since the affinities of CCK receptors for antagonists remain unaltered after solubilization, a high-affinity non-peptide CCK receptor antagonist radioligand such as [^{3}H](±)L-364,718 [6, 8, 9] may have advantages over agonist ligands such as [^{125}I]CCK in studies with solubilized CCK receptors. In the present report, the binding of [^{3}H](±)L-364,718 to solubilized CCK receptors is characterized.

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† Abbreviations: CCK, cholecystokinin; L-364,718, 3*S*(−)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepine-3-yl)-1*H*-indole-2-carboxamide; PEG, polyethylene glycol; EGTA, ethylene-glycol-bis-(aminoethylether)tetra-acetate: HEPES, *N*-2-hydroxyethyl piperazine-*N*′-2-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; BSA, bovine serum albumin; and SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

METHODS

Solubilization of CCK receptors. Pancreatic membranes were prepared from male Sprague–Dawley rats (200–350 g) as described previously [10]. Dissected pancreas were homogenized in 100 vol. of 10 mM HEPES, 1 mM EGTA and 5 mM $MgCl_2$ (pH 6.5). The homogenates were centrifuged at 50,000 *g*, and the pellets were washed by resuspension and centrifugation. Solubilization procedures were similar to those described by Szecowka *et al.* [7]. To solubilize the receptors, the washed membrane pellets were homogenized gently in 50 ml of ice-cold solubilization buffer (10 mM HEPES, 1 mM EGTA, 5 mM $MgCl_2$, 1% digitonin, 0.2 mM PMSF and 1 mM benzamidine HCl, pH 6.5) for each gram of original pancreas wet weight (87 ± 7 mg protein). They were shaken gently for 1 hr at 4° and then centrifuged at 150,000 *g* for 1 hr. The supernatant fractions were used for binding studies after appropriate dilution with binding assay buffer.

Binding assays. [^{3}H](±)L-364,718 (64.4 Ci/mmol) were prepared by Dr. A. Rosegay and Mr. H. T. Meriwether of Merck Sharp & Dohme Research Laboratories. The binding assay buffer contained 10 mM HEPES (pH 6.5), 1 mM EGTA, 5 mM $MgCl_2$, 118 mM NaCl, 4.7 mM KCl, 2 mg/ml BSA, 0.14 mg/ml bacitracin, 0.2 mg/ml soybean trypsin inhibitors and appropriate concentrations of digitonin. Initially, [^{3}H](±)L-364,718 binding studies were conducted using various concentrations of digitonin, as well as different receptor concentrations to

determine optimal conditions. In subsequent assays, a digitonin concentration of 0.05% was routinely employed. Solubilized receptors prepared from each gram of pancreas were diluted in 2000 ml of binding assay buffer (approximately 20–30 μg solubilized receptor protein/ml). For binding assays, 980 μl of solubilized receptors was added to triplicate tubes containing 10 μl of either buffer (for total binding), or unlabeled (±)L-364,718 (0.3 μM final concentration for nonspecific binding), or various concentrations of displacers and 10 μl of [^{3}H](±)L-364,718 (0.4 nM final concentration unless indicated otherwise). The incubation was at 37° for 30 min or as indicated in the association rate study. After incubation, the receptor–[^{3}H](±)L-364,718 complex was precipitated using methods similar to that previously described [7] by adding ice-cold 0.2 ml bovine γ-globulin (6 mg/ml), 1 ml PEG (20%) and 0.2 ml potassium iodide (30 mg/ml). The mixture was immediately vortexed and filtered through Glass fibers GF/B and then washed with 2 × 2 ml of 10% PEG. The radioactivity on the filters was measured using a liquid scintillation counter.

The amount of radioactivity precipitable by PEG in the absence of solubilized receptors (blank value) was less than 0.5% of the total radioactivity.

RESULTS

Effect of digitonin concentration on the binding of [^{3}H](±)*L-364,718 to solubilized pancreatic CCK receptors.* The specific binding of [^{3}H](±)L-364,718 was dependent on the final digitonin concentration in the incubation medium. The optimal digitonin concentration was between 0.025 and 0.05% (Fig. 1). The percentage of total added radioactivity specifically bound was 5.5 ± 0.22 and 4.3 ± 0.87 at digtonin concentrations of 0.025 and 0.05% respectively. The percentage of nonspecific binding in terms of total added radioactivity was 0.70 ± 0.17 and 0.68 ± 0.02 at these two digitonin concentrations (Fig. 1). Routinely, a digitonin concentration of 0.05% was employed. This experiment did not distinguish between whether digitonin directly affected [^{3}H](±)L-364,718 binding or the degree of precipitation of the [^{3}H](±)L-364,718–receptor complex.

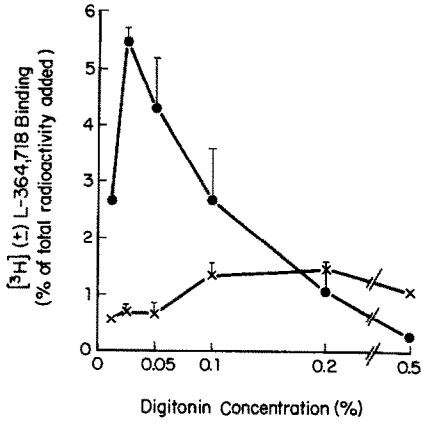

Fig. 1. Effect of digitonin concentration on the binding of [^{3}H](±)L-364,718 to solubilized receptors. [^{3}H](±)L-364,718 (0.4 nM) was incubated with solubilized receptors (14 μg/ml) at the indicated final concentrations of digitonin. Specific binding (●) and nonspecific binding (×) are expressed as percentage of total radioactivity added (25,000 cpm). The points are means ± SEM from three separate experiments. Values without SEM are means of two experiments.

Effect of solubilized receptor concentration on [^{3}*H*](±)*L-364,718 binding.* The specific binding of [^{3}H](±)L-364,718 to solubilized pancreatic receptors increased linearly with receptor concentration over the range of 0.125 to 1 mg/ml of original tissue (7–56 μg protein/ml) (Fig. 2). Routinely, 20–30 μg protein was used. The amount of nonspecific binding increased very little with increasing receptor concentrations (Fig. 2).

Kinetics of [^{3}H](±)*L-364,718 binding to solubilized pancreatic CCK receptors.* Specific [^{3}H](±)L-364,718 binding to solubilized pancreatic receptors reached steady state after incubation for 30 min. The rate constant of association (K_1) was calculated to be 0.17 ± 0.02 $min^{-1}nM^{-1}$ (Fig. 3). The rate of dissociation of [^{3}H](±)L-364,718 binding was determined by adding unlabeled (±)L-364,718 at equilibrium to prevent rebinding. The rate constant for dissociation (K_{-1}) was calculated to be 0.045 ± 0.005 min^{-1} (Fig. 4). The dissociation constant (K_d) calculated from K_{-1}/K_1 was 0.26 nM which is comparable to the value obtained from equilibrium studies (see below).

Saturation of [^{3}H](±)*L-364,718 binding to solubilized pancreatic receptors.* The binding of [^{3}H](±)L-364,718 to solubilized receptors was saturable (Fig. 5). The nonspecific binding increased linearly with ligand concentration, whereas specific

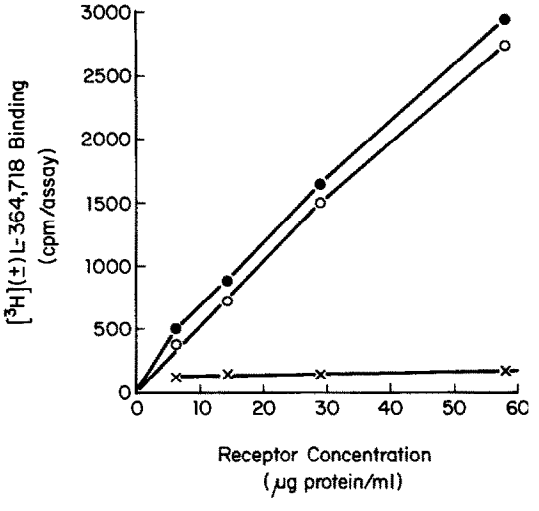

Fig. 2. [^{3}H](±)L-364,718 binding to solubilized receptors as a function of increasing receptor concentrations. Various concentrations of solubilized receptors were incubated with 0.4 nM [^{3}H](±)L-364,718 for 30 min as described in Methods. Nonspecific binding was defined in the presence of 0.3 μM unlabeled (±)L-364,718. Specific binding (○) is the difference between total binding (●) and nonspecific binding (×). Each point represents the mean of triplicate determinations. The experiments were repeated three times. In all experiments the specific binding was linear up to at least 30 μg/ml.

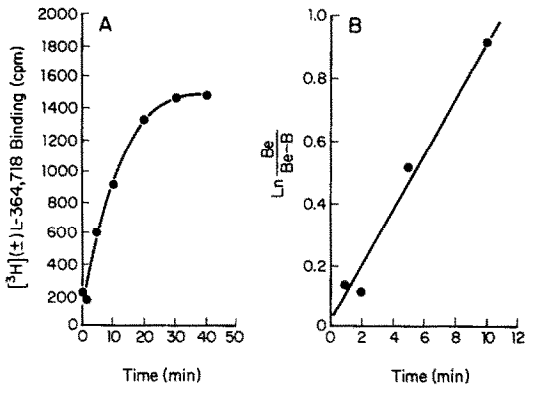

Fig. 3. Time course of association of [³H](±)L-364,718 binding. The association of [³H](±)L-364,718 binding to solubilized pancreatic receptors (0.5 mg original tissue wet weight) was determined at various time intervals as described in Methods. Specific binding was defined as the difference between binding obtained in the presence and absence of 0.3 μM unlabeled (±)L-364,718. The points shown are those obtained in a single experiment performed in triplicate. The experiments were repeated three times and the mean rate constant of association was $0.17 \pm 0.02\ min^{-1}\ nM^{-1}$. (A) Specific [³H](±)L-364,718 binding as a function of time. (B) Pseudo first-order kinetic plots of initial specific [³H](±)L-364,718 binding. On the ordinate, (B) is the amount of specific [³H](±)L-364,718 binding at time t and (B_e) is the amount of specific [³H](±)L-364,718 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 $K_1 = (k_{ob} - k_{-1})/[[^3H](\pm)L\text{-}364{,}718]$, was $0.21\ min^{-1}\ nM^{-1}$. K_{-1} is the first-order rate constant for dissociation (from Fig. 4) and [[³H](±)L-364,718] is the concentration of radioligand used in the experiment (0.4 nM).

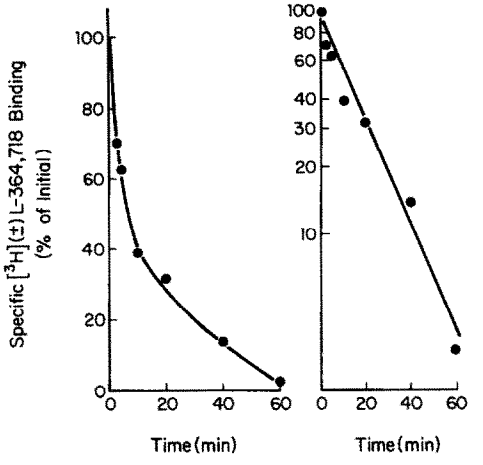

Fig. 4. Dissociation of specific [³H](±)L-364,718 binding to solubilized rat pancreatic receptors. [³H](±)L-364,718 binding assays were performed as described in Methods. For dissociation studies, [³H](±)L-364,718 was first allowed to associate for 30 min at 37°, whereupon 0.3 μM unlabeled (±)L-364,718 was added to prevent rebinding of dissociated [³H](±)L-364,718. The dissociation reaction was measured at various time intervals after the addition of unlabeled (±)L-364,718 by rapid filtration following PEG precipitation as described in Methods. The points shown were obtained in a single experiment performed in triplicate. The experiments were repeated four times. The mean rate constant of dissociation was $0.045 \pm 0.005\ min^{-1}$. The left panel is a linear plot, and the right panel is a semilog plot of B/B_e vs 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-364,718. The dissociation rate constant (k_{-1}) was calculated according to the formula, $k_{-1} = 2.3 \times$ slope was $0.053\ min^{-1}$.

binding reached maximal at approximately 3 nM. The ratio of total binding to nonspecific binding was about 10 at a [³H](±)L-364,718 concentration of 0.4 nM which was used for routine binding assays. Scatchard analysis of these data indicated a single class of binding sites with a dissociation constant (K_d) of 0.53 ± 0.15 nM and a B_{max} of 181 ± 56 fmol/mg tissue (3.1 ± 1.0 pmol/mg protein) (Fig. 5). The maximal number of binding sites for [³H](±)L-364,718 in membrane-bound receptor is 270 ± 25 fmol/mg tissue (3.1 ± 0.29 pmol/mg protein) [6]. The percentage yield for solubilized binding sites was thus 70% compared to membrane-bound sites.

Inhibition of [³H](±)*L-364,718 binding by CCK receptor agonists and antagonists.* Specific [³H](±)L-364,718 binding to solubilized receptors was inhibited by both (−) and (+)-enantiomers of L-364,718 (Table 1). The affinity of the more biologically active (−)-enantiomer [8, 9] ($K_i = 0.15$ nM) was approximately 70 times greater than the (+)-enantiomer, thus demonstrating the stereoselectivity of the radioligand binding. Other CCK antagonists including asperlicin [11] CBZ-CCK(26–32)amide [6], dibutyryl-cGMP [12], benzotript and proglumide [13] also effectively displaced [³H](±)L-364,718 binding (Table 1). The potencies of the various antagonists in inhibiting specific [³H](±)L-364,718 binding in solubilized receptors agreed well with their potencies in inhibiting specific [³H](±)L-364,718 binding in membrane-bound receptors (Table 1). However, the K_i values of the CCK agonists, caerulein and CCK-8, for inhibition of specific ([³H](±)L-364,718 binding to solubilized receptors were 15–100 times higher than their K_i values determined in membrane-bound receptors (Table 1).

The Hill coefficients for both agonists and antagonists in displacing [³H](±)L-364,718 to solubilized receptors approximated unity. In contrast, as reported previously [6], when membrane-bound receptors were employed the Hill coefficients of agonists, but not antagonists were appreciably less than unity (Table 1).

CCK-8-desulfate and gastrin (1–3 μM) were inactive in displacing [³H](±)L-364,718 binding to solubilized receptors or membrane-bound receptors.

Molecular size of digitonin-solubilized [³H](±)*L-364,718 binding sites estimated by gel filtration on Sepharose 6B.* [³H](±)L-364,718 binding to fractions of the digitonin-solubilized protein obtained by gel filtration through Sepharose 6B column demonstrated a single prominent peak (fraction 37) (Fig. 6). This peak corresponded to an apparent molecular weight of 400,000 daltons and a Stokes radius of 57 Å when compared to standard protein markers (Fig. 6).

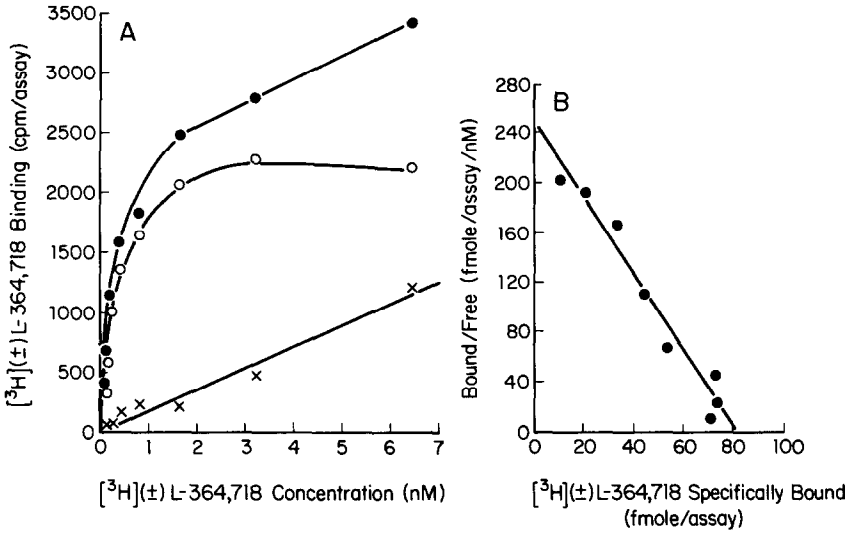

Fig. 5. [^{3}H](±)L-364,718 binding as a function of increasing concentrations of [^{3}H](±)L-364,718. The binding assays were performed as described in Methods using various concentrations of [^{3}H](±)L-364,718 (0.05 to 6.4 nM). The points shown are means of triplicate determinations in a single experiment. This experiment was repeated three times, and the calculated mean ± SEM for K_d and B_{max} are given in the text. The mean correlation coefficient for the Scatchard plots was 0.96 ± 0.02. (A) Key: (●) total binding, (×) nonspecific binding and (○) specific binding. Nonspecific binding was defined using 0.3 μM unlabeled (±)(L-364,718. Specific binding is the difference between total and nonspecific binding. (B) Scatchard plot for specific [^{3}H](±)L-364,718 binding.

The [^{3}H](±)L-364,718 binding peak was abolished when [^{3}H](±)L-364,718 binding was determined in the presence of an excess (3×10^{-7} M) of unlabeled (±)L-364,718 (Fig. 6A).

DISCUSSION

L-364,718 is an extremely potent, competitive antagonist of CCK receptors which has high selectivity for peripheral tissues [8, 9]. The utility of [^{3}H](±)L-364,718 as a new radioligand for the study of CCK receptors in rat pancreatic membranes is the subject of another report [6]. In the present studies, the binding of [^{3}H](±)L-364,718 to solubilized CCK receptors from rat pancreas is characterized.

Using digitonin-solubilized pancreatic CCK recep-

Table 1. Displacement of specific [^{3}H](±)L-364,718 binding by various CCK agonists and antagonists in solubilized and membrane-bound receptors

	Solubilized		Membrane-bound†	
Displacers	K_i (nM)	n_H*	K_i (nM)	n_H*
Agonists				
Caerulein	29 ± 5.9‡	0.8 ± 0.06	2.0 ± 0.28	0.4 ± 0.01
CCK-8	200 ± 50‡	0.9 ± 0.06	2.0 ± 0.45	0.4 ± 0.02
CCK-8-desulfate	>800		>800	
Gastrin	>1,400		>2,000	
Antagonists				
(−)L-364,718	0.15 ± 0.072	1.0 ± 0.03	0.036 ± 0.003	0.9 ± 0.02
(+)L,364,718	10 ± 2.3	0.8 ± 0.05	4.5 ± 0.4	0.8 ± 0.01
Asperlicin	590 ± 140	0.9 ± 0.03	312 ± 100	0.7 ± 0.1
CBZ-CCK(26–32)amide	12,000	1.1	7,500 ± 900	0.8 ± 0.1
Dibutyryl-cGMP	17,000 ± 7,700	1.3 ± 0.4	25,000 ± 9,300	1.3 ± 0.1
Benzotript	68,000 ± 10,000	1.2 ± 0.1	41,000 ± 1,000	1.0 ± 0.1
Proglumide	380,000 ± 90,000	1.0 ± 0.04	510,000 ± 12,000	0.8 ± 0.1

Values are means ± SEM from at least three 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 radioactive ligand.

* n_H = Hill coefficient.

† Data from Ref. 6.

‡ $P < 0.005$ (solubilized vs membrane-bound).

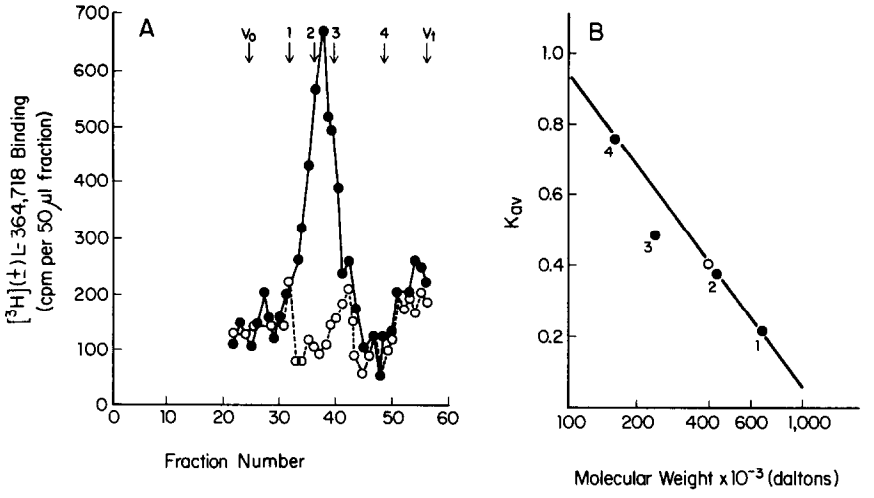

Fig. 6. Gel filtration of digitonin-solubilized pancreatic receptors on Sepharose 6B. (A) Sepharose 6B column (1.4 × 72 cm) was equilibrated with 0.1% digitonin, 1 mM EGTA, 5 mM $MgCl_2$ and 10 mM HEPES, pH 6.5. Five milliliters of digitonin-solubilized receptors (1 g original tissue in 50 ml of 1% digitonin) was applied to the column. Fractions of 2 ml each were collected. Aliquots (50 µl) from each fraction were diluted to 980 µl and used for [³H](±)L-364,718 binding as described in Methods. The points are the means of triplicate determinations. Key: (●) total binding, and (○) nonspecific binding determined in the presence of 3×10^{-7} M unlabeled (±)L-364,718. Arrows indicate column void volume (V_0) and column volume (V_t): (1) thyroglobulin, (2) ferritin, (3) catalase, (4) aldolase. (B) Calibration curve of standard molecular weight markers (●) run on the same column described in panel A. The open circle indicates the position of elution of solubilized [³H](±)L-364,718 binding sites. $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume.

tors [7], [³H](±)L-364,718 recognized a single class of binding sites with a high affinity ($K_d = 0.53$ nM). The binding of [³H](±)L-364,718 was stereospecific in that the more biologically active (−)-enantiomer demonstrated greater potency than the (+)-enantiomer as a displacing agent. The rank order of potencies of both CCK agonists and antagonists in competing for [³H](±)L-364,718 binding to solubilized receptors correlated well with their rank order of potencies in displacing [¹²⁵I]CCK or [³H](±)L-364,718 to membrane-bound receptors [6, 8] and their reported biological activities in peripheral tissues [4, 8, 11–13]. Collectively, these data indicate that [³H](±)L-364,718 represents a new antagonist radioligand for studying interactions with solubilized CCK receptors.

The absolute potencies of CCK antagonists in displacing [³H](±)L-364,718 binding in solubilized pancreatic CCK receptors were similar to those observed in membrane CCK receptors. However, the CCK agonists (caerulein and CCK-8) were approximately 10–100 times less potent in solubilized compared to membrane preparations. These results agree with those reported by Szecowka *et al.* [7] who also found that digitonin solubilization of CCK receptors decreased the affinity of agonists, but not antagonists, for the receptors.

The present studies demonstrated an additional difference in the binding characteristics of agonists in displacing [³H](±)L-364,718 binding to solubilized as compared to membrane-bound receptors. When membrane-bound receptors were employed, the Hill coefficients of agonists were appreciably less than unity. A shift of the Hill coefficients for agonist binding towards unity was observed when solubilized receptors were employed. The data may indicate that solubilization results either in the conversion of a high-agonist state to a low-affinity conformation or a selective inactivation of high-affinity sites.

The solubilization of membrane-bound CCK receptors which retained ligand specificity for [¹²⁵I]CCK by Szecowka *et al.* [7] has provided the first important step toward further purification and characterization of peripheral CCK receptors. However, in view of the decreased affinity of CCK agonists for solubilized receptors, the use of an antagonist ligand such as [³H](±)L-364,718 offers some advantages. In addition to retaining high ligand specificity, [³H](±)L-364,718 is less readily precipitable than [¹²⁵I]CCK by PEG in the absence of receptors (0.5% vs 6–9% of total radioactivity), thus yielding a higher ratio of total binding to nonspecific binding and blank value. Additionally, the high affinity of [³H](±)L-364,718 permits the use of simple filtration techniques, rather than centrifugation methods, for separation of free and bound ligand.

Previous studies using photoaffinity labeling or covalent cross-linking of [¹²⁵I]CCK-33 to receptors followed by SDS–PAGE revealed a major molecular weight of 80,000–95,000 daltons with additional proteins of 200,000 and 120,000–140,000 daltons [14–17]. However, the estimated molecular weight of the CCK receptor under nondenaturation conditions has not been reported. In the present study, the molecular weight of [³H](±)L-364,718 binding sites was estimated to be 400,000 daltons by gel filtration on

Sepharose 6B. Although the reason for the differences in the estimated CCK receptor molecular weight observed in the present and previous studies is unknown, several explanations are possible. These include: CCK receptors may be oligomers with subunits which become dissociated under denatured conditions; the CCK receptor may not be a globular protein as is assumed in the estimation of molecular weight by gel filtration; or digitonin may bind to the solubilized CCK receptor and contribute to the molecular weight estimation.

In conclusion, the present studies demonstrate that [^{3}H](±)L-364,718 binds to solubilized CCK receptors with very high affinity and specificity. The characteristics of [^{3}H](±)L-364,718 binding to solubilized receptors may offer some advantages over [^{125}I]CCK in assaying solubilized CCK receptors during purification studies.

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REFERENCES

1. J. A. Williams, *Biomed. Res.* **3**, 107 (1982).
2. H. Sankaran, I. D. Goldfine and J. A. Williams, *J. biol. Chem.* **255**, 1849 (1979).
3. R. B. Innis and S. H. Snyder, *Proc. natn. Acad. Sci. U.S.A.* **77**, 6917 (1980).
4. R. T. Jensen, G. F. Lemp and J. D. Gardner, *Proc. natn. Acad. Sci. U.S.A.* **77**, 2079 (1980).
5. L. J. Miller, S. A. Rosenzweig and J. D. Jamieson, *J. biol. Chem.* **256**, 12417 (1981).
6. R. S. L. Chang, V. J. Lotti, T. B. Chen and K. A. Kunkel, *Molec. Pharmac.* **30**, 212 (1986).
7. J. Szecowka, I. D. Goldfine and J. A. Williams, *Regulat. Peptides* **10**, 71 (1985).
8. R. S. L. Chang and V. J. Lotti, *Proc. natn. Acad. Sci. U.S.A.* **83**, 4923 (1986).
9. B. E. Evans, M. G. Bock, K. E. Rittle, R. M. DiPardo, W. L. Whitter, D. F. Veber, P. S. Anderson and R. M. Freidinger, *Proc. natn. Acad. Sci. U.S.A.* **83**, 4918 (1986).
10. R. S. L. Chang, V. J. Lotti and T. B. Chen, *Biochem. Pharmac.* **33**, 2334 (1984).
11. R. S. L. Chang, V. J. Lotti, R. L. Monaghan, J. Birnbaum, E. D. Stapley, M. A. Goetz, G. Albers-Schonberg, A. A. Patchett, J. M. Liesch, O. D. Hensens and J. P. Springer, *Science* **230**, 177 (1985).
12. S. R. Peikin, C. L. Costernbader and J. D. Gardner, *J. biol. Chem.* **254**, 5321 (1979).
13. W. F. Hahne, R. T. Jensen, G. F. Lemp and J. D. Gardner, *Proc. natn. Acad. Sci. U.S.A.* **78**, 6304 (1981).
14. M. Svoboda, M. Lambert, J. Furnelle and J. Christophe, *Regulat. Peptides* **4**, 163 (1982).
15. S. A. Rosenzweig, L. J. Miller and J. D. Jamieson, *J. Cell Biol.* **96**, 1288 (1983).
16. L. Madison, S. A. Rosenzweig and J. D. Jamieson, *J. biol. Chem.* **259**, 14818 (1984).
17. C. Sakamoto, I. D. Goldfine and J. A. Williams, *Biochem. biophys. Res. Commun.* **118**, 623 (1984).