The Biologically Crucial C Terminus of Cholecystokinin and the Non-peptide Agonist SR-146,131 Share a Common Binding Site in the Human CCK1 Receptor

EVIDENCE FOR A CRUCIAL ROLE OF MET-121 IN THE ACTIVATION PROCESS*

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The cholecystokinin (CCK) receptor-1 (CCK1R) is a G protein-coupled receptor, which mediates important central and peripheral cholecystokinin actions. Our aim was to progress in mapping of the CCK1R binding site by identifying residues that interact with the methionine and phenylalanine residues of the C-terminal moiety of CCK because these are crucial for its binding and biological activity, and to determine whether CCK and the selective non-peptide agonist, SR-146,131, share a common binding site. Identification of putative amino acids of the CCK1R binding site was achieved by dynamics-based docking of the ligand CCK in a refined three-dimensional model of the CCK1R using, as constraints, previous results that identified contact points between residues of CCK and CCK1R (Kennedy, K., Gigoux, V., Escrieu, C., Maigret, B., Martínez, J., Moroder, L., Frehle, D., Gully, D., Vaysse, N., and Fourmy, D. (1997) J. Biol. Chem. 272, 2920–2926 and Gigoux, V., Escrieu, C., Fehrentz, J. A., Poirot, S., Maigret, B., Moroder, L., Gully, D., Martínez, J., Vaysse, N., and Fourmy, D. (1999) J. Biol. Chem. 274, 20457–20464). By this approach, a series of residues forming connected hydrophobic clusters were identified. Pharmacological and functional analysis of mutated receptors indicated that a network of hydrophobic residues including Cys-94, Met-121, Val-125, Phe-218, Ile-329, Phe-330, Trp-326, Ile-352, Leu-356, and Tyr-360, is involved in the binding site for CCK and in the activation process of the CCK1R. Within this hydrophobic network, the physico-chemical nature of residue 121 seems to be essential for CCK1R functioning. Finally, the biological properties of mutants together with dynamic docking of SR-146,131 in the CCK1R binding site demonstrated that SR-146,131 occupies a region of CCK1R binding site which interacts with the C-terminal amidated tripeptide of CCK, i.e. Met-Asp-Phe-NH₂. These new and important insights will serve to better understand the activation process of CCK1R and to design or optimize ligands.

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The choceystokinin (CCK) receptor-1 (CCK1R) is a G protein-coupled receptor, which mediates important central and peripheral cholecystokinin actions. Our aim was to progress in mapping of the CCK1R binding site by identifying residues that interact with the methionine and phenylalanine residues of the C-terminal moiety of CCK because these are crucial for its binding and biological activity, and to determine whether CCK and the selective non-peptide agonist, SR-146,131, share a common binding site. Identification of putative amino acids of the CCK1R binding site was achieved by dynamics-based docking of the ligand CCK in a refined three-dimensional model of the CCK1R using, as constraints, previous results that identified contact points between residues of CCK and CCK1R (Kennedy, K., Gigoux, V., Escrieu, C., Maigret, B., Martínez, J., Moroder, L., Frehle, D., Gully, D., Vaysse, N., and Fourmy, D. (1997) J. Biol. Chem. 272, 2920–2926 and Gigoux, V., Escrieu, C., Fehrentz, J. A., Poirot, S., Maigret, B., Moroder, L., Gully, D., Martínez, J., Vaysse, N., and Fourmy, D. (1999) J. Biol. Chem. 274, 20457–20464). By this approach, a series of residues forming connected hydrophobic clusters were identified. Pharmacological and functional analysis of mutated receptors indicated that a network of hydrophobic residues including Cys-94, Met-121, Val-125, Phe-218, Ile-329, Phe-330, Trp-326, Ile-352, Leu-356, and Tyr-360, is involved in the binding site for CCK and in the activation process of the CCK1R. Within this hydrophobic network, the physico-chemical nature of residue 121 seems to be essential for CCK1R functioning. Finally, the biological properties of mutants together with dynamic docking of SR-146,131 in the CCK1R binding site demonstrated that SR-146,131 occupies a region of CCK1R binding site which interacts with the C-terminal amidated tripeptide of CCK, i.e. Met-Asp-Phe-NH₂. These new and important insights will serve to better understand the activation process of CCK1R and to design or optimize ligands.

Cholecystokinin (CCK) is a neuropeptide that has a wide spectrum of biological actions. CCK is composed of several molecular variants, the octapeptide (CCK-8: Asp-Tyr(SO₃H)-Met-Gly-Trp-Met-Asp-Phe-NH₂) being the major fully active one (1, 2). Two CCK receptors have been characterized pharmacologically, biologically and subsequently cloned, the CCK1 receptor (abbreviated CCK1R, previously named CCKA receptor) and the CCK2 receptor (abbreviated CCK2R, previously named CCK-B/gastrin receptor), which both belong to the superfamilly of G protein-coupled receptors (3, 4). The CCK1R and CCK2R can exist in several conformational states, which bind CCK with high, low, and very low affinities, respectively, and share the functional coupling to phospholipase C, via binding to heterotrimeric GTP-binding protein(s) (5–7). CCK1R-mediated effects include control of gallbladder contraction, pancreatic exocrine secretion, gastric emptying and gut motility, and satiety (8, 9). The wide spectrum of biological functions regulated by the CCK1R makes it a candidate target for a therapeutic approach in a number of diseases related to nutrient assimilation. This led a number of academic and pharmaceutical research groups to design specific and highly potent agonists and antagonists for this receptor (8, 9).

Pharmacological studies have shown that chemically distinct molecules such as peptides, peptoids, and non-peptides can bind to the CCK1R with very close affinities (8, 9). On the other hand, within each chemical family of CCK1R ligands, compounds having close structures are agonists, partial agonists, or antagonists, indicating that appropriate modifications within the pharmacophore switches an agonist to an antagonist and vice versa. This can be illustrated with both peptide and non-peptide ligands of CCK1R. For instance, JMV 179, a CCK heptapeptide analogue in which the C-terminal amidated phenylalanine and the l-tryptophan have been replaced by a phenethyl ester moiety and a D-tryptophan, respectively, is a full CCK1R antagonist (10). This antagonist has been converted to JMV 180, a peptide exhibiting dual agonistic/antagonistic activity, by exchanging the D-tryptophan for an L-tryptophan (7, 11). Another interesting example came from the discovery of the non-peptide CCK1R agonist, SR-146,131, by...
chemical modification of the CCK1R antagonist, SR-27,897 (12, 13) (Fig. 1). These examples, which could probably be extended to multiple G protein-coupled receptors, raise the important questions of whether closely related ligands having opposite biological activities share the same binding site and of which intrinsic mechanism(s) at the binding site level govern(s) G protein-coupled receptor functioning.

One of our recent objectives has been to define the agonist binding site on the CCK1R and to identify interactions between critical residues of that binding site and chemical functions of the pharmacophoric domain of CCK (Figs. 1 and 3). Amino acids within three regions of the CCK1R were identified as belonging to the binding site for CCK. Trp-39 and Gln-40, located at the top of transmembrane helix I, were shown to interact directly with the N-terminal portion of CCK (14). Met-195 and Arg-197, located in the second extracellular loop, were then shown to interact with the sulfated tyrosine (15, 16). More recently, Arg-336 and Asn-333, at the top of helix VI, were demonstrated to pair with the Asp carboxylate and the C-terminal amide of CCK, respectively (17). The first two identified amino acids of the CCK1R (Trp-39 and Gln-40) contribute weakly to CCK1R affinity for and response to CCK, whereas all others play a more critical role because of their interaction with residues of CCK, which are essential for both binding and biological activity of CCK. However, contact points within the CCK1R binding site for other key residues of CCK such as the Trp, Met, and Phe residues have not yet been identified.

To progress in the mapping of the CCK1R binding site(s), the three-dimensional model of the CCK1R-CCK complex was optimized, leading to the identification of putative amino acids involved in the interaction with the Met and Phe residues of the ligand CCK. Mutation of candidate residues and extensive characterization of the resulting mutants allowed us to position the C-terminal biological part of CCK in hydrophobic pockets formed by aromatic and nonaromatic amino acids located in the upper half of transmembrane helices III, V, VI, and VII. Our data, therefore, refute the model of the CCK1R-CCK complex proposed by other investigators in which the C terminus of CCK interacts with an amino acid residue (Trp-39) of helix I (18). Furthermore, binding site for the non-peptide agonist SR-146,131 was identified and experimentally validated as overlapping with that of the C-terminal tripeptide of CCK. Finally, the role of Met-121 located on helix III in the activation of the CCK1R by agonists was demonstrated.

Experimental Procedures

Materials—The C-terminal nonapeptide analogue (Nle)-CCK-9 (Fig. 1) was synthesized as described previously (19). The other analogues of Fig. 1 were synthesized on an ACT 396 synthesizer by applying the Fmoc (N-ter-butyloxycarbonyl)/N-(9-fluorenyl)methoxycarbonyl chemistry and chloro- and thiophenoxyphenyl)-5-(2-cyclohexylethyl)thiazol-2-ylcarbamoyl]-5,7-dimethyl-yl-indol-1-yl-1-acetic acid (SR-146,131), were donated by Sanofi-Synthelabo (Toulouse, France) (12, 15). 125I Na and radioiodinated as described previously (20). The specific activity of the radioiodinated peptide was 1600–2000 Ci/mmol. All other chemicals were obtained from commercial sources.

Computer Modeling of the CCK1R and CCK1R-CCK Complex—The model of empty CCK1R was built using the transmembrane (TM) helical arrangement found in the bacteriorhodopsin crystal structure as starting point (21). It was then modified according to the rhodopsin crystal structure (22, 23) and to the mutant data base “input/output” information scheme defined in the Viseur program (24). Extracellular and intracellular loops connecting the transmembrane helices were then added to the preliminary seven-helix bundle, and the structural model was optimized by the use of simulated annealing procedures. The entire system was finally relaxed and submitted to a 1 ns molecular dynamics with possible translational and rotational movements of individual TM helices taken into account. The final model respects most transmembrane arrangements found in the recent x-ray structure of rhodopsin (23). For docking of CCK ligand into the CCK1R binding site, experimental data that identified contact points between residues Trp-39 and Gln-40 and the N-terminal moiety of CCK as a first constraint to place CCK within the CCK1R groove (14). In a first step, manual docking was achieved by taking into account molecular electrostatic potentials at the top of the receptor groove. The resulting complex was submitted to annealing molecular dynamics calculations. The resulting theoretical positioning of CCK into the CCK1R binding site was experimentally validated by two-dimensional site-directed mutagenesis. By doing so, Met-195–Arg-197, Arg-336, and Asn-333 were shown to belong the CCK1R binding site and to interact with the sulfated tyrosine of CCK, the Asp-8 carboxylate, and the C-terminal amide, respectively (15–17). The program package (Insight II, Discover, Homology, Biopolymer) from Molecular Simulations Inc. (San Diego, CA) was used for all the calculations.

Site-directed Mutagenesis and Transfection of COS-7 Cells—Mutant receptor cDNAs were constructed by oligonucleotide-directed mutagenesis (QuikChange® site-directed mutagenesis kit, Stratagene, France) using the human CCK1R cDNAs cloned into pRFENeo vector as template (25). Oligonucleotides were designed to include a silent restriction site to facilitate analysis of mutant constructs by restriction endonuclease digestion. The presence of the desired and the absence of undesired mutations were confirmed by automated sequencing of both cDNA strands (Applied Biosystems).

COS-7 cells (5 × 10⁴) were plated onto 10-cm culture dishes and grown in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum (complete medium) in a 5% CO₂ atmosphere at 37 °C. After overnight incubation, cells were transfected with 2.5 μg plate of pRFENeo vectors containing the cDNA for the wild-type or mutated CCK1 receptors, using a modified DEAE-dextran method. Cells were transferred to 24-well plates at a density of 80,000–150,000 cells/well 24 h after transfection.

| Abbreviation | Primary structure |
|--------------|------------------|
| (Nk)•CCK     | Arg-Asp-Tyr(SO₄H)·Thr-Gly-Trp-Nle-Asp-Phe-CONH₂ |
| (Mn)•CCK     | Asp-Tyr(SO₄H)·Thr-Gly-Trp-Met-Asp-Phe-CONH₂ |
| (Gln)•CCK    | Arg-Asp-Tyr(SO₄H)·Thr-Gly-Trp-Gln-Asp-Phe-CONH₂ |
| (Ala)•CCK    | Arg-Asp-Tyr(SO₄H)·Thr-Gly-Trp-Ala-Asp-Phe-CONH₂ |
| SR-27,897    | ![SR-27,897](https://example.com/sr27897.png) |
| SR-146,131   | ![SR-146131](https://example.com/sr146131.png) |

Fig. 1. Ligands used to map the CCK1R binding site.
Receptor Binding Assay—Approximately 24 h after the transfer of transfected cells to 24-well plates, the cells were washed with phosphate-buffered saline, pH 6.95, 0.1% BSA and then incubated for 60 min at 37 °C in 0.5 ml of Dulbecco’s modified Eagle’s medium, 0.1% BSA with either 71 pmol I125-1-BH-(Thr,Nle)-CCK-9 or 1.83 nm [3H]SR-27,887 in the presence or the absence of competing agonists or antagonists. The cells were washed twice with cold phosphate-buffered saline, pH 6.95, containing 2% BSA, and cell-associated radioactivity was collected with 0.1 N NaOH added to each well. The radioactivity was directly counted in a γ counter (Auto-Gamma, Packard, Dohmeau Grove, IL) or added to scintillator and counted for the tritiated radioligand.

Inositol Phosphate Assay—Approximately 24 h after the transfer to 24-well plates, the following overnight incubation in complete medium containing 2 µCi/ml [methyl-3H]inositol, the transfected cells were washed with Dulbecco’s modified Eagle’s medium and then incubated for 30 min in 1 ml/well Dulbecco’s modified Eagle’s medium containing 20 mM LiCl at 37 °C. The cells were washed with PI buffer at pH 7.45: phosphate-buffered saline containing 153 mM NaCl, 20 mM HEPES, 2 mM CaCl2, 1.2 mM MgSO4, 1 mM EGTA, 10 mM LiCl, 11.1 mM glucose, and 0.5% BSA. The cells were then incubated for 60 min at 37 °C in 0.3 ml of PI buffer with or without ligands at various concentrations. The reaction was stopped by adding 1 ml of methanol/chloroformic acid to each well, and the content was transferred to a column (Dowex AG 1-X8, formate form, Bio-Rad) for the determination of inositol phosphates. The columns were washed twice with 3 ml of distilled water and twice with 3 ml of 50% ethanol. Sodium tetraborate, 10 mM ammonium formate, 10 mM formic acid. 0.5 ml of the eluted fraction was added to scintillator, and β radioactivity was counted.

Membrane Preparation—Approximately 65 h after transfection, the cells were washed three times with phosphate-buffered saline, pH 6.95, scraped from the plate in 10 mM Hepes buffer, pH 7.0, containing 0.01% soybean trypsin inhibitor, 0.1% bacitracin, 0.1% phenylmethylsulfonyl fluoride and frozen in liquid N₂. After thawing at 37 °C, the cells were subjected to another cycle of freeze/thawing and then centrifuged at 25,000 × g for 20 min. The membrane pellet was resuspended in 50 mM Hepes buffer, pH 7.0, containing 115 mM NaCl, 5 mM MgCl₂, 0.01% soybean trypsin inhibitor, 0.1% bacitracin, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (binding buffer); aliquoted; and stored at −80 °C until use. Membrane protein concentrations were determined using the Bio-Rad protein assay kit. To assess the effect of GTPγS on CCK binding, membranes from transfected COS-7 cells (0.4–4 µg of proteins) were incubated with 71 pmol [125]I-BH-(Thr,Nle)-CCK-9 in the absence or in the presence of increasing concentrations GTPγS in binding buffer for 120 min at 25 °C. Nonspecific binding was measured in the presence of 1 µM CCK.

RESULTS

Importance of Methionine 7 and Phenylalanine 9 of CCK for Binding and Activation of the CCKR—Previous structure-activity studies using synthetic replicates of CCK and pancreatic acini from rodents, a biological model naturally expressing CCKR, have clearly shown the importance of both Met and Phe residues for binding and activity of CCK; however, no such study has been reported for human CCKR in any expression system (26). Therefore, we first determined to what extent the Met and Phe side chains contribute to the affinity of CCK for human CCKR expressed in COS-7 cells and to its capacity to induce production of total inositol phosphates. As shown in Fig. 2, replacement of Met-7 in CCK by an Ala residue caused 4000- and 3900-fold decrease in affinity and potency, respectively. In contrast, replacement of Met-7 by Nle did not affect affinity and potency of CCK. Furthermore, exchange of Phe for Ala was found to induce a 4900- and 2700-fold decrease in affinity and potency of the analogues, respectively.

Identification of Candidate Amino Acid Residues of the CCKR for Interaction with Nle/Met-7 and Phe-9 of CCK Using Molecular Modeling—Considering the major anchoring points of CCK inside the receptor discovered in our previous studies, it appears that the Nle/Met-7 residue is located in the vicinity of a hydrophobic pocket constituted by residues Leu-50, Ile-51, Leu-53, Pro-101, Val-125, Met-121, Ileu-352, and Leu-356. In the model of the (WT)-CCKR, both Nle-7 or Met-7 side chains are positioned in the same way. The Phe-9 aromatic side chain of CCK is also positioned into a well defined cavity delineated by Phe-330 at the bottom, and Pro-177, Val-125, Leu-214, Ile-329 around Phe-330, which itself belongs to a large aromatic cluster constituted by the side chains of Cys-94, Phe-130, Trp-166, Phe-170, Phe-218, Phe-323, Phe-322, Trp-326, and Tyr-360. These two pockets are connected via the Val-125 side chain so that helix movements changing the structure of one of them may have consequences on the other. Examination of the organization of the two clusters in the three-dimensional model of the CCKR/CCK complex suggests that not only a single, but several hydrophobic residues are contributing to the binding energy between Nle/Met-7 or Phe side chains of CCK and the CCKR. As a consequence and unlike the charged residues of the binding pocket that were characterized previously, a mutation of only one of these amino acid residues of the CCKR was not expected to induce changes in affinity and biological potency of the CCKR to an extent comparable with effects caused by replacements of Nle/Met or Phe in CCK.

Effects of CCKR Mutations on CCKR Expression and Affinity for the Non-peptide Antagonist SR-27,897—Among all residues forming hydrophobic clusters around the Nle/Met-7 and Phe-9 side chains, in a first instance those in closest contact were chosen for mutagenesis experiments (Fig. 3). These were exchanged for amino acids lacking the chemical functions thought to be responsible for the interactions. In addition, Met-121 and Ile-329, which appear to be important for the equilibrium within the hydrophobic clusters surrounding Nle/Met-7 and Phe-9 of CCK, were each exchanged for more bulky and hydrophobic residues, namely Val and Phe, respectively. In a first series of experiments, COS-7 cells expressing

Fig. 2. Importance of Met-7 and Phe-9 of CCK for binding (a) and production of inositol phosphates (b), α, COS-7 cells expressing the human wild-type CCKR were incubated with 125 I-BH-(Thr,Nle)-CCK-9 alone or in the presence of increasing concentrations of competition peptide as described under Experimental Procedures. Specific binding in each assay is expressed as percentage of specific binding.
mutated receptors were assayed for binding of the non-peptide antagonist \[^{3}H\]SR-27,897. Indeed, binding of this antagonist, unlike that of an agonist, offers the advantage of allowing detection of CCK1R independent of the coupling state to G protein(s), thus yielding accurate expression levels of all mutants (15, 17). Ligand binding data are summarized in Table I. No binding of \[^{3}H\]SR-27,897 was found with the (I329F)-CCK1R mutant, a result that could be interpreted at this stage as does the wild-type receptor; however, the affinity of either the high or low affinity component, or both, was modified. For instance, (L356A)-CCK1R and (I329A)-CCK1R showed minor changes in their high affinity sites relative to the (WT)-CCK1R, but a 5.5–8.5-fold decrease in affinity of the low affinity sites, respectively. A third category of mutants, i.e. (L50A), (I51A), (L53A), (V125A), and (W326A)-CCK1R, exhibited binding features that were very similar to those of the (WT)-CCK1R because radioligand binding to two affinity classes of binding sites was observed, both with affinities similar to those of their equivalents in the (WT)-CCK1R. Finally, two mutants, i.e. (I329F)- and (I352A)-CCK1R, did not bind \[^{125}I\]BH-(Thr,Nle)-CCK-9, even when the radioligand concentration was increased up to 250 pM.

The maximal number of binding sites for CCK were either similar as in the mutants (C94L), (M121V), (M121A), (F218A), (F330A)-CCK1R, or lower (all other mutants) to expression levels calculated from binding experiments with the non-peptide antagonist \[^{3}H\]SR-27,897.

Effects of Mutations on CCK1R Functional Coupling to Phospholipase-C—The biological function of the mutated receptors was evaluated by determining inositol phosphate accumulation in transfected COS-7 cells upon (Nle-7)-CCK stimulation. Table II summarizes potency (D\(_{50}\)) and efficacy (E\(_{max}\)) of the different mutants in response to (Nle-7)-CCK stimulation. The mutants (L50A), (I51A), (L53A), (M121A), and (F218A)-CCK1R were found to exhibit biological potencies very similar to that of the (WT)-CCK1R. However, the biological efficacy of some of these mutants was affected. Indeed, maximal production of inositol phosphates by the mutants (I51A), (M121A), and (F218A)-CCK1R reached 63, 48, and 35% of that achieved by the (WT)-CCK1R. Conversely, the mutants (C94L), (I352A), (L356A), (V125A), (W326A), (I329F), (F330A), and (Y360F)-CCK1R showed, respectively, a 28-, 29-, 2.3-, 4.3, 38-, 468-, 2.6-, and 32-fold decrease in their potency to stimulate inositol phosphate production if compared with the (WT)-CCK1R. The biological efficacy of (C94L)- and (F330A)-CCK1R was reduced to 45 and 57% of that of the (WT)-CCK1R. It is worthy to note that (I329F)-CCK1R, which failed to bind both the CCK and SR-27,897 radioligands, was capable of inducing production of inositol phosphates after (Nle-7)-CCK stimulation, although its potency was 507-fold decreased compared with the (WT)-CCK1R. This result suggests that the absence of binding was likely because of a drastically reduced affinity of the mutant for CCK and SR-27,897. Indeed, this explanation was indirectly confirmed with experiments, which showed that the potency of SR-27,897 to inhibit CCK-induced production of inositol phosphates by COS-7 cells...
GraphPad Prism program (Software). The mutation factors (Fmut) were calculated as
\[ \frac{K_d}{K_d} \text{protein(s) coupling} \] 
was further evaluated by experiments, the importance of the side chain of residue 121 in the CCK1R for G
this residue is located in the upper part of helix III. Indeed, the crucial role in the coupling of CCK1R to G
this analogue was used because of its high stability compared with Met in terms of possible oxidation on handling (19). The use of this analogue was supported by previous findings that confirmed its full biological potency. This was also confirmed in the present study, as shown in Fig. 2, where replacement of Met-7 with Nle was found to be without any effect on affinity and activity of the ligand. According to the three-dimensional model of the CCK1R-CCK complex, Nle (or Met) of CCK is inserted into a hydrophobic pocket including residues Leu-50, Ile-51, Leu-53, Cys-94, Met-121, Val-125, Ile-352, and Leu-356. To analyze whether the biological efficacy of mutated receptors was de-

**TABLE I**

| CCK1R | SR 27897 binding | CCK binding |
|-------|------------------|-------------|
|       | Kd (nM) Fmut pmol/10^6 cells | Bmax | Kd (nM) Fmut pmol/10^6 cells | Bmax |
| WT    | 3.3 ± 0.5 1.0 | 9.2 ± 1.6 | 1.20 ± 0.05 | 1.0 |
| L50A  | 3.1 ± 0.7 0.9 | 6.5 ± 0.5 | 1.1 ± 0.05 | 1.0 |
| I51A  | 6.4 ± 3.8 1.9 | 7.9 ± 0.9 | 2.0 ± 0.5 | 1.7 |
| L53A  | 1.9 ± 0.6 0.6 | 5.6 ± 1.5 | 1.4 ± 0.7 | 1.2 |
| C94L  | 7.4 ± 2.6 2.8 | 4.9 ± 0.9 | 74.0 ± 5.0 | 62 |
| M121V | 6.2 ± 1.4 1.9 | 6.5 ± 3.4 | 21.0 ± 2.2 | 1.8 |
| M121A | 8.9 ± 2.6 2.7 | 10.0 ± 3.3 | 3.1 ± 0.3 | ND |
| I352A | 5.4 ± 1 | 1.6 | 4.7 ± 0.2 | No binding |
| L356A | 4.0 ± 0.3 | 1.2 | 5.1 ± 0.4 | 0.97 ± 0.03 |
| V125A | 3.4 ± 0.5 | 1.0 | 2.6 ± 0.4 | 0.50 ± 0.14 |
| F218A | 5.3 ± 1.6 | 1.6 | 0.6 ± 1.2 | 2.8 ± 0.6 |
| W326A | 3.2 ± 0.1 | 1.0 | 7.9 ± 0.8 | 0.25 ± 0.12 |
| I329A | 5.0 ± 0.6 | 1.5 | 4.4 ± 0.3 | 0.57 ± 0.05 |
| F330F | 7.1 ± 2.8 | 1.1 | No binding |
| F330A | 3.0 ± 0.5 | 0.9 | 4.2 ± 0.1 | 12.5 ± 3.3 |
| Y360F | 2.8 ± 0.3 | 0.8 | 6.2 ± 0.1 | 2.7 ± 1.4 |

**TABLE II**

| CCK1R | (Nle-7)-CCK-induced inositol phosphate |
|-------|---------------------------------------|
|       | EC50 Fmut pmol/106 cells |
| WT | 3.3 ± 0.5 |
| L50A | 3.1 ± 0.7 |
| I51A | 6.4 ± 3.8 |
| L53A | 1.9 ± 0.6 |
| C94L | 7.4 ± 2.6 |
| M121V | 6.2 ± 1.4 |
| M121A | 8.9 ± 2.6 |
| I352A | 5.4 ± 1 |
| L356A | 4.0 ± 0.3 |
| V125A | 3.4 ± 0.5 |
| F218A | 5.3 ± 1.6 |
| W326A | 3.2 ± 0.1 |
| I329A | 5.0 ± 0.6 |
| F330F | 7.1 ± 2.8 |
| F330A | 3.0 ± 0.5 |
| Y360F | 2.8 ± 0.3 |

expressing (I329F)-CCK1R was 625-fold decreased if compared with the (WT)-CCK1R (1500 nM versus 24 nM, n = 2, not shown). Finally, and very surprisingly, the mutant (M121V)-CCK1R failed to induce production of inositol phosphate upon stimulation with (Nle-7)-CCK, whereas (M121A)-CCK1R retained the ability to couple to phospholipase C, even though with a moderate efficacy (48% of that of (WT)-CCK1R; see Table II and Fig. 4). These data suggest that Met-121 plays a crucial role in the coupling of CCK1R to G protein(s), although this residue is located in the upper part of helix III. Indeed, the importance of the side chain of residue 121 in the CCK1R for G protein(s) coupling was further evaluated by experiments, which showed that the nonhydrolyzable analogue of GTP, GTP\(\gamma\)S, did not dissociate binding of 125I-BH-(Thr,Nle)-CCK-9 to (M121V)-CCK1R, whereas it dissociated binding of CCK to (M121A)- and (WT)-CCK1R to extents that agree with the biological efficacies of these receptors (Fig. 4).
Results from all the CCK1R complexes are expressed as percentage of maximal inositol phosphate production obtained in COS-7 expressing the wild-type CCK1R after stimulation by (Nle-7)-CCK. Potency (D50) and efficacy (E(max)) for the different complexes were: (WT)-CCK1R-(Nle-7)-CCK, D50: 1.3 ± 0.1 nM, E(max): 100; (WT)-CCK1R-(Met-7)-CCK, D50: 1.0 ± 0.1 nM, E(max): 100; (WT)-CCK1R-(Gln-7)-CCK, D50: 12.5 ± 2.4 nM, E(max): 95; (M121V)-CCK1R-(Nle-7)-CCK, D50: 12.4 ± 1.1 nM, E(max): 83; (M121V)-CCK1R-(Gln-7)-CCK, D50: 22.0 ± 4.5 nM, E(max): 44; (M121Q)-CCK1R-(Nle-7)-CCK, D50: 1.8 ± 1.5 nM, E(max): 95. Results are from three to five individual determinations.

Effects of Nle/Met exchange in CCK and Met-121 mutations on CCK1R-mediated production of inositol phosphates. Inositol production assays were conducted as described under "Experimental Procedures." Results from the CCK1R-CCK complexes are expressed as percentage of maximal inositol phosphate production obtained in COS-7 expressing the wild-type CCK1R after stimulation by (Nle-7)-CCK. Potency (D50) and efficacy (E(max)) for the different complexes were: (WT)-CCK1R-(Nle-7)-CCK, D50: 12.5 ± 2.4 nM, E(max): 95; (WT)-CCK1R-(Met-7)-CCK, D50: 1.0 ± 0.1 nM, E(max): 100; (WT)-CCK1R-(Gln-7)-CCK, D50: 12.5 ± 2.4 nM, E(max): 95; (M121V)-CCK1R-(Nle-7)-CCK, D50: 12.4 ± 1.1 nM, E(max): 83; (M121V)-CCK1R-(Gln-7)-CCK, D50: 22.0 ± 4.5 nM, E(max): 44; (M121Q)-CCK1R-(Nle-7)-CCK, D50: 1.8 ± 1.5 nM, E(max): 95. Results are from three to five individual determinations.

Effects of Nle/Met exchange in CCK and Met-121 mutations on CCK1R-mediated production of inositol phosphates. Inositol production assays were conducted as described under "Experimental Procedures." Results from all the CCK1R complexes are expressed as percentage of maximal inositol phosphate production obtained in COS-7 expressing the wild-type CCK1R after stimulation by (Nle-7)-CCK. Potency (D50) and efficacy (E(max)) for the different complexes were: (WT)-CCK1R-(Nle-7)-CCK, D50: 1.3 ± 0.1 nM, E(max): 100; (WT)-CCK1R-(Met-7)-CCK, D50: 1.0 ± 0.1 nM, E(max): 100; (WT)-CCK1R-(Gln-7)-CCK, D50: 12.5 ± 2.4 nM, E(max): 95; (M121V)-CCK1R-(Nle-7)-CCK, D50: 12.4 ± 1.1 nM, E(max): 83; (M121V)-CCK1R-(Gln-7)-CCK, D50: 22.0 ± 4.5 nM, E(max): 44; (M121Q)-CCK1R-(Nle-7)-CCK, D50: 1.8 ± 1.5 nM, E(max): 95. Results are from three to five individual determinations.

Results are from all the CCK1R complexes are expressed as percentage of maximal inositol phosphate production obtained in COS-7 expressing the wild-type CCK1R after stimulation by (Nle-7)-CCK. Potency (D50) and efficacy (E(max)) for the different complexes were: (WT)-CCK1R-(Nle-7)-CCK, D50: 1.3 ± 0.1 nM, E(max): 100; (WT)-CCK1R-(Met-7)-CCK, D50: 1.0 ± 0.1 nM, E(max): 100; (WT)-CCK1R-(Gln-7)-CCK, D50: 12.5 ± 2.4 nM, E(max): 95; (M121V)-CCK1R-(Nle-7)-CCK, D50: 12.4 ± 1.1 nM, E(max): 83; (M121V)-CCK1R-(Gln-7)-CCK, D50: 22.0 ± 4.5 nM, E(max): 44; (M121Q)-CCK1R-(Nle-7)-CCK, D50: 1.8 ± 1.5 nM, E(max): 95. Results are from three to five individual determinations.

Effects of Nle/Met exchange in CCK and Met-121 mutations on CCK1R-mediated production of inositol phosphates. Inositol production assays were conducted as described under "Experimental Procedures." Results from all the CCK1R complexes are expressed as percentage of maximal inositol phosphate production obtained in COS-7 expressing the wild-type CCK1R after stimulation by (Nle-7)-CCK. Potency (D50) and efficacy (E(max)) for the different complexes were: (WT)-CCK1R-(Nle-7)-CCK, D50: 1.3 ± 0.1 nM, E(max): 100; (WT)-CCK1R-(Met-7)-CCK, D50: 1.0 ± 0.1 nM, E(max): 100; (WT)-CCK1R-(Gln-7)-CCK, D50: 12.5 ± 2.4 nM, E(max): 95; (M121V)-CCK1R-(Nle-7)-CCK, D50: 12.4 ± 1.1 nM, E(max): 83; (M121V)-CCK1R-(Gln-7)-CCK, D50: 22.0 ± 4.5 nM, E(max): 44; (M121Q)-CCK1R-(Nle-7)-CCK, D50: 1.8 ± 1.5 nM, E(max): 95. Results are from three to five individual determinations.

Effects of CCK1R Mutations on SR-146,131-induced Production of Inositol Phosphates—SR-146,131 is an agonist having high affinity and specificity for the CCK1R. Although of non-peptidic nature, this compound exhibits some structural similarities with the C-terminal part of CCK docked into the three-dimensional model of the CCK1R (Fig. 1). This observation raises the interesting question of whether these structural similarities imply an overlapping of the binding sites for the two ligands, SR-146,131 and CCK. A way to address this question would be to analyze the effects on binding of SR-146,131 by the mutation of those residues in the receptor which were found to be involved in CCK binding. However, as no labeled SR-146,131 was available, these effects were determined by measuring SR-146,131-stimulated production of inositol phosphates. The results (Table III) revealed that residues Met-195 and Arg-197, which were shown previously to pair with the C-terminal amide and the carboxylate side chain of Asp-8 of CCK, respectively, are likely involved in recognition of SR-146,131 because their mutation caused a 120- and 126-fold decrease in potency of inositol phosphate responses, respectively. Among the residues of the receptor that were shown to interact with the Nle-Met-7 and Phe residues, several seem to be involved in SR-146,131-induced inositol phosphate production. In fact, the mutants (C94L), (I352A), (L356A), (V125A), (W326A), (I329A), (I329F), and (F330A)-CCK1R responded to

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**Fig. 5. Effects of Nle/Met exchange in CCK and Met-121 mutations on CCK1R on CCK1R-mediated production of inositol phosphates.** Inositol production assays were conducted as described under "Experimental Procedures." Results from all the CCK1R complexes are expressed as percentage of maximal inositol phosphate production obtained in COS-7 expressing the wild-type CCK1R after stimulation by (Nle-7)-CCK. Potency (D50) and efficacy (E(max)) for the different complexes were: (WT)-CCK1R-(Nle-7)-CCK, D50: 1.3 ± 0.1 nM, E(max): 100; (WT)-CCK1R-(Met-7)-CCK, D50: 1.0 ± 0.1 nM, E(max): 100; (WT)-CCK1R-(Gln-7)-CCK, D50: 12.5 ± 2.4 nM, E(max): 95; (M121V)-CCK1R-(Nle-7)-CCK, D50: 12.4 ± 1.1 nM, E(max): 83; (M121V)-CCK1R-(Gln-7)-CCK, D50: 22.0 ± 4.5 nM, E(max): 44; (M121Q)-CCK1R-(Nle-7)-CCK, D50: 1.8 ± 1.5 nM, E(max): 95. Results are from three to five individual determinations.
The aim of the present study was to advance in the knowledge of the binding site of CCK1 receptor for CCK, by identifying amino acid residues that interact with two crucial residues of the CCK, namely Met and Phe for which no receptor partner was yet identified. For this purpose, approaches of
molecular modeling and experimental site-directed mutagenesis of the CCK1R were combined.

For modeling experiments, a structural model of the CCK1R-CCK complex was generated in which amino acid residues of the binding site for CCK were shown previously to be involved by pharmacological and functional analysis of CCK1R mutants using several peptidic and non-peptidic ligands (14–17). In this structural model, the C-terminal portion of CCK was strongly constrained in the receptor groove because of interactions between Arg-336 and the carboxylate of the penultimate Asp residue of CCK, and between Asn-333 and the C-terminal CCK amide. These interactions restrict considerably possible movements of the C-terminal part of CCK within the bottom of the receptor groove. In fact, after dynamic docking, two closely linked hydrophobic clusters appeared as likely candidates for interactions with the Nle/Met 7 and Phe side chains of the ligand, which are so critical for binding affinity and biological potency of the neuropeptide.

This CCK1R-CCK structural model was well supported by a large set of experimental data and their physicochemical interpretation. The observation that mutation of residues such as Leu-50, Ile-51, Leu-53, and Val-125 affect only slightly recognition of CCK by the receptor is in full agreement with their expected low contribution to the stability of CCK1R-CCK complex. The moderate decrease in affinity and potency (10- and 3-fold) caused by mutation of Phe-330 was consistent with a T-shaped interaction between the aromatic rings of CCK Phe-9 and receptor Phe-330. The positioning of the C terminus of CCK was furthermore in agreement with the dramatic effect of the Ile-329 → Phe mutation on CCK-induced CCK1R activation, which likely results from a reduced ability of the mutant to bind CCK. Indeed, in the three-dimensional model of (I329F)-CCK1R-CCK complex, exchange of Ile-329 for a Phe residue causes rotation of the amide of CCK away from Asn-333, its partner in (WT)-CCK1R. Consequently, the interaction between the CCK amide and the carboxamide of Asn-333 is lost (data not shown). The similar properties of (I329F)-CCK1R and the previously characterized (N333A)-CCK1R support this explanation (17). Indeed, the (N333A)-CCK1R mutant mediates CCK-induced inositol phosphate production with a 1350-fold lower potency than (WT)-CCK1R and with an efficacy 60% of that of (WT)-CCK1R (17). Two sets of experimental data obtained with the receptor mutated at residues Ile-352 and Met-121 further validate the location of the C-terminal portion of CCK. Exchange of Ile-352 for an Ala caused a 232-fold shift in the potency of CCK1R to induce inositol phosphates, a result that was likely the result of an important decrease in the binding affinity of (I352A)-CCK1R for CCK. This result agrees with observations from the three-dimensional receptor model, which suggest that, in the empty receptor, Ile-352 of TM7 interacts with Ile-329 of TM6 and that, upon docking of CCK, the Nle/Met-7 side chains disrupt the interaction between these two residues.

To confirm the existence of the hydrophobic cluster surrounding Nle/Met of CCK, we predicted that mutation of Met-121 to Val will decrease binding affinity of the CCK1R for CCK because of presence of bulky isopropyl side chain of Val residue, whereas mutation of Met-121 to Ala will not significantly affect binding. Experimental data confirmed the modeling predictions because mutants (M121V)- and (M121A)-CCK1R bound CCK with affinities that were 16- and 1.8-fold lower than that of the (WT)-CCK1R, respectively. Proximity between Met-121 and Met/Nle of CCK was further documented by the set of data showing that Met-7 of CCK and Met-121 of the CCK1R were interchangeably to yield an active CCK1R-CCK complex (see below).

The present study also provided important new data concerning the comparison between the binding site for the non-peptide agonist SR-146,131 and the binding site for CCK. Both the experimental data obtained with this compound and the CCK1R mutants and its dynamic docking to the three-dimensional receptor model agree with a positioning of the non-peptide agonist into the part of the binding site that interacts with the C-terminal tripeptide of CCK, Met-Asp-Phe-NH₂. Note that the sulfur atom and the carbonyl group have a position similar to that of Nle/Met of CCK toward Met-121 of the CCK1R.
photoaffinity labeling experiments (18, 32, 33). In the photoaffinity labeling experiments with a CCK photoprobe in which the C-terminal Phe residue was replaced by benzophenylalanine, Trp-39 at the top of helix I was identified, supporting the hypothesis that the C terminus of CCK was in close proximity of the first helix (32). Based on these findings, a model was proposed of the peptide-occupied CCK1R in which CCK resembles an hairpin lying at the receptor extracellular surface with the C-terminal Phe of CCK being in contact with Trp-39 of the receptor (18). It is very likely that the large amount of energy required to generate the covalent bond from a p-nitrophenylalanine (or benzophenone) (30 min of UV irradiation at a wavelength of 300 nm) significantly affected the conformation of both the CCK1R and CCK, leading to movement of CCK within its binding site. On the other hand, by definition, photoaffinity labeling using structurally modified CCK could not identify amino acids of the receptor in interaction with native CCK.

Finally, with the current study, we succeeded in providing the first data related to the process of CCK1R activation following agonist binding. Indeed, we found that an exchange of Met-121 for a Val residue leads to a CCK1R mutant that is unable to induce inositol phosphate accumulation upon (Nle-7)-CCK stimulation. With this mutant, 30% of biological activity is recovered upon stimulation by (Met-7)- and (Gln-7)-CCK and 84% upon stimulation by SR-146,131. From a general point of view, results with mutants at position 121 show that ascribing a functional role to a residue must take into account the fact that impact of mutations on pharmacological and functional properties of a receptor can depend on the nature of the amino acid by which a critical residue is substituted as well as on the ligand used to analyze the mutants. Direct involvement of the sulfur atom of Met-7 of CCK or Met-121 of CCK1R in the mechanism of receptor activation can be ruled out by results demonstrating that the Gln side chain can mimic the Met side chain. However, the presence of a polar atom within the hydrophobic cluster surrounding the Met-121/Met-7 residues seems to be required for CCK1R activation. Indeed, in terms of production of inositol phosphates, the relative efficacies of the mutated complexes were (M121V)-CCK1R(Nle-7)-CCK = 0, < (M121V)-CCK1R(Met-7)-CCK < (M121A)-CCK1R(Nle-7)-CCK < (M121A)-CCK1R(Met-7)-CCK. The more the side chain of residues in the vicinity of position 121 is hydrophobic, the less efficient is phospholipase C activation. In agreement with this view, efficacy of mutant M121A represents ~50% of that of (WT)-CCK1R, a value in accordance with moderate “hydrophobic weight” of the methyl of Ala relative to isopropyl of Val. The much higher efficacy of SR-146,131 compared with (Met-7)-CCK in stimulating functional coupling of (M121V)-CCK1R to phospholipase C can tentatively be ascribed to the presence of two polar elements, i.e., a sulfur atom and a carbonyl moiety in the vicinity of position 121 of the receptor. Another major support for the peculiar role of the residues in position 121 of the receptor and 7 of the ligand was derived from molecular dynamic modeling of the different complexes, which show the importance of the amino acid side chains in these positions for the correct positioning of the C-terminal part of CCK, particularly toward Phe-330 of the CCK1R. An interaction between the aromatic ring of CCK Phe-9 and that of Phe-330 (T-shape) seems to be important for CCK1R full activation. This view is in line with the 60% decrease in efficacy to stimulate inositol phosphate production caused by an exchange of Phe-9 with Ala in CCK (Fig. 2). Furthermore, stimulation of inositol phosphate production was strongly affected by mutation of residue Phe-330, as it was affected by mutations of Cys-94 and Phe-218. In addition to their role in CCK-induced production of inositol phosphates, binding results also argue in favor of a role of the residues Met-121, Cys-94, Phe-218, and Phe-330 in the conformational stability of CCK1R. Indeed, the data showed that exchange of Met-121 for a Val or an Ala converted the whole CCK1R population into a single, relatively high, affinity state as did mutation of residues Cys-94, Phe-218, and Phe-330. All these data can be interpreted by considering the prevailing model for G protein-coupled receptor activation, which is the allosteric ternary complex formed between the receptor R, the agonist L, and G protein(s). According to this model, in absence of any agonist stimulation, R is believed to undergo spontaneous conformational changes, however, with the inactive conformation being energetically the most stable. Binding of an agonist would either induce or stabilize active receptor species (R*), or both (34–36). Accordingly, Met-121, Phe-330, Cys-94, and Phe-218 would represent key residues allowing the receptor either to be stabilized in an inactive conformation in absence of ligand and to undergo proper conformational changes for G protein(s) coupling and phospholipase C activation in presence of the agonist.

Further investigations are obviously required to determine precisely how certain residues such as those pointed out in this study regulate affinity state and/or activation of the CCK1R. In light of recent data with rhodopsin and β2-adrenergic receptor, we hypothesize that these residues play a pivotal role for helix movements upon CCK binding thereby enabling G protein(s) to efficiently couple with previously buried region of that receptor (37, 38). Several conserved regions at the bottom of helix III (such as (D/E)-R-Y motif), helix VII (such as N-P-X-Y-Y), and helix VI near the third intracellular loop have been shown to be directly involved in activation process of G protein-coupled receptors (39–42).

To summarize, the current study has provided much information regarding positioning of the C-terminal part of CCK, which triggers the biological activity of the peptide, in the CCK1R. SR-146,131, a non-peptide agonist, was found to occupy this critical region of CCK1R binding site. Data showing the importance of several amino acid residues involved in this region of CCK1R binding site will be used to investigate CCK1R functioning.

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The Biologically Crucial C Terminus of Cholecystokinin and the Non-peptide Agonist SR-146,131 Share a Common Binding Site in the Human CCK1 Receptor: EVIDENCE FOR A CRUCIAL ROLE OF MET-121 IN THE ACTIVATION PROCESS

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