Evidence for a Direct Interaction between the Penultimate Aspartic Acid of Cholecystokinin and Histidine 207, Located in the Second Extracellular Loop of the Cholecystokinin B Receptor*

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Recently, we reported that the mutation of His207 to Phe located in the second extracellular loop of the cholecystokinin B receptor strongly affected cholecystokinin (CCK) binding (Silvente-Poirot, S., Escrieut, C., and Wank, S. A. (1998) Mol. Pharmacol. 54, 364–371). To characterize the functional group in CCK that interacts with His207, we first substituted His207 to Ala. This mutation decreased the affinity and the potency of CCK to produce total inositol phosphates 302-fold and 456-fold without affecting the expression of the mutant receptor. The screening of 1-alanine-modified CCK peptides to bind and activate the wild type and mutant receptors allowed the identification of the interaction of the C-terminal Asp8 of CCK with His207. The H207A-CCKBR mutant, unlike the wild type receptor, was insensitive to substitution of Asp8 of CCK to other amino acid residues. This interaction was further confirmed by mutating His207 to Asp. The affinity of CCK for the H207D-CCKBR mutant was 100-fold lower than for the H207A-CCKBR mutant, consistent with an electrostatic repulsion between the negative charges of the two interacting aspartic acids. Peptides with neutral amino acids in position eight of CCK reversed this effect and displayed a gain of affinity for the H207D mutant compared with CCK. To date, this is the first report concerning the identification of a direct contact point between the CCK receptor and CCK.

Cholecystokinin (CCK) is found throughout the gastrointestinal tract and the central nervous system where it functions both as a hormone and a neurotransmitter (1). CCK exists physiologically in multiple forms processed from a 115-amino acid preprohormone. Post-translational processing of CCK involved sulfation of tyrosine at position seven from the C terminus and amidation of the C-terminal phenylalanine. In the gastrointestinal system, CCK regulates motility, pancreatic exocrine secretions and growth, gastric emptying, and inhibition of gastric acid secretion. In the nervous system, CCK is implicated in anxiogenesis, satiety, analgesia, and regulation of dopamine release. The actions of CCK are mediated by two pharmacologically distinct receptor subtypes, the CKA and the CCKB receptors. The longer sulfated forms (CCK-58, CCK-39, CCK-33, and CCK-8) bind to the CCKAR and CCKBR with similar nanomolar affinities. The nonsulfated peptides as well as shorter C-terminal fragments like CCK-4 and CCK-5 still bind with nanomolar affinities the CCKBR but display very low affinities for the CCKAR. Gastrin, a related family peptide that shares with CCK the C-terminal pentapeptide also discriminates the two subtypes by presenting similar affinity as CCK-8 for the CCKBR but micromolar affinity for CCKAR. Studies using synthesized CCK fragments have shown that the C-terminal sulfated and amidated octapeptide Asp-Tyr(SO3H)-Met-Gly-Tyr-Met-Asp-Phe-NH2 retains the full spectrum of biological activity (2–5). The cloning of the cDNA coding for these receptors has shown that CCKA and CCKB receptors belong to the superfamily of seven transmembrane G-protein-coupled receptor and that they have approximately 50% homology (6, 7). Due to the important physiological roles of CCK acting through these two receptor subtypes and, therefore, its possible implication in associated disorders, there has been considerable interest in the identification of ligands that selectively activate or inhibit the CCKAR and CCKBR. However, to date none of these compounds have been used as a therapeutic agent (8–13). The characterization of the interactions between the key pharmacophores of CCK and their partners in the receptor would certainly help in a more rational approach to the design of new molecules or the modification of preexisting molecules to optimize their properties. Despite numerous studies concerning the identification of amino acids involved in the binding site of agonist and antagonist ligands for the different classes of G-protein-coupled receptors (14, 15), very few studies concerned the identification of the functional groups in the ligand that directly interact with the identified amino acid in the receptor. However such studies are essential to model the binding pocket accurately. One way to address this issue is through the use of complementary substitutions in the ligand and the receptor as previously reported for G protein-coupled receptors for neurotransmitters and peptide receptors (16–20). We successfully used this approach for the CCKA receptor subtype to demonstrate that two amino acids Trp289 and Gln414, located in the N-terminal domain of the CCKAR, interact with the residues Arg7 and Asp18 of CCK (21). Recently, we identified an-
other interaction between Met139, located in the second extracellular loop of CCKAR, and the aromatic ring of sulfated tyrosine of CCK (Tyr^3), an important pharmacophore for conferring high affinity and activity of the peptide (22). To date, no such study has been reported for the CCKBR. We demonstrated by mutational analysis of the CCKBR that the extra- cellular domains of the CCKBR are also implicated in the high affinity binding of CCK and gastrin (23, 24). We showed that the mutation of the residue His207, located in the second extra- cellular loop of the CCKBR induced a dramatic decrease in CCK binding (24). Regarding the important role of the second extracellular loop for the high affinity binding of CCK in the CCKAR, the present study was undertaken to determine the amino acid within CCK that interacts with His207 in the CCKBR. The screening of modified CCK peptides for their binding and biological activity toward the wild type receptor and a variety of His^207.CCKBR receptor mutants permitted us to demonstrate that His207 directly interacts with the C-terminal aspartic acid of CCK, an essential residue for this family of peptides.

**EXPERIMENTAL PROCEDURES**

**Material**—The sulfated C-terminal nonapeptide of CCK ([Thr^28, Nle^31]-CCK25–33 (Thr,Nle)CCK1–9s), the tetrapeptide CCK30–33 (CCK6–9), and the sulfated heptapeptide ([Nle^28,31]-CCK27–33 (CCK3–9s) were synthesized as described previously (25). The sulfated and nonsulfated C-terminal octapeptide (CCK2–9s and CCK2–9) were from NeoSystem, Strasbourg, France. 125I^2Na was from Amersham Pharma- cia Biotech. (Thr^28, Nle^31)-CCK25–33 was conjugated with Bolton-Hunter reagent, purified, radioiodinated as described previously (26), and referred to as 125I-BH-(Thr,Nle)CCK1–9s.

**Synthesis of Modified CCK Peptides**—The l-amine-modified peptides were synthesized by solid phase synthesis on a Pioneer Perseptive Biosystems apparatus using Fmoc-peptide amide linker-polyethylene glycol flow continuous resin. Fmoc amino acid side chains were protected with the acid-labile protecting groups (Asp: t-butyl; Tyr: t-butyl; Trp, t-butyloxycarbonyl). Coupling reactions were carried out with a 4-fold excess of Fmoc-protected amino acid residues, reagent HBTU (2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophos- phate), and diisopropylethylamine for 1 h. At the end of the synthesis, the Fmoc gas protecting group was removed, and the peptidyl resin was solubilized in a mixture of acetonitrile/water/trifluoroacetic acid (50/50/0.1, v/v/v), and lyophilized. These peptides were purified by reverse phase high performance liquid chromatography on a Micromass, Waters, Millipore, 150 mm C18 (15 μm, 100 × 150 mm) with UV detection at 214 nm with a flow rate of 50 ml/min of water/trifluoroacetic acid (0.1%) and acetonitrile/trifluoroacetic acid (0.1%). The purity of the peptides was checked by analytical reverse phase HPLC with a Beckman instrument on a Delta-Pak C18 analytical column. The peptide structure was assessed by mass spectroscopy on a Platform II (Micromass, Manches- ter, UK) quadrupole mass spectrometer fitted with an electrospray interface.

**Construction of Mutant Receptor cDNAs**—Mutant receptor cDNAs were constructed by oligonucleotide-directed mutagenesis (Quick- Change™ site-directed mutagenesis kit, Stratagene, France) using the rat CCKBR cDNAs as template. Oligonucleotides were designed to include a silent restriction site to facilitate analysis of mutant con- structs by restriction endonuclease digestion. Mutations were con- firmed by DNA sequencing using an automated sequencer (Applied Biosystems).

**Transfection of Wild Type and Mutant Receptor cDNAs into Mammalian Cells**—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum. Two μg of the CCKBR and mutant receptor cDNAs subconfluent in pCIL-SRαs were transiently transfected into COS-7 cells using the DEAE/dextran method as de- scribed previously (23). 24 h after transfection, the transfected cells were transferred to 24-well culture plates, seeded at a density of approximately 1 × 10^4 cells/well, and assayed for 125I-BH-CCK-9 binding or inositol phosphate hydrolysis.

**Binding of 125I-BH-CCK-9 and 125I-HL-365,260 to Transfected COS-7 Cells**—Twenty-four h after the transfer of transfected cells to 24-well plates, the cells were washed once with cold phosphate-buffered saline, pH 7.4, containing 0.1% bovine serum albumin and incubated in Dul- becco’s modified Eagle’s medium containing 0.1% bovine serum albu- min for 60 min at 37 °C with either 50 pM (WT-CCKBR) or 500 pM (H207A-CCKBR) of 125I-BH-(Thr,Nle)CCK1–9s with and without increasing concentrations of unlabeled peptides. Cell-associated 125I-BH-(Thr,Nle)CCK1–9s was separated from free radioligand by washing 2× with phosphate-buffered saline containing 2% bovine serum albumin. Cell-associated 125I-BH-(Thr,Nle)CCK1–9s was collected with 0.5 ml of 0.1 N NaOH added to each well, and radioactivity was detected in a γ counter. Nonspecific binding (determined in presence of 1 μM CCK-8) was subtracted from total binding. Plasma membranes from transfected COS cells were prepared as described in Kennedy et al. (21) and incubated (50–100 μg) with 500 pM [^3H]HL-365,260 for 45 min at 37 °C in the presence of increasing L-365,260 concentrations (27). After incubation, the membranes were washed and filtered through Whatman GF/C glass fiber filters over a vacuum-filtering manifold. Filters were counted in a β counter. Binding assays were performed in duplicate in at least three separate experiments. Binding data were deter- mined using the nonlinear, least squares curve-fitting computer program, Ligand (28) and GraphPad Prism Program (Software). Kd values were calculated as Kd = IC50/1 + [labeled ligand]/Kd of labeled ligand.

**Measurement of Total Inositol Phosphate Accumulation**—Twenty- four h after COS-7 cell transfection, the transfected cells were trans- fected with 24-well cell culture plates and incubated overnight with the modified Eagle’s medium with 2 μg/well of myo-[^3H]inositol (18.6 Ci/mmol) (NEN Life Science Products). After the aspiration of the medium containing the myo-[^3H]inositol, the cells were incubated at 37 °C for 20 min with 1 μl of Dulbecco’s modified Eagle’s medium containing 20 μl NaCl. The cells were washed with PI buffer, pH 7.45 (20 mM Heps, 155 mM NaCl, 2 mM CaCl2, 1.2 mM MgSO4, 1 mM EGTA, 10 μM LiCl, 11.1 mM glucose, and 0.5% bovine serum albumin), incubated 1 h at 37 °C with PI buffer containing the indicated concentations of peptides. The reaction was stopped with 1 μl of methanol/HCl added to each well, and the content was transferred to a AG 1-X8 (formate form) column (Bio-Rad). Each column was washed twice with 3 ml of water followed by 2 ml of 5 mM sodium tetraborate, 60 mM sodium formate. Total inositol phosphates were eluted from the column with 2 ml of 1 M ammonium formate, 100 mM formic acid. myo-[^3H]inositol phosphate β radioactivity was detected in a liquid scintillation counter (Packard Instrument Co.). EC50 was calculated using Graph- Pad Prism program software.

**RESULTS**

**Effect of the H207A Mutation on Binding and Biological Activity of the CCKBR**—In a recent work, we have shown (24) that the mutation of His^207 in the CCKBR to phenylalanine (the equivalent amino acid in the CCKAR) resulted in a marked decrease in CCK affinity. Upon transient expression of the H207F –CCKBR mutant into COS cells, there was no detectable binding of radiolabeled CCK, and there was a 3,044-fold reduction in CCK-stimulated inositol phosphate production com- pared with the WT-CCKBR. All together these results sug- gested that His^207 was crucial for conferring high affinity to CCK (24). The present study was undertaken to identify the amino acid residue within CCK that interacts with His^207.

We first tried a potentially less drastic exchange by mutating His^207 to Ala. This mutation resulted in undetectable binding of 125I-BH-(Thr,Nle)CCK1–9s to the H207A-CCKBR (1 μM). By contrast, significant 125I-BH-(Thr,Nle)CCK1–9s binding was observed in the presence of 500 pM [^3H]L-365,260 for 45 mn at 37 °C. This was 1.52 pmol/10^6 cells. Compared with the WT-CCKBR, which had an affinity of 0.51 (±0.15) nM and a maximal binding capacity of 4.35 (±1.52) pmol/10^6 cells. The H207A-CCKBR mutant displayed a decrease in CCK2-9s affinity of 456-fold despite a similar level of cell surface expression. This mutant also retained similar efficacy compared with WT-CCKBR (11-fold over basal) for CCK2-9s-stimulated increase in total IP.
production, although the EC₅₀ was 302-fold higher than the WT-CCKBR (EC₅₀: 145 (±79) nM versus 0.48 (±0.3) nM). The decrease in CCK2-9s potency to stimulate IP production correlates well with the decrease in CCK2-9s affinity observed for the H207A-CCKBR mutant (302-fold versus 456-fold). On the basis of the marked shift observed in CCK2-9s affinity and potency when His²⁰⁷ was mutated to Ala, we hypothesized that His²⁰⁷ might interact with a crucial amino acid of CCK.

**Structure-Function Studies of CCK in COS Cells Expressing the WT-CCKBR—**Since no previous CCK structure-function studies have been reported for the CCKBR in any expression systems, we first determined what extent each amino acid of CCK contributes to its affinity and potency for stimulation of total IP in the WT-CCKBR transiently expressed in COS-7 cells. For that, we tested different fragments of CCK and synthesized CCK octapeptides modified by replacing each single amino acid by an L-alanine. We expected similar affinity and potency decreases resulting from alanine substitutions in the receptor and in the ligand. In this first screening, the L-alanine-modified peptides were synthesized in a nonsulfated form for the convenience of the synthesis. As shown in Table I, the exchange of C-terminal amino acids. As shown in Table I, the exchange of Trp⁶ by Ala induces a dramatic decrease in both the affinity and potency. It was difficult to determine an accurate Kᵢ because [Ala⁶]CCK2-9 bound the WT-CCKBR with a very low affinity. Consistent with this result, the EC₅₀ of [Ala⁶]CCK2-9 was reduced 13,975-fold. These results are not in favor of an interaction of Trp⁶ with His²⁰⁷, because Trp⁶ substitution results in a large decrease in the affinity and potency of the modified peptide for the WT-CCKBR, which does not correlate with that of CCK2-9s for the H207A-CCKBR mutant. The affinities of [Ala⁴]CCK2-9 and [Ala⁵]CCK2-9 were reduced 5,224- and 5,008-fold, respectively, which correlated with the 4,629- and 6,837-fold decrease in potencies observed for these two peptides, whereas the exchange of Asp⁸ by Ala induces a less important decrease in the affinity and potency of [Ala⁴]CCK2-9 with a shift of 1,191- and 935-fold, respectively (Table I). Considering that these peptides are not sulfated, the decrease in the affinities and potencies of [Ala⁴]CCK2-9, [Ala⁵]CCK2-9, and [Ala⁶]CCK2-9 for the WT-CCKBR are closed to the 456- and 302-fold reduction in CCK2-9s affinity and potency observed for the H207A-CCKBR mutant, suggesting that these residues could interact with His²⁰⁷.

**Demonstration That His²⁰⁷ of the CCKBR Interacts with Asp⁸ of CCK—**We subsequently tested the equivalent sulfated compounds [Ala⁴]CCK2-9s, [Ala⁵]CCK2-9s, and [Ala⁶]CCK2-9s on the WT-CCKBR and H207A-CCKBR to determine if these residues interact with His²⁰⁷. We hypothesized that removing the functional group in CCK that interacts with His²⁰⁷ should not further affect the affinity and potency of the modified peptide for the H207A-CCKBR mutant compared with CCK2-9s, because the mutation should have already disrupted the interaction. On the contrary, exchanging a functional group that does not interact with His²⁰⁷ should induce an additional decrease in the affinity and potency of the modified peptide for the H207A-CCKBR mutant compared with CCK2-9s.

### Table I

| Peptides                  | Binding to WT-CCKBR | Inositol phosphate production |
|--------------------------|---------------------|-------------------------------|
|                          | Kᵢ  (±S.E.) | EC₅₀ (±S.E.) | E₅₀ (±S.E.) | Kᵢ  (±S.E.) | EC₅₀ (±S.E.) | E₅₀ (±S.E.) |
| CCK1–9s                  | 0.43 (±0.12) | 0.9 (±0.13) | 1 (±0.12) | 0.43 (±0.12) | 0.9 (±0.13) | 1 (±0.12) |
| CCK2–9s                  | 0.71 (±0.23) | 1.6 (±0.25) | 2 (±0.23) | 0.71 (±0.23) | 1.6 (±0.25) | 2 (±0.23) |
| CCK3–9s                  | 12.82 (±1.23) | 28 (±0.23) | 56 (±0.23) | 12.82 (±1.23) | 28 (±0.23) | 56 (±0.23) |
| [Ala⁷]CCK2–9             | 1191 (±708) | 6708 (±633) | 68 (±633) | 1191 (±708) | 6708 (±633) | 68 (±633) |
| [Ala⁸]CCK2–9             | 935 (±4629) | 90 (±4629) | 90 (±4629) | 935 (±4629) | 90 (±4629) | 90 (±4629) |
| [Ala⁹]CCK2–9             | 13,975 (±2351) | 633 (±68) | 13,975 (±2351) | 633 (±68) |

Cholecystokinin Binding Site of the Rat CCKB Receptor

| Peptides                  | Binding to H207A-CCKBR | Inositol phosphate production |
|--------------------------|------------------------|-------------------------------|
|                          | Kᵢ  (±S.E.) | EC₅₀ (±S.E.) | E₅₀ (±S.E.) | Kᵢ  (±S.E.) | EC₅₀ (±S.E.) | E₅₀ (±S.E.) |
| CCK1–9s                  | 0.43 (±0.12) | 0.9 (±0.13) | 1 (±0.12) | 0.43 (±0.12) | 0.9 (±0.13) | 1 (±0.12) |
| CCK2–9s                  | 0.71 (±0.23) | 1.6 (±0.25) | 2 (±0.23) | 0.71 (±0.23) | 1.6 (±0.25) | 2 (±0.23) |
| CCK3–9s                  | 12.82 (±1.23) | 28 (±0.23) | 56 (±0.23) | 12.82 (±1.23) | 28 (±0.23) | 56 (±0.23) |
| [Ala⁷]CCK2–9             | 1191 (±708) | 6708 (±633) | 68 (±633) | 1191 (±708) | 6708 (±633) | 68 (±633) |
| [Ala⁸]CCK2–9             | 935 (±4629) | 90 (±4629) | 90 (±4629) | 935 (±4629) | 90 (±4629) | 90 (±4629) |
| [Ala⁹]CCK2–9             | 13,975 (±2351) | 633 (±68) | 13,975 (±2351) | 633 (±68) |

**TABLE I**

**Affinities and potencies of CCK peptides, CCK fragments, and L-alanine-modified CCK peptides for the WT-CCKBR transiently expressed in COS cells**

*Kᵢ* values were calculated from competition curves of **₁²⁵I-BH-(Thr,Nle)CCK1–9s binding by the indicated peptides. EC₅₀ values were calculated from dose-response curves of total IP production stimulated by the indicated peptide. Results are expressed as mean ± S.E. of three to five separate experiments from different batches of transfected cells. The factor *F*ₘₕₐₓ₉ₐₚ represents the effect of the mutation on the affinity or on the potency of the peptides tested relative to CCK2-9s. The efficacies of the peptides tested to stimulate total inositol phosphate production are expressed as the percent of the maximal increase obtained using 1 × 10⁻⁶ M CCK2-9s and referred to as *E*₅₀. ND, not determined.
interact with His207. In contrast to that observed with [Ala]CCK2-9s and [Ala]CCK2-9s, the affinity and potency of [Ala]CCK2-9s for the H207A-CCKBR mutant were similar to that CCK2-9s but were decreased 460- and 151-fold, respectively, for the WT-CCKBR (Fig. 1, right, and Table II). Thus, [Ala]CCK2-9s, unlike CCK2-9s, [Ala]CCK2-9s, and [Ala]CCK2-9s, is insensitive to the H207A mutation. These results and the fact that [Ala]CCK2-9s had a similar affinity and potency for the H207A-CCKBR mutant and the WT-CCKBR suggest a direct interaction between Asp8 of CCK and His207 (Fig. 1 and Table II). To confirm this interaction, these data were analyzed according to the mutant cycle methodology of Hidalgo and MacKinnon (29) and Schreiber and Fersht (30). ω values, defined as $\omega = K_{m} (WT:WT) \times K_{m} (mut:mut)/K_{m} (WT:\\ mut) \times K_{m} (mut:WT)$, determined if two modified or mutated residues are independent of one another (ω will be unity) or if these residues interact (ω will deviate from unity). As shown in Table II, the large ω value for the [Ala]CCK2-9s/H207A-CCKBR pair supports that Asp8 interacts specifically with His207. This contrasts with the ω value for the [Ala]CCK2-9s/H207A-CCKBR pair that is near unity, suggesting that Met7 does not interact with His207. Similarly, our inability to determine ω value for the [Ala]CCK2-9s/H207A-CCKBR pair is compatible with their independence. Together these results provide further support for a direct and specific interaction of Asp8 with His207.

We subsequently tested the effect of exchanging Asp8 of CCK2-9s with other amino acid residues on the ability of the modified peptide to bind and stimulate total IP production to both wild type and mutated receptor (Table III). For the WT-CCKBR, the exchange of Asp8 to Gln reduced the affinity and the potency of Gln8/CCK2-9s, 199- and 148-fold, respectively. These values are similar to that obtained when Asp8 was exchanged to Ala, indicating that the length of the side chain at this position is as crucial as the presence of the carboxylic group. Similarly, the exchange of Asp8 by Phe in CCK2-9s reduced the affinity and the potency of the modified peptide Phe8/CCK2-9s to the same extent (Table III). CCK peptide with an Asp8 to Leu substitution ([Leu]CCK2-9s) bound to the WT-CCKBR with an affinity lower than the other peptides modified at position 8. Moreover, this peptide did not stimulate IP production, even at a concentration of 10^{-4} M as with the WT-CCKBR, indicating that unlike the WT-CCKBR, the H207A-CCKBR mutant is insensitive to the changes introduced at position 8 of CCK (Table III). It should be noted that all these modified peptides had a 1.6- to 2-fold lower efficacy for stimulating total IP production in the H207A-CCKBR mutant compared with the WT-CCKBR. This is most likely due to the additive effects of the changes introduced both in the mutated receptor and in the modified peptides, whereas CCK2-9s had the same efficacy on both wild type and mutated receptor.
Because we have previously shown that the sulfated Tyr³ of CCK interacts with Met¹⁹⁵, located near His²⁰⁷ in the second extracellular loop of the CCKA receptor (22), we tested a CCK ligand modified at position 3, [Ala³]CCK2-9, to further demonstrate that the effect of substituting His²⁰⁷ on CCK affinity and potency was specific for Asp⁸. The effect of mutating His²⁰⁷ and substituting Tyr³ of CCK was additive. [Ala³]CCK2-9 displayed an affinity and a potency for the H₂₀⁷D-CCKBR mutant (~90% of WT-CCKBR) that were further reduced 11- and 13-fold compared with the WT-CCKBR mutant (~80% of WT-CCKBR). Moreover, the affinity and potency of [Ala³]CCK2-9 was reduced 100- and 246-fold compared with the WT-CCKBR (Table III).

To further demonstrate the interaction of Asp⁸ of CCK with His²⁰⁷ in the CCKBR, we mutated His²⁰⁷ to Asp. If Asp interacts with the residue 207 in the receptor, the introduction of a negative charge at position 207 should further decrease CCK2-9s affinity and potency compared with the H₂₀⁷A-CCKBR mutant because of the repulsive effect between the two negatively charged residues. After transient expression of the H₂₀⁷D-CCKBR mutant in COS cells, [¹²⁵I-BH-(Thr,Nle)]CCK2-9s 300 (±1.5) 100 1870 (±352) 13 246 70

| Peptide | EC₅₀ (±SEM) | E_max (±SEM) | F_CCK2-9s | F_WT | E_CCK2-9s | E_WT |
|---------|-------------|--------------|-----------|-------|----------|-------|
| WT-CCKBR | 145 (±79) | 100 | 1 | 16218 (±969) | 67 | 1 |
| [Glu¹]CCK2-9s | 572 (±112) | 60 | 4 | ND | 9 | ND |
| [Ala³]CCK2-9s | 418 (±121) | 50 | 3 | 1350 (±412) | 40 | 0.08 |
| [Phe¹]CCK2-9s | 300 (±79) | 50 | 2 | 595 (±44) | 54 | 0.03 |

Indirect effect. The effect of a mutation may directly influence the binding pocket by substituting a residue that directly binds the ligand. Alternatively, the mutation may indirectly affect the binding pocket by causing a conformational change that disrupts a distal binding site. One way to address this question is to characterize the functional group in the ligand that interacts with the mutated receptor amino acid by using modified ligands. We have recently shown by mutational analysis of the CCKBR that one residue, His²⁰⁷, located in the second extracellular loop of the CCKBR when mutated, affects CCK2-9s
high affinity binding (24). Regarding the direct role of the second extracellular loop of the CCKAR for the high affinity binding of CCK (22), the aim of the present study was to characterize the functional group in CCK that interacts with His^{207} in the CCKBR and thus to determine if His^{207} is likely to be directly involved in the CCK binding pocket.

We first showed that the mutation of His^{207} to Ala decreased 456-fold the affinity of CCK2-9s, and 302-fold, its potency to stimulate total IP production compared with the wild type CCK receptor. The similar expression of the mutated receptor and wild type receptor at the cell surface as well as the similar biological efficacy of CCK2-9s for the two receptors indicated that the mutation did not introduce a gross conformational change in the receptor. The mutation of His^{207} to Ala, albeit less drastic than to Phe, still affected the affinity and potency of CCK2-9s, suggesting that this residue might interact with an important amino acid of CCK. In a preliminary approach to identifying the putative amino acid of CCK interacting with His^{207}, we first screened L-alanine-substituted analogues of CCK octapeptide at each position for their ability to bind and stimulate IP production at the wild type receptor. We expected similar affinity and potency decreases resulting from Ala substitutions in the receptor and in the ligand. This strategy permitted us to identify three residues at the C terminus of CCK, Met^{7}, Asp^{8}, and Phe^{9}, as being putative partners of His^{207}, since the affinities and potencies of the corresponding modified peptides for the WT-CCKBR were close to that of CCK2-9s for the H207A-CCKBR mutant. Among these residues, Asp^{8} was identified as the only residue interacting with His^{207}, since the H207A-CCKBR mutant displayed similar affinities and potencies for [Ala^{7}]CCK2-9s and CCK2-9a, whereas its affinity and potency for [Ala^{7}]CCK2-9s and [Phe^{9}]CCK2-9s were importantly decreased. The interaction between Asp^{8} and His^{207} was confirmed by the fact that the H207A-CCKBR mutant was unable to distinguish between the native ligand and a series of analogues modified at position 8, whereas the WT-CCKBR was highly sensitive to changes introduced at this position. Moreover, the peptides modified at position 8 displayed similar affinities and potencies for the wild type and mutated receptor, whereas the analogues modified at positions 7 and 9 bound to and activated H207A-CCKBR mutant with decreased affinities and potencies relative to the wild type receptor.

Further support for an interaction of Asp^{8} with His^{207} was brought by mutating His^{207} to Asp. The drastic effect observed on CCK2-9s affinity and potency when His^{207} was substituted to Asp compared with Ala (100-fold) is consistent with an electrostatic repulsion between the negative charges of the Asp^{8} of the ligand and the one introduced at position 207 in the receptor. In addition, the severe loss of affinity and potency of [Glu^{5}]CCK2-9s for the H207D-CCKBR mutant is in accordance with an increase of the repulsive effect due to an increase of the length of the side chain while maintaining the carboxylic function in position 8. In agreement with this result, when the repulsive force was removed by substituting Asp^{8} of the ligand for a neutral amino acid such as Ala or Phe, the potency of the two modified peptides for the mutant H207D were increased accordingly 12- and 27-fold compared with CCK2-9s. This gain of function is a strong evidence for the direct interaction between His^{207} of the CCKBR and Asp^{8} of CCK. Moreover, since the affinity and potency of the two peptides [Ala^{7}]CCK2-9s and [Phe^{9}]CCK2-9s for the H207A and H207D-CCKBR mutants were similar, it is likely that the repulsive effect observed was caused by the direct repulsion of the negative charges of the aspartic residues of the ligand and the receptor and is not due to a non-specific interaction of Asp^{207} with another residue of the receptor. All together these results argue that the changes introduced in the mutated receptor and in the ligand affect the same bond.

However, the present work does not allow one to conclude about the nature of this interaction. Imidazol-carboxylate interactions such as the one found here between the carboxyl side chain of CCK and His^{207} of the CCKBR are often found as an important stabilizing element in protein structures (31–33), enzyme reactions (34–36), and ligand-receptor recognition (37, 38). Such interactions can be due to a salt bridge between imidazole and carboxylate ions or to a network of hydrogen bonds linking the imidazole and carboxylate side chain together. Such an interaction might be considered as the initialization step of a proton pumping mechanism, which could be hypothesized in the activation process of G protein-coupled receptors (37). The implication of Asp^{8} of CCK in CCKBR activation, in addition to its role in ligand binding, was previously reported for the stimulation of acid secretion from dispersed gastric cells (39). Consistent with this result, we observed in the present paper that the substitution of Asp^{8} for Leu completely abolished total inositol phosphate production both in the wild type CCKBR and in the H207A-CCKBR mutant while maintaining micromolar affinities for both receptors.

Direct interactions of peptides with G protein-coupled receptors have been documented in a limited number of cases. However, it appears that the direct involvement of the extracellular domains in the peptide binding pocket is not unique to the CCKA and CCKB receptors subtypes. A few reports have indicated that the extracellular domains of the peptide receptors are direct contact sites for the peptide ligands. For example, it has been reported that in the AT1 angiotensin receptor, Asp^{281} of the extracellular loop 3 and His^{183} of the extracellular loop 2, respectively, bind Arg^{2} and Asp^{3} of angiotensin II (19). Similarly, Tyr^{115} of the extracellular loop 1 of the vasopressin V1A receptor interacts with Arg^{6} of arginine-vasopressin (40). Despite the fact that CCK presents a similar high affinity for both the CCKA and the CCKB receptor subtypes, the mutagenesis data obtained in this study clearly indicate a different anchoring of CCK in the CCKBR compared with the CCKAR (21, 22). These results are also consistent with a recent study in which we showed that the reciprocal mutation in the CCKBR of the two residues corresponding to Trp^{228} and Gln^{15} in the CCKAR (21) that directly interact with the N terminus of CCK2-9s have no effect on CCK2-9s binding in the CCKBR receptor (24).

In conclusion, using site-directed mutagenesis of the CCKB receptor together with the analysis of the binding and biological potency of different modified CCK analogues, we have shown in this study that His^{207} is directly involved in the binding pocket of CCK by interacting with the penultimate Asp^{8} residue of CCK. This study represents an important step in the molecular characterization of the CCK binding site especially since this is the first report concerning the identification of a direct contact point between the CCKB receptor and CCK. The importance of this study is reinforced by the fact that it involves an important amino acid of CCK.

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