Pergamon

Biochemical Pharmacology, Vol. 47, No. 8, pp. 1339–1343, 1994.
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0006-2952/94 $6.00 + 0.00

0006-2952(93)E0097-R

CHARACTERIZATION OF CLONED HUMAN CHOLECYSTOKININ-B RECEPTOR AS A GASTRIN RECEPTOR

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(*Received* 27 *July* 1993; *accepted* 30 *November* 1993)

Abstract—The cholecystokinin (CCK)-B receptor cloned from human brain was characterized as a gastrin receptor by using heterologous expression systems of COS-7 cells and *Xenopus* oocytes. ^{125}I-gastrin binding to human CCK-B receptor expressed in COS-7 was time-dependent, saturable and also specific, as well as ^{125}-I-CCK-8. The binding of ^{125}I-gastrin was inhibited by CCK-8 about 10-fold more potently than by gastrin. The rank order of potency of several antagonists to ^{125}I-gastrin binding was YM022 > CI-988 > L-365,260 > L-364,718. Addition of GTPγS, a nonhydrolysable analog of GTP, dose-dependently inhibited ^{125}I-gastrin binding, and lowered the gastrin binding affinity, Gastrin (10^{-9}–10^{-7} M) also evoked a Ca^{2+}-dependent Cl^- current in *Xenopus* oocytes expressing CCK-B receptors. These results suggest that the pharmacological profile of the cloned human CCK-B receptor using ^{125}I-gastrin is closely parallel to that reported in gastric mucosa, and that the receptor transduces cellular signals of gastrin as well as those of CCK-8.

Key words: cloned CCK-B receptor; cholecystokinin; gastrin; YM022

Gastrin is a peptide hormone produced and secreted by gastric antral G cells. This hormone exerts its physiological activities through its receptor in gastric mucosa, causes the secretion of gastric acid from gastric mucosa [1, 2] and the release from gastric mucosal cells of histamine which is also a potent stimulator of acid secretion [3], as well as proliferation of enterochromaffin-like cells in the stomach which are rich in histamine [4]. Gastrin shares a common C-terminal sequence (Gly-Trp-Met-Asp-Phe-NH_2) with the CCK† family of peptides, a neuropeptide family widely distributed in the central nervous system and gastrointestinal tract, and has an affinity for the receptors of these neuropeptides. CCK receptors with a high affinity for gastrin are distributed in brain, and classified as the CCK-B subtype [5, 6]. Based on binding studies using CCK/gastrin radiolabeled ligands, pharmacological profiles of the CCK-B receptor in brain are reported to closely resemble those of the gastrin receptor in gastric mucosa [7, 8]. It is therefore of interest whether the brain CCK-B receptor is identical to the gastric gastrin receptor. Recently, the cDNA of an identical CCK-B receptor has been isolated from human brain and gastric mucosa [9]. Furthermore, Southern blot hybridization of human genomic DNA indicates that a single gene encodes both the brain and the gastric mucosa CCK-B receptor [10]. These findings suggest that the receptor, termed "CCK-B/gastrin receptor", may mediate the physiological activities of gastrin in gastric mucosa as well as those of the CCK family of peptides in the central nervous system. It has been reported that the pharmacological profile of ^{125}I-CCK-8 binding to this receptor is consistent with that reported in brain, and that this receptor is functional for CCK-8. However, it remains to be determined whether ^{125}I-gastrin binding to the cloned CCK-B receptor mimics the pharmacological profile of ^{125}I-gastrin binding in gastric mucosa, and whether the cloned receptor can transduce cellular signals of gastrin. In this study, we isolated the entire coding region of human CCK-B receptor cDNA using RT-PCR. Monkey kidney fibroblast COS-7 cells transfected with this cDNA were characterized by ligand binding to the CCK-B receptor using ^{125}I-gastrin and ^{125}I-CCK-8. Cellular signals of gastrin through the cloned CCK-B receptor were studied using a heterologous expression system in *Xenopus* oocytes.

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† Abbreviations: CCK, cholecystokinin; RT-PCR, reverse transcriptase-polymerase chain reaction.

MATERIALS AND METHODS

Materials. YM022 [11], CI-988, L-365,260 and L-364,718 were chemically synthesized in our institute. Other materials were purchased from the following sources: CCK-8 (fragment 26-33), gastrin (human gastrin I) and GTPγS from the Sigma Chemical Co. (St Louis, MO, U.S.A.); and ^{125}I-gastrin (3-[^{125}I]-iodotyrosyl^{12}-gastrin I, human; ~74 TBq/mmol) and ^{125}I-CCK-8 (CCK-8 sulfated, ^{125}I-labeled with Bolton-Hunter reagent; ~74 TBq/mmol) from Amersham International (Amersham, U.K.).

Isolation of human CCK-B receptor cDNA using RT-PCR. Human poly (A)$^+$ RNA (Clontech, Palo Alto, CA, U.S.A.) was converted to first strand cDNA using random hexamer and avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, Mannheim, Germany). Human CCK-B

receptor cDNA was amplified from human brain cDNA with sense primer (5′-CCATG GAGCT GCTAA AGCTG-3′) and antisense primer (5′-CTCAG CCAGG CCCTA GCGTG-3′) using *Taq* polymerase (Toyobo, Osaka, Japan) for 30 cycles (denaturation at 94°, 1 min, annealing at 60°, 2 min and extension at 72°, 3 min). The corresponding DNA fragment (~1.3 kb) was cloned into pCRII plasmid vector (Invitogen, San Diego, CA, U.S.A.) and confirmed by terminator cycle sequencing (Applied Biosystem, Foster City, CA, U.S.A.).

Expression of human CCK-B receptor in COS-7 cells. The entire coding region of the human CCK-B receptor was subcloned, as a *Hind*III/*Xba*I fragment, into eukaryotic expression vector pCDM8 (Invitogen, San Diego, CA, U.S.A.). COS-7 cells were plated in 10-cm culture dishes and grown in Dulbecco's modified Eagle medium containing 10% fetal bovine serum. After overnight incubation, cells were transfected with pCDM8 vector containing human CCK-B receptor cDNA using a DEAE-dextran method. Cells were exposed to DNA/DEAE-dextran for 3 hr, shocked with 15% (v/v) glycerol in HEPES-buffered saline for 2 min, and used for binding assay about 48 hr later.

Binding experiment. Transfected COS-7 cells were rinsed with ice-cold 25 mM HEPES-buffered Hanks' balanced salt solution (pH 7.4) containing 0.1% (w/v) bovine serum albumin, scraped from the plate, centrifuged (400 *g*), and suspended in the same solution. Suspended cells (0.25 mL; 1–2 × 10^5 cells for ^{125}I-gastrin or 0.5–1 × 10^5 cells for ^{125}I-CCK-8) were incubated at 37° with 100 pM of ^{125}I-gastrin or ^{125}I-CCK-8. Cells were washed with ice-cold 50 mM Tris buffer (pH 7.4) containing 0.01% bovine serum albumin by filtration on glass fiber filters (Filtermat A; Pharmacia, Turku, Finland) using a cell harvester. Filters were measured for γ radioactivity. Nonspecific binding was determined in the presence of 10^{-6} M of gastrin or CCK-8. Competition studies of CCK/gastrin ligands were performed at 60 min.

In experiments to study the effects of GTPγS on gastrin binding, membrane preparations of COS-7 cells were used as a receptor source. The transfected COS-7 cells were rinsed with ice-cold lysis buffer (10 mM HEPES buffer, pH 7.4, containing 130 mM NaCl, 5 mM $MgCl_2$, and 0.25 mg/mL bacitracin), and homogenized in the lysis buffer with 50 strokes of a Dounce homogenizer (type B). The homogenate was centrifuged at 38,000 *g* for 30 min. The pellet was suspended at 2–3 mg/mL of protein and stored at −20° until use. Radiolabeled ligands were incubated with membrane (50–100 μg of protein) in 0.25 mL of lysis buffer supplemented with 0.1% bovine serum albumin. Other procedures were performed as described above.

Expression of human CCK-B receptor in Xenopus *oocytes.* The pCRII plasmid containing CCK-B receptor cDNA was linearized with *Not*I, *in vitro* transcribed by using T7 RNA polymerase (Stratagene, La Jolla, CA, U.S.A.) in the presence of the cap analog 5′7meGppp5′G, and purified with a Sephadex G-50 spin column. *Xenopus* oocytes were injected with 50 nL (~15 ng) of transcribed RNA. At 3–5 days, oocytes were voltage clamped at −70 mV, ligands were applied to the constantly perfused bath, and ligand-dependent Cl^- current was measured.

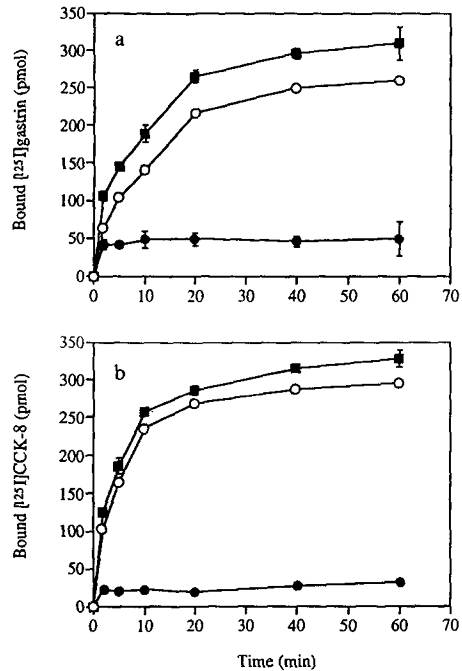

Fig. 1. Kinetics of ^{125}I-gastrin (a) and ^{125}I-CCK-8 (b) binding to human CCK-B receptor. COS-7 cells were transfected with pCDM8 containing the human CCK-B receptor cDNA, and incubated at 37° with 100 pM ^{125}I-gastrin and ^{125}I-CCK-8 in the absence (■) or presence (●) of 1 μM gastrin and CCK-8, respectively. The difference between them represents specific binding (○). The data represent the mean ± SE of three determinations.

Analysis of data. Results are expressed as the mean ± SE or the mean with 95% confidence limits. IC_{50} values, the concentration required to inhibit specific binding by 50%, were computed by logit–log analysis.

RESULTS

We attempted to amplify a cDNA encoding the human CCK-B receptor using RT-PCR with human brain poly $(A)^+$ RNA as a template. RT-PCR was carried out using a set of nucleotide primers (20-mer) based on 5′ and 3′ nucleotide sequences of the entire coding region of the canine gastrin receptor cDNA [12]. As a result, we obtained a DNA fragment encoding an amino acid sequence of human CCK-B receptor as described previously [9, 10]. Both ^{125}I-gastrin and ^{125}I-CCK-8 bound to COS-7 transfected with human CCK-B receptor cDNA, but not to untransfected COS-7. As shown in Fig. 1a, ^{125}I-gastrin bound to human CCK-B receptor expressed in COS-7 in a time-dependent manner, with saturation at 40–60 min after the addition of ^{125}I-gastrin. Nonspecific binding of ^{125}I-gastrin, as assessed in the presence of 10^{-6} M gastrin, was 10–20% of total binding at 60 min. Thus, the highly

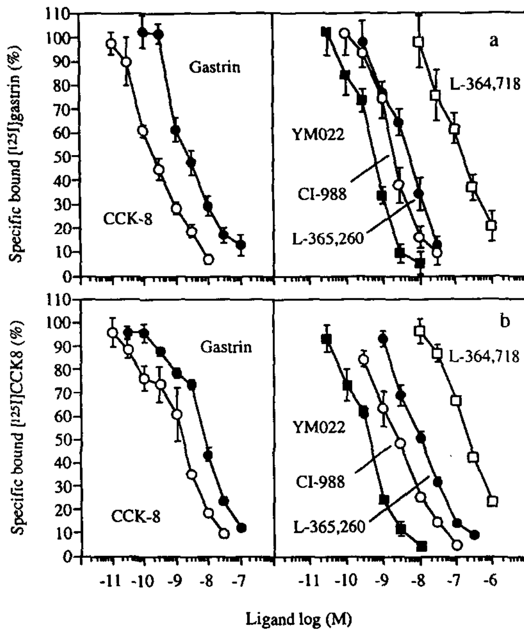

Fig. 2. Inhibition of ^{125}I-gastrin (a) and ^{125}I-CCK-8 (b) binding to human CCK-B receptor by CCK/gastrin ligands. COS-7 cells were transfected with pCDM8 containing the human CCK-B receptor cDNA, and incubated at 37° for 60 min with 100 pM ^{125}I-gastrin and ^{125}I-CCK-8 in the presence of the indicated concentrations of CCK-8, gastrin, YM022, CI-988, L-365,260 and L-364,718. The data represent the mean ± SE of four to six determinations.

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

Ligand	IC_{50} (nM)* ^{125}I-gastrin	^{125}I-CCK-8
Gastrin	2.8 (2.5–3.1)	7.4 (7.0–7.9)
CCK-8	0.21 (0.20–0.22)	0.81 (0.73–0.89)
YM022	0.56 (0.52–0.61)	0.34 (0.32–0.36)
CI-988	2.0 (1.8–2.1)	2.6 (2.3–2.8)
L-365,260	4.5 (4.0–5.0)	10.3 (9.9–10.7)
L-364,718	187 (158–222)	231 (222–240)

* IC_{50} values were analysed from data in Fig. 2. Numbers in parentheses show 95% confidence limits.

efficient expression of the CCK-B receptor enabled us to detect ^{125}I-gastrin binding with low nonspecific binding. In addition, ^{125}I-CCK-8 binding also exhibited time-dependent kinetics with less non-specific binding than ^{125}I-gastrin (Fig. 1b). In equilibrated states, specific binding of ^{125}I-gastrin and ^{125}I-CCK-8 were 0.29 ± 0.05 (N = 9) and 1.05 ± 0.05 (N = 9) fmol/10^5 cells, respectively.

The competition curves of CCK/gastrin ligands for ^{125}I-gastrin binding to CCK-B receptor in COS-7 are shown in Fig. 2a, and their IC_{50} values are summarized in Table 1. Gastrin inhibited ^{125}I-gastrin binding in a dose-dependent manner, with an IC_{50} of 2.8 nM. CCK-8 also inhibited this binding with about 10-fold greater potency. All CCK-B or gastrin receptor-selective antagonists, YM022 [11, 13], CI-988 (PD134308) [14] and L-365,260 [15], inhibited the binding dose-dependently. YM022, a gastrin antagonist developed in our Institute, was the most potent competitor of this binding (IC_{50} = 0.56 nM). In contrast, the CCK-A receptor-selective antagonist L-364,718 (MK-329 or devazepide) [16] was a weak inhibitor. The relative potencies of gastrin, CCK-8, CI-988, L-365,260 and L-364,718 correlated well with those previously reported for ^{125}I-gastrin binding sites in membrane preparations of gastric mucosa [7, 8]. Furthermore, all CCK/gastrin ligands inhibited ^{125}I-CCK-8 binding to the CCK-B receptor with similar potencies to those for ^{125}I-gastrin binding (Fig. 2B and Table 1). These data suggest that ^{125}I-CCK-8 and ^{125}I-gastrin may bind to an identical component of the CCK-B receptor.

To determine whether gastrin binding affinity of the CCK-B receptor is affected by interaction with G-proteins, ^{125}I-gastrin binding to the CCK-B receptor was measured in the presence of GTPγS, a nonhydrolysable analog of GTP, using membrane preparations of COS-7 expressing the receptor. As shown in Fig. 3A, GTPγS produced a dose-dependent decrease in ^{125}I-gastrin binding. The maximal decrease by GTPγS occurred at 10^{-7} M. These results support the possibility that GTPγS reduces affinity of gastrin for the CCK-B receptor. To determine further this possibility, competition experiments were performed in the absence and presence of GTPγS. The competition curve of gastrin was shifted lower by 10^{-7} M GTPγS (Fig. 3B). These results suggest that the binding of gastrin to the CCK-B receptor induced dissociation of G-proteins from the receptor.

The cloned human CCK-B receptor is reported to transduce cellular signals of CCK-8, such as an increase in free cytosolic calcium and in the level of inositol-1,4,5-trisphosphate [10]. To determine whether the cloned CCK-B receptor is functional for gastrin, we measured a cellular signal, Ca^{2+}-dependent Cl^- current, in *Xenopus* oocytes injected with a capped *in vitro* transcript of the CCK-B receptor cDNA (Fig. 4). Gastrin at 10^{-9}–10^{-7} M produced the long-lasting oscillatory Cl^- current characteristically elicited by G-protein-coupled receptors. In contrast, oocytes injected with vehicle showed no response to 10^{-6} M gastrin. The receptors to which agonist binding mobilizes intracellular Ca^{2+} in their native environment can evoke Ca^{2+}-dependent Cl^- current when expressed in oocytes; gastrin has also been shown to mobilize intracellular Ca^{2+} in gastric parietal cells [17] as well as to evoke Ca^{2+}-dependent Cl^- current. Our findings therefore suggest that the CCK-B receptor can transduce cellular signals of gastrin in addition to those of CCK-8.

DISCUSSION

Molecular cloning and genomic Southern blot analysis of the human brain CCK-B receptor suggested the possibility that the brain CCK-B and

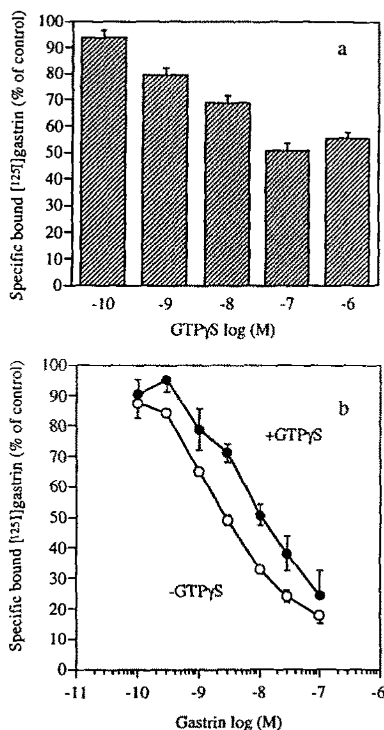

Fig. 3. GTPγS sensitivity of gastrin binding to human CCK-B receptor. (a) Inhibitory effect of GTPγS against ^{125}I-gastrin binding to the CCK-B receptor. Membranes prepared from transfectants were incubated with 100 pM ^{125}I-gastrin in the presence of the indicated concentrations of GTPγS. The data represent the means ± SE of four determinations. (b) Competition curves of gastrin against ^{125}I-gastrin binding in the absence (○) or presence (●) of 10^{-7} M GTPγS. The data represent the mean ± SE of six determinations.

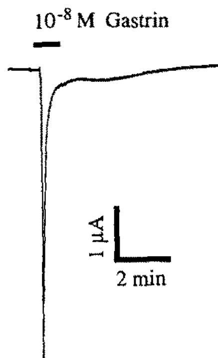

Fig. 4. Cl$^-$ current evoked by gastrin in *Xenopus* oocytes expressing human CCK-B receptor. *Xenopus* oocytes were injected with *in vitro* transcribed RNA of human CCK-B receptor, and exposed to 10^{-8} M gastrin. Experiments were repeated several times in different oocytes, with similar results.

gastric gastrin receptor are identical [9, 10]. However, it had not been determined whether this CCK-B receptor manifests the pharmacological and physiological characteristics of the gastric gastrin receptor. In this paper, we present findings which support this hypothesis. Firstly the rank order of potency of CCK/gastrin ligands to complete ^{125}I-gastrin binding to the human CCK-B receptor was closely parallel to that of membrane preparations of gastric mucosa [7, 8]. Secondly, the relative potencies of antagonists to compete ^{125}I-gastrin binding to this receptor correlated well with those needed to inhibit gastrin-induced acid secretion and histamine release from gastric mucosa [2, 18]. Thirdly the gastrin binding affinity of the CCK-B receptor was affected by the addition of GTPγS, an observation consistent with the concept that G-protein is involved in the gastrin receptor signaling pathway [19, 20]. Fourthly gastrin evoked a Ca^{2+}-dependent Cl^- current in *Xenopus* oocytes expressing the CCK-B receptor at physiological concentrations (10^{-9}–10^{-7} M). These concentrations of gastrin have been shown to mobilize intracellular Ca^{2+} in gastric parietal cells [17], and to induce acid secretion and histamine release from gastric mucosa [2, 3, 19].

The present study supports the possibility that the CCK-B receptor regulates physiological activities in gastric mucosa as well as in the central nervous system. However, the sensitivity of agonist binding affinities to guaninenucleotide were reported to be greater in gastric mucosa than in brain [8]. Thus, G-protein coupling mechanism of the gastrin receptor in gastric mucosa may be different from that of the CCK-B receptor in brain although the gastrin receptor may be identical to the CCK-B receptor. Inositol-1,4,5-trisphosphate formation and acid secretion of gastric parietal cells in response to gastrin have been reported to be partially sensitive to pertussis toxin, which inactivates G_i and G_o subtypes through ADP-ribosylation [19, 20]. The gastrin binding affinity is also sensitive to pertussis toxin [19]. These findings indicate possible coupling of gastrin receptor to G-protein subtypes both sensitive (G_i/G_o) and insensitive (G_q) to pertussis toxin. Furthermore, G_i and G_o have been shown to couple with and to be activated by the amino acid sequence containing B-B-X-B or B-B-X-X-B (B, basic amino acid; X, another amino acid) in its C-terminal [21]; indeed, the putative amino acid sequence of the cloned CCK-B receptor contains this sequence in the C-terminal of the putative third intracellular loop: Lys-Arg-Val-Val-Arg (residue 329–333). In contrast, there is no information on the G-protein coupled to CCK-B receptor in brain. To investigate the function of the CCK-B receptor in gastric mucosa and brain, it will be important to elucidate which G-protein subtypes couple with the CCK-B receptor in each of the tissues.

Acknowledgements—We thank Gensei Kon, Akito Nishida and Keiji Miyata for helpful discussions.

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