Multiple Affinity States of Different Cholecystokinin Receptors*

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We transfected COS cells with cDNA for rat cholecystokinin-A (CCK-A) and different CCK-B receptors and measured binding of [125I]-CCK-8, [3H]L-364,718 and [3H]L-365,260 to characterize the different affinity states for each type of CCK receptor. Rat CCK-A and CCK-B receptors, canine CCK-B receptors and canine mutant CCK-B (M-CCK-B) receptors in which the leucine in position 355 was replaced by valine each existed in three different affinity states for CCK-8, high affinity, low affinity, and very low affinity. In rat CCK-A and probably CCK-B receptors, most were in the very low affinity state, whereas with canine CCK-B and M-CCK-B receptors, most were in the low affinity state. Studies with CCK receptor agonists, CCK-8, gastrin, and CCK-JMV-180, in conjunction with CCK receptor antagonists, L-364,718 and L-365,260, showed a different pattern of affinities for these ligands at the different CCK receptors. Thus, each transfected CCK receptor can exist in three different affinity states for CCK-8 and has a characteristic pattern of interaction with different ligands. This ability to exist in multiple affinity states is an intrinsic property of the CCK receptor molecule itself.

Receptors for CCK1 have been divided into two classes based on their pharmacologic properties, amino acid sequences, and tissue distribution (1–5). CCK-A receptors have a high affinity for CCK-8 and the CCK-A-specific receptor antagonist, L-364,718 and a low affinity for gastrin and the CCK-B-specific receptor antagonist, L-365,260. CCK-B receptors have a high affinity for CCK-8, gastrin, and L-365,260 and a low affinity for L-364,718. CCK-A receptors and CCK-B receptors have been cloned and found to have 50% amino acid identity (2, 4, 5). In addition, receptors that were initially referred to as "gastrin receptors" have been cloned and found to be identical to CCK-B receptors (5). (Gastrin receptors are now usually referred to as CCK-B receptors.) CCK-A receptors are located in pancreas, gallbladder, and certain localized areas of the central nervous system, whereas CCK-B receptors are located in gastric parietal cells, gastrointestinal smooth muscle, and a variety of different areas in the central nervous system (1, 3).

In a previous study (6) using pancreatic acini or COS cells that had been transfected with pancreatic CCK-A receptors, we showed that pancreatic CCK-A receptors exist in three different affinity states for CCK-8, high affinity, low affinity, and very low affinity. The CCK receptor antagonist, L-364,718, had the same affinity (Kd ~ 1 nm) for each different state of the receptor in pancreatic acini and in COS cells (6). Measurements of binding of [125I]-CCK-8 detected receptors in the high and under certain conditions the low affinity state, whereas measurements of binding of [3H]L-364,718 detected receptors in the low and very low affinity states (6).

Previous measurements of ligand binding to CCK-B receptors using intact cells, membranes, or cells transfected with CCK-B receptors have been somewhat limited and have not recognized multiple affinity states of the receptors (2, 7–11). On the other hand, measurements of binding of [3H]L-365,260, the CCK-B-specific receptor antagonist, have generated data that make it highly likely that CCK-B receptors, like CCK-A receptors, exist in multiple affinity states for CCK-8 (9). In particular, measurement of inhibition of binding of [3H]L-365,260 by CCK-8 gave a broad dose-inhibition curve with a Hill coefficient of less than 1.0 (9). Moreover, the value of IC50 was much greater than that obtained from measurements of the ability of CCK-8 to inhibit binding of [125I]-CCK-8 in the same preparation (9), raising the possibility that the radiolabeled receptor antagonist bound to a very low affinity state of the receptor.

In the present study, we have transfected COS cells with CCK-B receptors (as well as with CCK-A receptors for comparison) and have used ligand binding techniques to determine the number of different affinity states for the different CCK receptors. Our findings indicate that both rat and canine CCK-B receptors, like CCK-A receptors, exist in three different affinity states for CCK-8.

**EXPERIMENTAL PROCEDURES**

**Materials**

Bovine serum albumin (fraction V), soybean trypsin inhibitor, DEAE-dextran, chloroquine, Dulbecco's modified Eagle's medium, fetal bovine serum, and bacitracin were from Sigma; CCK-426-33) (CCK-8), CCK-JMV-180, and gastrin-1 were from Research Plus Inc., Bayonne, NJ. BME amino acids (100x concentrated) and BME vitamin solution (100x concentrated) were from Life Technologies, Inc.; [3H]Bolton-Hunter-labeled CCK-8(1010CCK-8, 2200 Ci/mmol), [3H]L-364,718 (86 Ci/mmol), and [3H]L-365,260 (71 Ci/mmol) were from DuPont NEN. Noyesil-50 was from William F. Nye, New Bedford, MA, and L-364,718 and L-365,260 were gifts from Dr. Ben E. Evans, Merck, Sharp and Dohme Laboratories.

Unless stated otherwise, the standard incubation solution contained 24 mM HEPES pH 7.4, 120 mM NaCl, 7.2 mM KCl, 2.2 mM NaH2PO4, 6 mM sodium pyruvate, 7 mM sodium fumarate, 0.5 mM MgCl2, 1.2 mM CaCl2, 6 mM sodium glutamate, 14 mM glucose, 2 mM glutamine, 0.01% (w/v) trypsin inhibitor, 1% (v/v) essential amino acid mixture, 1% (w/v) bovine serum albumin, 1% (v/v) vitamin mixture, and 0.1% (w/v) bacitracin.

**Methods**

**Cell Preparation**—COS-7 cells were plated in 175-cm2 culture flasks and grown in Dulbecco's modified Eagle's medium, 10% fetal bovine serum, in an incubator with 5% CO2, 95% air at 37 °C. The plasmid,
pCDL-SRa (containing cDNA for the rat CCK-A receptor cloned from rat pancreas (4)), cDNA for rat CCK-B receptor cloned from rat brain (2), or cDNA for canine CCK-B receptor cloned from canine brain (11) was transfected into COS-7 cells, using the DEAE-dextran chloroquine method (12) as described previously (6). Another plasmid, pcDNA I, containing cDNA for a mutant canine CCK-B receptor (canine M-CCK-B receptor), to which leucine in position 355 was replaced by valine (13), was also transfected into COS-7 cells using the same method. 48-72 h posttransfection, the transfected cells were washed 3 times with phosphate-buffered saline solution (137 mM NaCl, 3 mM KCl, 10 mM Na2HPO4, and 2 mM K2PO4, pH 7.4) containing 0.1% (w/v) bovine serum albumin, scraped from the flask, centrifuged (400 × g), and resuspended in standard incubation solution at a cell concentration of approximately 10⁶ cells/ml.

Binding of [3H]L-364,718—Binding of [3H]L-364,718 was measured as described previously (6). Incubations contained 300 μl of COS cell suspension and 80 pm [3H]L-364,718 in polypropylene tubes and, unless otherwise specified, were for 60 min (canine CCK-B receptor, canine M-CCK-B receptor), 90 min (rat CCK-B receptor), or 120 min (rat CCK-A receptor) at 37 °C. After the incubation, duplicate samples (100 μl) were added to microcentrifuge tubes containing 100 μl of silicon oil (Nyosil) and centrifuged at 10,000 × g for 45 s in a microcentrifuge (Beckman Microfuge). The portion of the microcentrifuge tube containing the cell pellet was cut off and added to a counting tube for measurement of radioactivity. Nonsaturable binding of [3H]L-364,718 was determined as the amount of radioactivity associated with the cells that were incubated with 80 pm [3H]L-364,718 plus 1 μM [3H]L-365,260 (100 μl of COS cell suspension and 400 pm [3H]L-364,718 or 400 pm [3H]L-365,260 in polypropylene tubes and, unless otherwise specified, were for 60 min at 37 °C). After the incubation, duplicate 100-μl samples were added to 5 ml of iced (4 °C) phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 10 mM Na2HPO4, and 2 mM K2PO4, pH 7.4). The cells were then filtered (0.5 μm) with Whatman GF/C glass fiber filters over a vacuum filtering manifold. COS cells retained on the filters were washed 3 times with 5 ml of the same solution. Filters were placed in scintillation vials containing liquid scintillation fluid (Aquasol, Du Pont NEN) and assayed for radioactivity. Nonsaturable binding of [3H]L-364,718 or [3H]L-365,260 was determined as the amount of radioactivity associated with COS cells that were incubated with 400 pm [3H]L-364,718 plus 1 μM [3H]L-365,260 or 400 pm [3H]L-365,260 plus 1 μM L365,260, respectively. Nonsaturable binding of [3H]L-364,718 with rat CCK-A receptors and canine CCK-B receptors was 6 ± 1 and 41 ± 4%, respectively. Nonsaturable binding of [3H]L-365,260 with canine M-CCK-B receptors was 20 ± 2%. All values given for bound [3H]L-364,718 and [3H]L-365,260 represent saturable binding, i.e., total binding minus nonsaturable binding.

Analysis of Data—Results are expressed as means ± S.E. Binding parameters (Kd dissociation constant and Bmax, ase, cells per site) were determined using a nonlinear least-squares curve-fitting program (LIGAND) (14). The number of classes of binding sites was determined by repetitive fits with increasing classes of sites and was established by determining the fewest number of classes that statistically best fit the data using the F-test and p < 0.05 as the level of significance. The value for sites/cell was calculated assuming that all cells expressed the transfected receptors. We know that this assumption is not valid, but we believe it is more convenient to express receptor number in this manner than as mol/mg of protein or DNA.

RESULTS—Fig. 2 illustrates the abilities of various ligands to inhibit binding of [125I]-CCK-8 and [3H]L-364,718 to COS cells that had been transfected with the rat CCK-A receptor (panel A), rat CCK-B receptor (panel B), canine CCK-B receptor (panel C), or canine M-CCK-B receptor (panel D). The transfected cells were incubated with 80 pm [125I]-CCK-8 (closed circles) or 400 pm [3H]L-364,718 or 400 pm [3H]L-365,260 (open circles) at 37 °C. Results given are means from at least three separate experiments. Vertical bars indicate S.E.

Fig. 1. Time courses for binding of [125I]-CCK-8 and [3H]L-364,718 or [3H]L-365,260 to COS cells that had been transfected with the rat CCK-A receptor (panel A), rat CCK-B receptor (panel B), canine CCK-B receptor (panel C), or canine M-CCK-B receptor (panel D). The transfected cells were incubated with 80 pm [125I]-CCK-8 (closed circles) or 400 pm [3H]L-364,718 or 400 pm [3H]L-365,260 (open circles) at 37 °C. Results given are means from at least three separate experiments. Vertical bars indicate S.E.

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RESULTS—Fig. 1 illustrates the time-course for binding of [125I]-CCK-8, [3H]L-364,718, and [3H]L-365,260 to COS cells that had been transfected with different CCK receptors. There was no detectable binding of [125I]-CCK-8, [3H]L-364,718, or [3H]L-365,260 to nontransfected COS cells. With transfected cells, binding of [125I]-CCK-8 reached a steady state by 90 min with rat CCK-A receptors (panel A), by 60 min with rat CCK-B receptors (panel B), by 45 min with canine CCK-B receptors (panel C), and by 60 min with canine M-CCK-B receptors (panel D). Binding of [3H]L-364,718 to rat CCK-A (panel A) and canine CCK-B (panel C) receptors as well as binding of [3H]L-365,260 to canine M-CCK-B (panel D) had reached a steady state by 15 min. With each radioligand tested, after saturable binding reached a steady state it remained constant for up to 120 min. With rat CCK-B receptors, we detected no saturable binding of [3H]L-364,718, and a small, but statistically significant amount of saturable binding of [3H]L-365,260. This saturable binding of [3H]L-365,260 was approximately 35% of total binding; and, therefore, it was not possible to obtain meaningful dose-response curves with this small amount of saturable binding. This binding of [3H]L-365,260, however, does appear to be to a state of the rat CCK-B receptor with a very low affinity for CCK-8 because detectable inhibition of binding occurred only with concentrations of CCK-8 of 1 μM or greater (data not shown).

Fig. 2 illustrates the abilities of various ligands to inhibit binding of [125I]-CCK-8 and [3H]L-364,718 to COS cells that had been transfected with rat CCK-A receptors. Table I gives the parameter values that result from analyzing the data in Fig. 2. Analysis of these results for

| Parameter Values | A | B | C | D |
|------------------|---|---|---|---|
| Kd (nM)          | 1.9 | 4.2 | 1.9 | 2.6 |
| Bmax (fmol/mg)   | 400 | 400 | 400 | 400 |

Table I

In agreement with a previous study using transfected CCK-A receptors (2), CCK-8 was more potent than gastrin, and L-364,718 was more potent than L-365,260 in inhibiting binding of [125I]-CCK-8 (Fig. 2). CCK-JMV-180 also inhibited binding of [125I]-CCK-8, but with a potency that was approximately one-third of that of CCK-8 (Fig. 2). Analysis of these results for binding of [125I]-CCK-8 indicates that [125I]-CCK-8 interacts with a single class of binding sites (Table I) that we have interpreted previously to reflect binding of the radioligand to the high affinity state of the CCK-A receptor (6). Compared with the affinity of CCK-8 for this state of the receptor (Kd (Kd = 1.9 nm), the affinity of CCK-JMV-180 (Kd = 9 nm) is 4-fold lower, and that of gastrin (Kd = 4.2 μM) is 2000-fold lower (Table I). The affinity of L-364,718 (Kd = 1.9 nm) for the high affinity state of the CCK-A receptor was 2000 times greater than that of L-365,260 (Kd = 2.6 μM).
Table I
Parameters that characterize ligand binding to the transfected rat CCK-A receptor

| Ligand            | High affinity Kd (nM) | Low affinity Kd (nM) | Very low affinity Kd (nM) |
|-------------------|-----------------------|----------------------|--------------------------|
| CCK-8             | 1.9 ± 0.1             | 330 ± 120            | 80,000 ± 5,000           |
| Gastrin           | 4,200 ± 350           | >10,000              | >100,000                 |
| CCK-JMV-180       | 9 ± 2                 | 30 ± 10              | 17,000 ± 2,000           |
| L-364,718         | 1.3 ± 0.1             | 1.5 ± 0.2            |                          |
| L-365,260         | 2,600 ± 170           | 13,000 ± 3,000       | 13,000 ± 3,000           |
| Sites/cell (x10^3)| 34 ± 4                | 434 ± 28             | 645 ± 32                 |

Fig. 2. Abilities of CCK-8, gastrin CCK-JMV-180, L-364,718, and L-365,260 to inhibit binding of [3H]-CCK-8 (upper panel) and [3H]-L-364,718 (lower panel) to COS cells that had been transfected with the rat CCK-A receptor. To measure binding of [3H]-CCK-8, transfected cells were incubated at 37 °C for 120 min with 50 pm [3H]-CCK-8 plus the indicated concentrations of CCK-8, gastrin, and CCK-JMV-180 (panel A), or L-364,718 and L-365,260 (panel B). To measure binding of [3H]-L-364,718, transfected cells were incubated at 37 °C for 60 min with 400 pm [3H]-L-364,718 plus the indicated concentrations of CCK-8, gastrin, and CCK-JMV-180 (panel C), or L-364,718 and L-365,260 (panel D). Results for saturable binding of each radiolabeled ligand are expressed as a percentage of the value measured with the radiolabeled ligand alone (i.e. percent control). Results given are means from at least three experiments. Vertical bars indicate S.E.

Table II
Parameters that characterize ligand binding to the transfected rat CCK-B receptor

| Ligand     | High affinity Kd (nM) | Low affinity Kd (nM) | Very low affinity Kd (nM) |
|------------|-----------------------|----------------------|--------------------------|
| CCK-8      | 0.52 ± 0.12           | 2.5 ± 1.0            |                          |
| Gastrin    | 1.6 ± 1.0             | 40 ± 17              |                          |
| CCK-JMV-180| 55 ± 14               | 1,100 ± 500          |                          |
| L-364,718  | 1,100 ± 230           | >100,000             |                          |
| L-365,260  | 35 ± 17               | 330 ± 160            |                          |
| Sites/cell (x10^3)| 40 ± 9                  | 43 ± 10               |                          |

In contrast to L-364,718, however, the affinities of L-365,260 for the very low affinity states were approximately 5 times less than that for the high affinity state (Table I).

The values in Table I for sites/cell agree with those determined in a previous study (6) and indicate that 3% of the transfected CCK-A receptors are in the high affinity state, 39% are in the low affinity state, and 58% are in the very low affinity state.

Fig. 2 illustrates the abilities of various ligands to inhibit binding of [3H]-CCK-8 to COS cells that had been transfected with the rat CCK-B receptor. Table II gives the parameter values that result from analysis of the data in Fig. 3. In agreement with a previous study (2), in inhibiting binding of [3H]-CCK-8, CCK-8 was slightly more potent than gastrin, and L-365,260 was substantially more potent than L-364,718. In contrast to comparable results obtained when measuring binding of [3H]-CCK-8 to transfected CCK-A receptors, where CCK-JMV-180 was 3-4 times less potent than CCK-8, with transfected rat CCK-B receptors CCK-JMV-180 was at least 100 times less potent than CCK-8. With each ligand tested, binding of [3H]-CCK-8 to rat CCK-B receptors was fit better by a two-site model than by a one-site model (Table II). Approximately half of the transfected receptors had a high affinity for CCK-8 (K_H = 0.52 nm), the high affinity state, and half had a lower affinity for CCK-8 (K_L = 2.5 nm, the low affinity state).

As indicated in Table II, the affinity of CCK-8 for the low affinity state of the rat CCK-B receptor was only 5 times less than its affinity for the high affinity state in contrast to it being approximately 100 times less with the rat CCK-A receptor (Table I). With gastrin and CCK-JMV-180, the corresponding differences between the affinities for the high and low affinity states of the rat CCK-B receptor were 25 and 20 times, respectively. The two CCK receptor antagonists also had different affinities for the two states of the rat CCK-B receptor. The affinities of L-365,260 and L-364,718 for the low affinity state were 9 and 91 times lower, respectively, than the corresponding affinities for the high affinity state.

With transfected rat CCK-B receptors, there was only a small amount of saturable binding of the radiolabeled receptor antagonist [3H]-L-365,260; however, with transfected canine CCK-B receptors there was substantial saturable binding of [3H]-L-364,718 (60% of total binding). This made it possible with the transfected canine CCK-B receptors to detect the high and low affinity states with [3H]-CCK-8 and the low and very low affinity states with [3H]-L-364,718 (Fig. 4, Table III).

In COS cells that had been transfected with canine CCK-B receptors, the effects of CCK-8 and gastrin on binding of [3H]-CCK-8 (Fig. 4) were similar to those reported previously by others (10), and the parameter values that characterize these effects (Table III) were essentially the same as those obtained with the rat CCK-B receptor (Fig. 3, Table II). With CCK-JMV-
L-365,260 to inhibit binding of \(^{3}H\)CCK-8 to COS cells that had been transfected with the canine CCK-B receptor. To measure binding of \(^{3}H\)CCK-8, transfected cells were incubated at 37°C for 90 min with 80 pm \(^{3}H\)CCK-8 plus the indicated concentrations of CCK-8, gastrin, and CCK-JMV-180 (panel A) or L-364,718 and L-365,260 (panel B). Results for saturable binding of \(^{3}H\)CCK-8 are expressed as a percentage of the value measured with \(^{3}H\)CCK-8 alone (i.e., percent control). Results given are means from at least three experiments. Vertical bars indicate S.E.

FIG. 3. Abilities of CCK-8, gastrin CCK-JMV-180, L-364-718, and L-365,260 to inhibit binding of \(^{3}H\)CCK-8 to COS cells that had been transfected with the rat CCK-B receptor. To measure binding of \(^{3}H\)CCK-8, transfected cells were incubated at 37°C for 90 min with 80 pm \(^{3}H\)CCK-8 plus the indicated concentrations of CCK-8, gastrin, and CCK-JMV-180 (panel A) or L-364,718 and L-365,260 (panel B). Results for saturable binding of \(^{3}H\)CCK-8 are expressed as a percentage of the value measured with \(^{3}H\)CCK-8 alone (i.e., percent control). Results given are means from at least three experiments. Vertical bars indicate S.E.

FIG. 4. Abilities of CCK-8, gastrin CCK-JMV-180, L-364-718, and L-365,260 to inhibit binding of \(^{3}H\)CCK-8 (upper panel) and \(^{3}H\)L-364,718 (lower panel) to COS cells that had been transfected with the canine CCK-B receptor. To measure binding of \(^{3}H\)CCK-8, transfected cells were incubated at 37°C for 60 min with 80 pm \(^{3}H\)CCK-8 plus the indicated concentrations of CCK-8, gastrin, and CCK-JMV-180 (panel A) or L-364,718 and L-365,260 (panel B). To measure binding of \(^{3}H\)L-364,718, transfected cells were incubated at 37°C for 60 min with 400 pm \(^{3}H\)L-364,718 plus the indicated concentrations of CCK-8, gastrin, and CCK-JMV-180 (panel C), or L-364-718 and L-365,260 (panel D). Results for saturable binding of each radiolabeled ligand are expressed as a percentage of the value measured with the radiolabeled ligand alone (i.e., percent control). Results given are means from at least three experiments. Vertical bars indicate S.E.

TABLE III

| Ligand | \(^{125}\)I-CCK-8 binding \(K_d\) \(nm\) | \(^{3}H\)L-364,718 binding \(K_d\) \(nm\) |
|--------|-------------------------------|-------------------------------|
| CCK-8  | 0.5 ± 0.2                     | >100,000                      |
| Gastrin| 0.5 ± 0.3                     | >100,000                      |
| CCK-JMV-180 | 400 ± 275                 | 80 ± 27                       |
| L-364,718 | 20 ± 29                      | 18 ± 2                       |
| L-365,260 | 800 ± 138                    | 100 ± 50                     |

S.E. calculated from the data illustrated in Fig. 4.

CCK-8, gastrin, and CCK-JMV-180 were substantially more potent in inhibiting binding of \(^{3}H\)L-364,718 to the canine CCK-B receptor than to the rat CCK-A receptor (compare Figs. 4 and 2). As indicated by Table III, the data for inhibition of binding of \(^{3}H\)L-364,718 by CCK-8 or gastrin (Fig. 4) were best fit by a two-site model and the values of \(K_d\) for the higher affinity state detected by \(^{3}H\)L-364,718 binding were essentially the same as those for the lower affinity state detected by binding of \(^{125}\)I-CCK-8. The closeness of these values indicates that they both reflect interaction of CCK-8, gastrin, and CCK-JMV-180 with the low affinity state of the canine CCK-B receptor. The second class of sites detected with \(^{3}H\)L-364,718 appeared to reflect a very low affinity state of the canine CCK-B receptor with affinities for CCK-8, gastrin, and CCK-JMV-180 that were much lower than the corresponding affinities for the low affinity state. The parameter values given in Table III indicate that 19% of the transfected canine CCK-B receptors are in the high affinity state, 65% are in the low affinity state, and 16% are in the very low affinity state.

With canine CCK-B receptors, both L-364,718 and L-365,260 were significantly more potent in inhibiting binding of \(^{3}H\)L-364,718 than in inhibiting binding of \(^{125}\)I-CCK-8 (Fig. 4). This resulted from these two receptor antagonists having higher affinities for the low and very low affinity states of the receptor than for the high affinity state (Table III). Moreover, in contrast to the inhibition of binding of \(^{3}H\)L-364,718 caused by CCK-8, gastrin, and CCK-JMV-180, that caused by L-364,718 or by L-365,260 was best fit by a one-site model indicating that the two states detected with CCK-8, gastrin, and CCK-JMV-180 have the same affinities for L-364,718 and for L-365,260 (Table III).

In agreement with a previous report (13), replacing the leucine in position 355 of the canine CCK-B receptor with valine reversed the potencies with which L-365,260 and L-364,718 inhibited binding of \(^{125}\)I-CCK-8 (Fig. 5). That is, with rat CCK-B receptors (Fig. 3) and canine M-CCK-B receptors (Fig. 5) L-365,260 was more potent than L-365,260 in inhibiting binding of \(^{125}\)I-CCK-8, whereas with wild type canine CCK-B receptors, L-364,718 was more potent than L-365,260 in inhibiting binding of \(^{125}\)I-CCK-8 (Fig. 4). Replacement of leucine-355 with valine in the canine CCK-B receptor also resulted in substantial saturable binding of \(^{3}H\)L-364,260 (Figs. 1 and 5). As a result, COS cells transfected with the canine M-CCK-B receptor, binding of \(^{125}\)I-CCK-8 detected the high and low affinity states of the receptor, and binding of \(^{3}H\)L-364,718 detected the low and very low affinity states of the receptor (Table IV). The parameter values in Table IV indicate that 8%
of the canine transfected M-CCK-B receptors in the high affinity state, 78% are in the low affinity state, and 14% are in the very low affinity state. The patterns (Fig 5) and parameter values (Table IV) with which CCK-8, gastrin, and CCK-JMV-180 inhibited binding of [3H]-CCK-8 and [3H]-CCK-718 were similar in the wild type and the mutant canine CCK-B receptors. In addition to the reversed relative potencies of L-364,718 and L-365,260, each of the three different states of the canine M-CCK-B receptor had the same affinities for L-365,260 and for L-364,718 (Table IV).

**DISCUSSION**

Several findings in the present study confirm previous observations by us (2, 6, 8, 10, 13, 16) and others (7, 9, 15, 17) using intact cells, membranes, or cultured cells transfected with a particular receptor. In terms of their abilities to inhibit binding of [3H]-CCK-8, CCK-8 is much more potent than gastrin at CCK-A receptors and slightly more potent than gastrin at CCK-B receptors. Similarly, L-364,718 is more potent than

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**Table IV**

| Ligand          | **[3H]-CCK-8 binding Kd** | **[3H]-L-365,260 binding Kd** |
|-----------------|---------------------------|-------------------------------|
|                 | High affinity | Low affinity | Low affinity | Very low affinity |
| CCK-8           | 0.4 ± 0.1     | 3 ± 1        | 7 ± 1        | >1,000,000       |
| Gastrin         | 0.8 ± 0.1     | 12 ± 7       | 16 ± 2       | >1,000,000       |
| CCK-JMV-180     | 100 ± 18      | 20 ± 6       | 45 ± 8       | 8,000 ± 6,100    |
| L-364,718       | 240 ± 30      | 240 ± 30     | 180 ± 10     | 180 ± 10         |
| L-365,260       | 19 ± 6        | 19 ± 6       | 17 ± 2       | 17 ± 2           |

**Sites/cell (×10⁵)**: 88 ± 11, 760 ± 140, 877 ± 55, 152 ± 15

**Figure 5**

Abilities of CCK-8, gastrin CCK-JMV-180, L-364-718, and L-365-260 to inhibit binding of [3H]-CCK-8 (upper panel) and [3H]-L-364,718 (lower panel) to COS cells that had been transfected with the canine M-CCK-B receptor. To measure binding of [3H]-CCK-8, transfected cells were incubated at 37 °C for 60 min with 80 pm [3H]-CCK-8 plus the indicated concentrations of CCK-8, gastrin, and CCK-JMV-180 (panel A), or L-364,718 and L-365-260 (panel B). To measure binding of [3H]-CCK-718, transfected cells were incubated at 37 °C for 60 min with 40 pm [3H]-CCK-718 plus the indicated concentrations of CCK-8, gastrin, and CCK-JMV-180 (panel C), or L-364-718 and L-365-260 (panel D). Results for saturable binding of each radiolabeled ligand are expressed as a percentage of the value measured with the radiolabeled ligand alone (i.e., percent control). Results given are means from at least three experiments. Vertical bars indicate S.E.

**Table V**

| Number of states | Rat CCK-A | Rat CCK-B | Canine CCK-B | Canine M-CCK-B |
|------------------|-----------|-----------|--------------|---------------|
| % Distribution   | (high/low/very low) | (high/low/very low) | (high/low/very low) | (high/low/very low) |
| Ligand used to detect very low affinity state | L-364a | L-365a | L-364a | L-365a |
| Agonist with highest affinity for low statea | JMV-180 CCK-8 | CCK-8 | CCK-8 | CCK-8 |
| Agonist with highest affinity for very low stateb | JMV-180 | JMV-180 | JMV-180 | JMV-180 |
| CCK-8 affinity ratio high/low | 174 | 5 | 10 | 10 |
| L-364,718 affinity ratio high/low | 1 | >100 | 0.1 | 1 |
| L-365,260 affinity ratio high/low | 5 | 9 | 0.1 | 1 |

a L-364 = [3H]-L-364,718; L-365 = [3H]-L-365,260; JMV-180 = CCK-JMV-180.
b Affinity was calculated as 1/Kd.

L-364,718 at CCK-A and canine CCK-B receptors, whereas L-365,260 is more potent than L-364,718 at rat CCK-B and canine M-CCK-B receptors. Except for our previous study using pancreatic acini and COS cells that had been transfected with CCK-A receptors (6) other measurements of binding of [3H]-CCK-718 or [3H]-L-365,260 have used broken cell preparations (9, 15). In both instances, however, measurements of binding of the radiolabeled receptor antagonist have detected receptors that have a very low affinity for CCK-8.

Table V summarizes the salient features of the different CCK receptors examined in the present study. Each receptor, when expressed in COS cells, exists in three different affinity states for CCK-8. These results directly demonstrate that the ability to exist in three different affinity states is an intrinsic property of the receptor molecule itself. Moreover, the previous findings that have demonstrated a very low affinity state of a particular CCK receptor in membrane preparations (9, 15) indicate that the very low affinity state of the different receptors is not a peculiar characteristic of CCK receptors expressed in COS cells.

With transfected CCK-A receptors most (58%) are in the very low affinity state, whereas with canine CCK-B receptors most (65 and 78%) are in the low affinity state (Table V). With transfected rat CCK-B receptors, nonsaturable binding of [3H]-L-365,260 was sufficiently high that dose-inhibition curves could not be performed with meaningful precision. On the other hand, measurements of binding of [3H]-L-365,260 to transfected rat CCK-B receptors did show clearly that these receptors can exist in a very low affinity state, and our results indicate that most of these transfected receptors, like the transfected rat CCK-A receptors, have to be in the very low affinity state for us to be able to detect any saturable binding of [3H]-L-365,260.

Of the three receptor agonists tested, CCK-8 had the highest affinity for the high affinity state of each transfected receptor. As indicated in Table V, CCK-8 also had the highest affinity for the low affinity state of each CCK-B receptor; however, CCK-JMV-180 had the highest affinity for the low affinity state of rat CCK-A receptors. We do not know the basis for CCK-JMV-180 having a higher affinity than CCK-8 for the low affinity state of the rat CCK-A receptor, but this is not peculiar to the transfected receptor because the low affinity state of the CCK-A
receptor in rat pancreatic acini also has a higher affinity for CCK-JMV-180 than for CCK-8 (18, 19). CCK-JMV-180 also had a higher affinity than CCK-8 for the very low affinity states of rat CCK-A and canine CCK-B receptors and may represent an important "lead compound" for developing ligands with higher affinities for the very low affinity states of the CCK receptors. Moreover, these findings with CCK-JMV-180 raise the possibility that CCK-8 is not the "preferred ligand" for the very low affinity state. That is, there may be an endogenous ligand, not yet discovered, that has a high affinity for the very low affinity state of the CCK receptor.

As shown in Table V, CCK-8, L-364,718, and L-365,260 differ in their relative affinities for the high and low affinity states of the various transfected CCK receptors. With CCK-8, the differences in affinities between the high and low affinity states were much greater for rat CCK-A receptors than for the various CCK-B receptors. L-364,718 had the same affinity for the high and low affinity states of rat CCK-A and canine M-CCK-B receptors, had a greater than 100-fold higher affinity for the high affinity state of rat CCK-B receptors and had a 10-fold lower affinity for the high affinity state of canine CCK-B receptors. In contrast, L-365,260 had the same affinity for the high and low affinity states of canine M-CCK-B receptors, a 5-9-fold greater affinity for the high affinity states of rat CCK-A and CCK-B receptors, and a 10-fold lower affinity for the high affinity state of canine CCK-B receptors.

A previous study (13) showed that the methyl group on the side chain of the leucine residue in position 355 of the canine CCK-B receptor is a critical determinant of the relative affinities of L-364,718 and L-365,260 for the canine CCK-B receptor. The present study illustrates that this previous study actually examined interaction of the receptor antagonist with the high and low affinity states of canine CCK-B receptors. The present study also shows that replacing leucine with valine in position 355 of the canine CCK-B receptor reverses the affinities of L-364,718 and L-365,260 for each of the three different affinity states of the receptor. As indicated in Table V, this modification of the canine CCK-B receptor also changes the relative affinities of both L-364,718 and L-365,260 for the high and low affinity states of the receptor. That is, in canine CCK-B receptors, both L-364,718 and L-365,260 have 10-fold higher affinities for the low affinity state of the receptor than for the high affinity states of the receptor. Replacing leucine 355 by valine alters the receptor in such a way that both L-364,718 and L-365,260 have the same affinity for the high and low affinity states of the receptor. It is also important to note that although the canine M-CCK-B receptor resembles the rat CCK-B receptor in that each state of both receptors has a higher affinity for L-365,260 than for L-364,718, the canine M-CCK-B receptor lacks other salient features of the rat CCK-B receptor. For example, with rat CCK-B receptors, both L-364,718 and L-365,260 have 10- to greater than 100-fold higher affinities for the high affinity state than for the low affinity state, but with canine M-CCK-B receptors, both ligands have the same affinities for the high and low affinity states. Thus, other residues besides the leucine in position 355 are important in influencing the interactions of different CCK receptor antagonists with the canine CCK-B receptor.

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