Antagonistic Effect of YM022, an Antiulcer Agent in Rats, on Human Cholecystokinin $(CCK)_B$/Gastrin Receptor

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ABSTRACT—We recently isolated a cDNA clone for the human cholecystokinin $(CCK)_B$/gastrin receptor and permanently expressed this receptor cDNA in NIH-3T3 cells. [^{125}I]CCK-8 specifically bound to the membrane of the transfectant, and this binding was displaced by unlabeled CCK-8 with an IC_{50} of 0.32 nM. Treatment of these cells with CCK-8 increased the intracellular Ca^{2+} concentration with an EC_{50} of 0.30 nM. Using these cells expressing functional human CCK_B/gastrin receptors, we investigated the pharmacological properties of (*R*)-1-[2,3-dihydro-1-(2′-methylphenacyl)-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl) urea (YM022), a potent and selective CCK_B/gastrin receptor antagonist in rats. YM022 potently inhibited [^{125}I]CCK-8 binding to the membrane with an IC_{50} of 55 pM and CCK-8-induced Ca^{2-} mobilization with that of 7.4 nM. On the other hand, its racemate and enantiomer more weakly inhibited this binding (IC_{50} of 110 pM and 11 nM, respectively) and Ca^{2+} mobilization (IC_{50} of 18 nM and 94 nM, respectively). These results indicate that YM022 stereoselectively recognizes the human CCK_B/gastrin receptor as a potent antagonist and that the established transfectant is useful for characterization of human CCK_B/gastrin-receptor ligands.

Keywords: YM022, Cholecystokinin (CCK)-8, CCK_B/gastrin receptor, Calcium mobilization, Stable expression

Cholecystokinin (CCK) is a gut-brain peptide that exerts a variety of physiological actions in the gastrointestinal tract and central nervous system through cell surface CCK receptors. On the basis of their pharmacological properties and specificities for ligand binding, these CCK receptors have been divided into two subtypes, namely, the CCK_A and CCK_B/gastrin receptors. The CCK_A receptor controls pancreatic secretion, gut mobility, while the CCK_B/gastrin receptor is involved in anxiety, analgesia and satiety in the central nervous system (1, 2). In the gastrointestinal tract, the CCK_B/gastrin receptor plays an important role in gastric histamine release and acid secretion (3) and enterochromaffin-like cell growth (4). These differences in pharmacological action aroused interest in antagonists for these subtypes, leading to the development of benzodiazepine derivatives L-364,718 (5) and L-365,260 (6) as selective CCK_A and CCK_B/gastrin antagonists, respectively.

We synthesized YM022 as a more potent and selective CCK_B/gastrin-receptor ligand than L-365,260. YM022 replaces the specific binding of radiolabeled ligand to rat brain CCK_B/gastrin receptor more potently than rat pancreatic CCK_A receptor and inhibits pentagastrin-induced gastric acid secretion (7) and prevents gastric and duodenal lesions in rats (8). These findings showed that YM022 acts as a selective rat CCK_B/gastrin-receptor antagonist both in vitro and in vivo and that this compound is a useful therapeutic agent for peptic ulcer disease.

Recently, the CCK_B/gastrin receptors were cloned from human brain (9, 10), dog parietal cells (11), rat brain (12) and *Mastomys natalensis* enterochromaffin-like cells (13). These receptors possess putative seven transmembrane domains, suggesting that they are rhodopsin-type G protein-coupled receptors. Expression studies have revealed that both human and dog receptors generate phosphoinositide breakdown leading to intracellular Ca^{2+} ($[Ca^{2+}]_i$) mobilization in response to a CCK agonist, CCK-8. Although the human and dog CCK_B/gastrin receptors share 90% amino acid homology and have similar affinities for CCK-8, the binding of L-365,260 and L-364,718 to these receptors differs by up to 20-fold. This difference in binding is due to the substitution of a single amino acid in the sixth transmembrane domain only (14). For the development of a human CCK_B/gastrin receptor

antagonist as an antiulcer drugs, it is therefore important to examine whether a CCK_B/gastrin ligand exerts the same actions in both humans and other species.

To determine whether YM022 is an antagonist for both the human and rat CCK_B/gastrin receptor, the functional human CCK_B/gastrin-receptor cDNA isolated in our laboratory (15) was permanently expressed in NIH-3T3 cells. We then investigated whether YM022 inhibited [^{125}I]CCK-8 binding and CCK-8-induced $[Ca^{2+}]_i$ mobilization in these human CCK_B/gastrin receptor-expressing cells.

MATERIALS AND METHODS

Materials

YM022 ((*R*)-1-[2,3-dihydro-1-(2′-methylphenacyl)-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl) urea), its racemate and *S*-enantiomer, 3*R*(+)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl)-*N*′-(3-methylphenyl) urea (L-365,260), and 3*S*(−)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl)-1*H*-indole-2-carboxamide (L-364,718) were chemically synthesized in our laboratory. [^{125}I]CCK-8 (CCK-8 sulfated, [^{125}I]labeled with Bolton-Hunter reagent; 74 TBq/mmol) was purchased from Amersham International (Amersham, UK) and sulfated CCK-8 (CCK-8) from Peptide Institute (Osaka). Fura-2/acetoxymethylester (fura-2/AM) was from Dohjin Chemicals (Kumamoto). Lipofectamine™, Opti-MEM® and geneticin were from Gibco (Grand Island, NY, USA). Other chemicals used were of analytical grade.

Expression of human CCK_B/gastrin receptor in NIH-3T3 fibroblasts

A stable transformed NIH-3T3 cell line was established as follows: The coding region of human CCK_B/gastrin receptor was subcloned into an expression vector, pEF-BOS (16), carrying a resistant gene against neomycin. After the expression plasmid DNA (2 μg) and Lipofectamine™ (15 μl) were incubated in 200 μl of Opti-MEM® for 30 min at 37℃, 800 μl of Opti-MEM® was added. The mixture was transfected into NIH-3T3 cells (4×10^5 cells) cultured on a 35-mm dish. After 6 hr of transfection, the medium was replaced with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (DMEM). NIH-3T3 cell clones were established by selection with 400 μg/ml geneticin.

Binding experiments

The NIH-3T3 cells permanently expressing the cDNA were washed with ice-cold lysis buffer [10 mM HEPES buffer (pH 7.4) containing 130 mM NaCl, 5 mM $MgCl_2$,

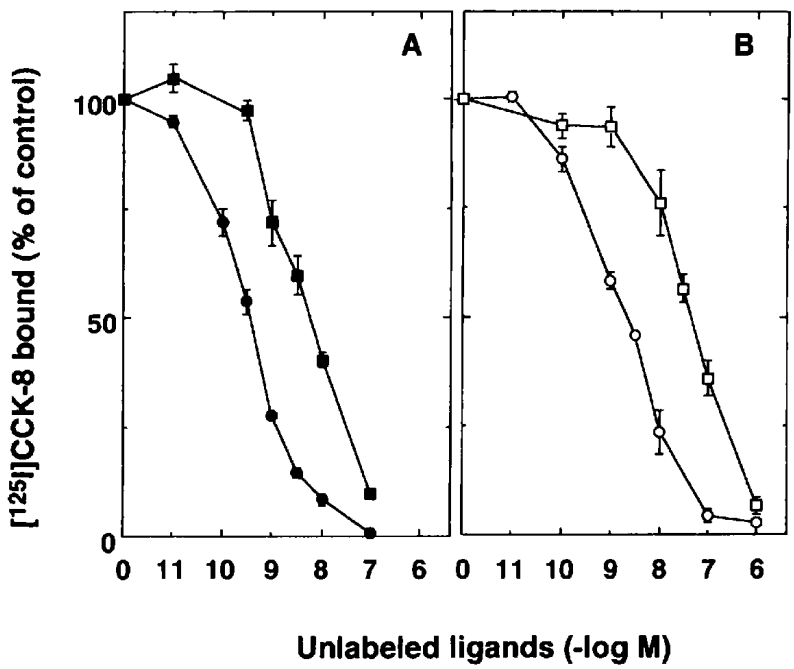

Fig. 1. Displacement of [^{125}I]CCK-8 binding to membrane of NIH-3T3 cells permanently expressing human CCK_B/gastrin receptor by CCK/gastrin ligands. Membranes of NIH-3T3 cells transfected with human CCK_B/gastrin receptor cDNA were incubated at 37℃ for 60 min with 0.1 nM [^{125}I]CCK-8 in the presence of the indicated concentrations of CCK/gastrin agonists (A) and antagonists (B). Ligands used were CCK-8 (●), gastrin (■), L-365,260 (○) and L-364,718 (□). The data shown are the means±S.E.M. for triplicate determinations.

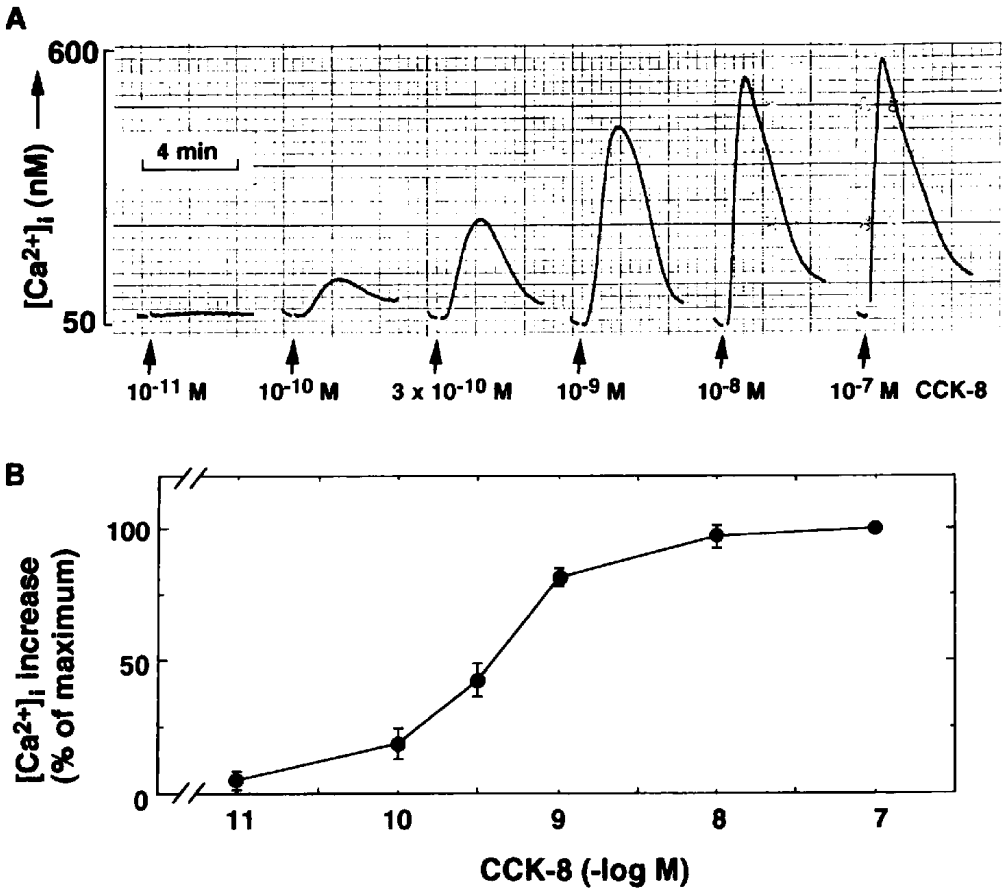

Fig. 2. Effect of CCK-8 on $[Ca^{2+}]_i$ mobilization in NIH-3T3 cells expressing human CCK_B/gastrin receptor. NIH-3T3 cells expressing human CCK_B/gastrin receptor were loaded with fura-2/AM and then treated with the indicated concentrations of CCK-8. A: The recordings shown are representative of three independent experiments that yielded similar results. B: The data shown are the means±S.E.M. for triplicate determinations. The basal $[Ca^{2+}]_i$ was 54±6.6 nM, and the maximal $[Ca^{2+}]_i$, which was increased by 100 nM CCK-8, was 575±65 nM (n=6).

and 0.25 mg/ml bacitracin] and then homogenized in the lysis buffer with 200 strokes of a Dounce homogenizer (Type B). The homogenate was centrifuged at 38,000×*g* for 30 min at 4℃. The pellet was resuspended in the lysis buffer and used as the crude membrane. Radioligand binding studies were carried out for 60 min at 37℃ in 250 μl of lysis buffer containing 0.1% bovine serum albumin. The binding reaction was started by the addition of 100 pM of [^{125}I]CCK-8 to 20 μg of protein from the crude membrane and terminated by rapid filtration on glass fiber filters (Filtermat A; Pharmacia, Turku, Finland) using a cell harvester. The filter was washed with ice-cold 50 mM Tris buffer (pH 7.4) containing 0.01% bovine serum albumin, and the radioactivity on the filter was counted with a gamma-counter (Packard, Meriden, CT, USA). Nonspecific binding was determined in the presence of 1 μM of unlabeled CCK-8. Analysis of binding data was performed as previously described (17).

Measurement of $[Ca^{2-}]_i$ mobilization

$[Ca^{2-}]_i$ in cells permanently expressing the human CCK_B/gastrin receptor was measured by fura-2 fluorometry. Cells cultured on a 13-mm diameter cover glass were loaded with 4 μM fura-2/AM in HEPES-buffered salt solution [HBSS: 20 mM HEPES (pH 7.4), 140 mM NaCl, 4 mM KCl, 1 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose and 0.05% bovine serum albumin] for 30 min at 37℃. After incubation, the cover glass was washed with HBSS and positioned in a quartz cuvette at 25℃ in a CAF/100 fluorescence spectrometer (Japan Spectroscopic Co., Tokyo). A final volume of 2 ml of HBSS with test reagents was stirred continuously at 1,000 rpm. The fluorescence was measured at excitation wavelengths of 340 and 380 nm and the emission wavelength of 500 nm. The level of $[Ca^{2+}]_i$ was calculated as described previously (17).

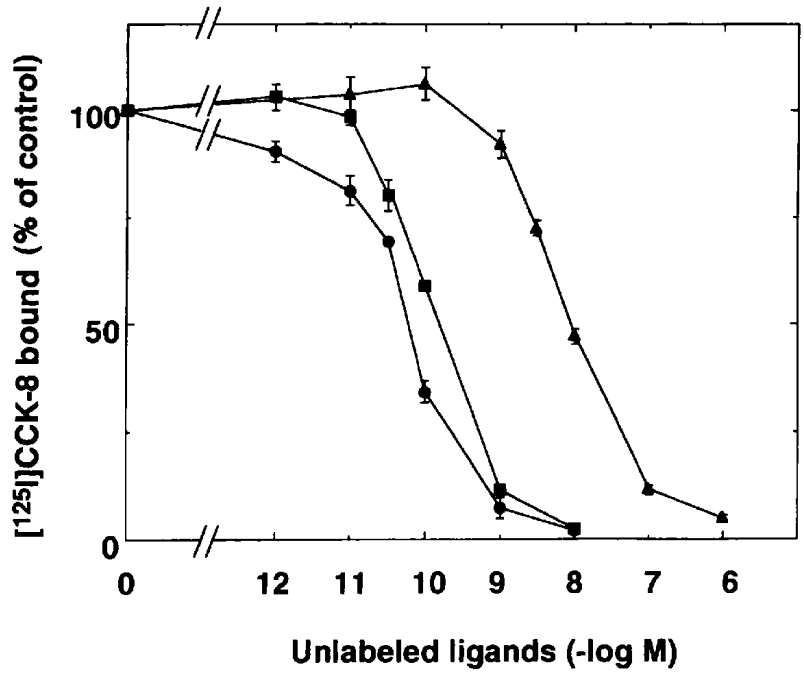

Fig. 3. Displacement of [^{125}I]CCK-8 binding to human CCK_B/gastrin receptor by YM022. The same membranes used in Fig. 1 were incubated at 37℃ for 60 min with 0.1 nM [^{125}I]CCK-8 in the presence of the indicated concentrations of YM022 (●), its racemate (■) and its enantiomer (▲). The data shown are the means±S.E.M. for triplicate determinations.

RESULTS

To investigate the biological properties of human CCK_B/gastrin receptor, we attempted to establish NIH-3T3 cells permanently expressing this receptor cDNA by selection with geneticin. To confirm the expression of human CCK_B/gastrin receptor, we examined [^{125}I]CCK-8 binding to the membrane from the selected cells. As shown in Fig. 1, [^{125}I]CCK-8 bound specifically to the membrane (67.8±2.1 fmol/mg protein), and unlabeled CCK-8 and gastrin I displaced the binding with an IC_{50} value of 0.32 and 4.4 nM, respectively. Furthermore, the CCK_B/gastrin antagonist L-365,260 more potently inhibited the binding (IC_{50}=1.8 nM) than the CCK_A antagonist L-364,718 (IC_{50}=37 nM). No specific binding was found in either untransfected NIH-3T3 cells or mock-transfected cells (data not shown).

Table 1. IC_{50} values of CCK/gastrin receptor ligands against [^{125}I]CCK-8 binding to human CCK_B/gastrin receptor

Ligands	IC_{50} (nM)	
CCK-8	0.32	(0.30–0.34)
Gastrin	4.4	(4.2–4.6)
YM022	0.055	(0.046–0.067)
Racemate	0.11	(0.10–0.12)
S-isomer	10.7	(9.8–11.7)
L-365,260	1.8	(1.7–1.9)
L-364,718	37.2	(30.1–46.2)

IC_{50} values were determined from the data in Figs. 1 and 3. Numbers in parentheses show the 95% confidence limits.

To elucidate whether the expressed human CCK_B/gastrin receptor could couple to intracellular signaling, we examined the effect of CCK-8 on $[Ca^{2+}]_i$ mobilization (Fig. 2). Treatment of the cells with CCK-8 at 0.1 to 100 nM increased $[Ca^{2+}]_i$ in a concentration-dependent manner with an EC_{50} value of 0.30 nM. No CCK-8-induced $[Ca^{2+}]_i$ mobilization was seen in either untransfected NIH-3T3 cells or mock-transfected cells (data not shown). These results indicate that the selected NIH-3T3 cells express a functional human CCK_B/gastrin receptor, which generates $[Ca^{2+}]_i$ mobilization in response to CCK-8.

Using these functional human CCK_B/gastrin receptor-expressing cells, we investigated the pharmacological properties of YM022, a potent and selective CCK_B/gastrin-receptor antagonist in rats (7). Figure 3 shows the displacement curves of [^{125}I]CCK-8 binding to the membrane by YM022, and Table 1 summarizes the IC_{50} values of CCK ligands for [^{125}I]CCK-8 binding. YM022 most potently inhibited this binding with an IC_{50} value of 55 pM, while its racemate and enantiomer inhibited the binding with values of 110 pM and 11 nM, respectively.

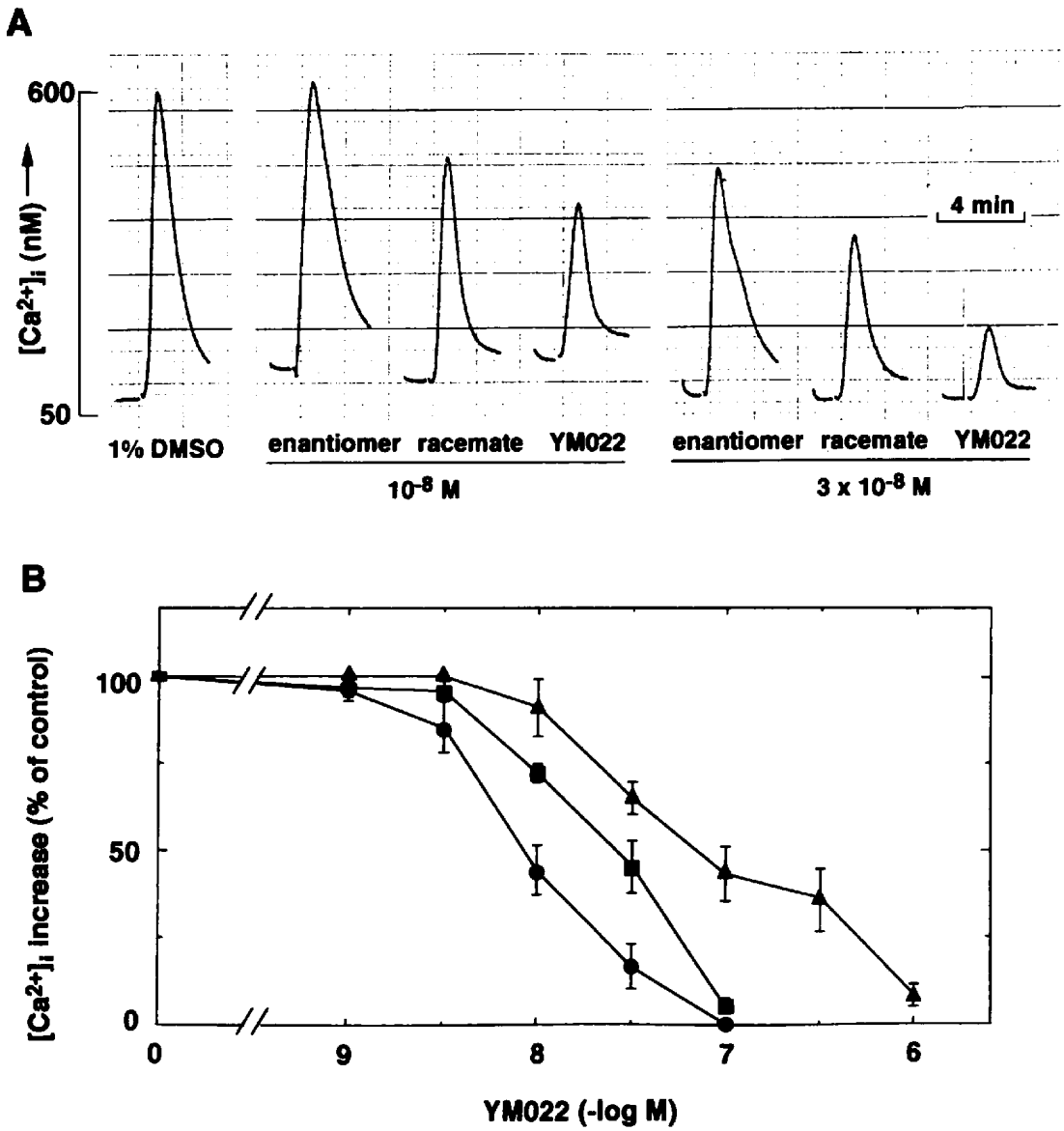

Fig. 4. Inhibitory effect of YM022 on CCK-8-induced $[Ca^{2+}]_i$ mobilization in NIH-3T3 cells expressing human CCK_B/gastrin receptor. Fura-2-loaded NIH-3T3 cells expressing human CCK_B/gastrin receptor were preincubated for 1 min with the indicated concentrations of YM022 (●), its racemate (■) or its enantiomer (▲) and then exposed to 10 nM CCK-8. A: The recordings shown are representative of three independent experiments that yielded similar results. B: The data shown are the means±S.E.M. for triplicate determinations.

The inhibitory effect of YM022 on [^{125}I]CCK-8 binding was about 33-fold more potent than that of L-365,260.

We next examined the effect of YM022 on $[Ca^{2+}]_i$ mobilization to determine whether YM022 acts on human CCK_B/gastrin receptor as an agonist or antagonist (Fig. 4). YM022 did not increase $[Ca^{2+}]_i$ mobilization at any concentration from 1 nM to 1 μM, but pretreatment with YM022 for 1 min dose-dependently inhibited 10 nM CCK-8-induced $[Ca^{2+}]_i$ mobilization with an IC_{50} value of 7.4 nM. The respective IC_{50} values of the racemate and enantiomer were 18 and 94 nM. These results indicate that YM022 and its enantiomer stereoselectively recognize human CCK_B/gastrin receptor as antagonists.

DISCUSSION

We have demonstrated that YM022, a rat CCK_B/gastrin antagonist, is also a potent antagonist for human CCK_B/gastrin receptor permanently expressed in NIH-3T3 cells. Preliminary experiments revealed that CCK-8 induced $[Ca^{2+}]_i$ mobilization in dihydrofolate reductase-deficient CHO cells but not in NIH-3T3 cells, which was

consistent with a previous report (18). We therefore transfected the human CCK_B/gastrin-receptor cDNA isolated in our laboratory (15) into NIH-3T3 cells. YM022 inhibited 0.1 nM [^{125}I]CCK-8 binding and 10 nM CCK-8-induced $[Ca^{2+}]_i$ mobilization in the transfected cells with IC_{50} values of 55 pM and 7.4 nM, respectively. These values were well-consistent with our previous reports that YM022 inhibited binding to rat brain CCK_B/gastrin receptor with a K_i of 68 pM (7) and $[Ca^{2+}]_i$ mobilization in rat GH3 cells with an IC_{50} of 4.4 nM (17). These results indicate that the binding sites for YM022 are conserved between human and rat CCK_B/gastrin receptor. On the other hand, YM022 weakly inhibited [^{125}I]CCK-8 binding with an IC_{50} of 730 pM in the cloned dog CCK_B/gastrin receptor (A. Miyake, unpublished observation), suggesting the dog receptor may not conserve the binding sites for YM022.

CCK_B/gastrin receptors have been cloned from humans (9, 10), rats (11), dogs (12) and *Mastomys natalensis* (13). All were shown to belong to a family of G protein-coupled receptors. It has been suggested that the ligand binding sites of G protein-coupled receptors exist within transmembrane domains. Importantly, recent developments in molecular biology have revealed that a single amino acid difference in the transmembrane domains can cause pharmacological variation between species of G protein-coupled receptors, such as 5-HT_{1B} (19) and NK_1 (20). Meanwhile, Beinborn et al. (14) clearly demonstrated a difference in ligand binding properties between human and dog CCK_B/gastrin receptors by transient expression of these receptors in COS cells. The human receptor binds L-365,260 with higher affinity than L-364,718 (IC_{50}=4.5 nM vs 147 nM, respectively), whereas in the dog receptor, this order of affinity is reversed (IC_{50}=80 nM vs 14 nM, respectively). The single amino acid substitution of valine in the sixth transmembrane domain of human receptor with a leucine, a dog counterpart, changes the binding property of the human receptor to that of the dog (14). The rat CCK_B/gastrin receptor, with a valine residue at this site, has higher affinity for L-365,260 than L-364,718 (6). These observations correlate well with the previous in vivo data that L-365,260 inhibited gastrin-stimulated acid secretion more strongly in rats than in dogs (6). Judging from the similarity in pharmacological properties and benzodiazepine structure, the valine residue in the sixth transmembrane domain may be essential for YM022 and L-365,260 binding to the human and rat CCK_B/gastrin receptors.

Comparison of the cDNA sequences of human and rat CCK_B/gastrin receptor shows that seven amino acids are different in their transmembrane domains. In the present study, these differences did not cause a dramatic change in affinity for CCK ligands similar to that above. However, the different residue(s) appeared to determine the stereoselectivity of YM022. YM022 and its *S*-isomer displaced [^{125}I]CCK-8 specific binding to human CCK_B/gastrin receptor with IC_{50} values of 55 pM and 11 nM, respectively. This stereoselectivity was firmly supported by the finding that the affinity of the racemate (IC_{50}=110 pM) was half that of YM022. We previously reported that YM022 and its racemate had the same affinity in the rat receptor as in the human homologue, with K_i values of 68 and 110 pM, respectively, but that its *S*-isomer (K_i =140 nM) had less affinity (7). The isomeric activity ratios of YM022 in the human and rat receptor were 200 and 2,100, respectively. In addition to stereoselectivity, ligand specificity was also slightly different between human and rat CCK_B/gastrin receptors. L-365,260 inhibited [^{125}I]CCK-8 binding to human CCK_B/gastrin receptor with an IC_{50} of 1.8 nM (Fig. 1 and Table 1); the inhibitory activity ratio of L-365,260 to YM022 was 33, which is one order smaller than the 279 obtained in rat receptor (7). Furthermore, the ratios of L-364,718 to L-365,260 were 21 and 105 in the human (Table 1) and rat (7) receptors, respectively. These results are well-consistent with the ratios of 22 and 33 reported in the human receptor expressed in COS cells (14, 15) and the ratio of 125 in the rat brain receptor (6). These values are summerized in Table 2. Although it remains to be clarified which residue(s) are involved in the stereoselectivity of YM022 or in the specificity of the antagonists, the human CCK_B/gastrin receptor-expressing NIH-3T3 cells established in the present study are useful for the development of human CCK_B/gastrin ligands.

In the view of clinical application, the healing of peptic ulcers is greatly assisted by antisecretory drugs such as histamine H_2 blockers and proton pump inhibitors. However, long-term treatment with antisecretory drugs leads to hypergastrinemia. Because gastrin is not only a

Table 2. Relative activities of CCK/gastrin receptor ligands against human and rat CCK_B/gastrin receptors

	Human	Rat	Human		Rat
		Ref. 13	Ref. 15	Ref. 14	Ref. 6
Ligands	IC_{50} (nM)	K_i (nM)	IC_{50} (nM)		IC_{50} (nM)
YM022	0.055 (1)	0.068 (1)	0.34 (1)		
S-isomer	10.7 (195)	140 (2100)			
L-365,240	1.8 (33) (1)	19 (280) (1)	10.3 (30) (1)	4.5 (1)	2 (1)
L-364,718	37.2 (680) (21)	2000 (29000) (105)	231 (680) (22)	147 (33)	250 (125)

IC_{50} or K_i values were cited from each reference. Numbers in parentheses show the ratios against YM022, and the underlined numbers in parentheses show those against L-365,240.

strong stimulant of gastric acid secretion (3) but also a growth factor of histamine-producing enterochromaffin-like cells (4), hypergastrinemia causes the relapse of peptic ulcer. It has been reported that treatment with a proton pump inhibitor induced gastric lesions and hyperplasia of enterochromaffin-like cells (21–23) and that these were inhibited by the CCK_B/gastrin antagonist PD136540 in rats (24). Taken together, these observations suggest that CCK_B/gastrin antagonists may prevent the relapse of peptic ulcers during therapy with the above antisecretory drugs.

We recently reported that the oral administration of YM022 in rats inhibited gastrin-induced acid secretion and gastric and duodenal lesion formation (7, 8). Furthermore, the affinity of YM022 for rat brain CCK_B /gastrin receptor was 930 times greater than that for rat pancreatic CCK_A receptor (7). The present study demonstrates that YM022 is also a potent human CCK_B/gastrin antagonist. These findings suggest that YM022 may be a useful drug for patients with peptic ulcer accompanied by hypergastrinemia without side effects on the CCK_A receptor.

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