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Evidence That Interspecies Polymorphism in the Human and Rat Cholecystokinin Receptor-2 Affects Structure of the Binding Site for the Endogenous Agonist Cholecystokinin*

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The cholecystokinin (CCK) receptor-2 exerts very important central and peripheral functions by binding the neuropeptides cholecystokinin or gastrin. Because this receptor is a potential therapeutic target, great interest has been devoted to the identification of efficient antagonists. However, interspecies genetic polymorphism that does not alter cholecystokinin-induced signaling was shown to markedly affect activity of synthetic ligands. In this context, precise structural study of the agonist binding site on the human cholecystokinin receptor-2 is a prerequisite to elucidating the molecular basis for its activation and to optimizing properties of synthetic ligands. In this study, using site-directed mutagenesis and molecular modeling, we delineated the binding site for CCK on the human cholecystokinin receptor-2 by mutating amino acids corresponding to that of the rat homolog. By doing so, we demonstrated that, although resembling that of rat homolog, the human cholecystokinin receptor-2 binding site also displays important distinct structural features that were demonstrated by susceptibility to several point mutations (F120A, Y189A, H207A). Furthermore, docking of CCK in the human and rat cholecystokinin receptor-2, followed by dynamic simulations, allowed us to propose a plausible structural explanation of the experimentally observed difference between rat and human cholecystokinin-2 receptors.

Although both receptors recognize sulfated CCK with comparable high affinity, the CCK2R has high affinity for both sulfated and non-sulfated gastrin (1, 2). CCK1R and CCK2R are seven-transmembrane spanning receptors that belong to the superfamily of G protein-coupled receptors and have ~50% homology (3–5). CCK1Rs are mainly found in the peripheral organs, where they regulate pancreatic secretion and gallbladder and gastrointestinal motility, but are also found in some areas of the central nervous system, where they regulate satiety and analgesia. CCK2Rs are predominantly throughout the central nervous system, where they regulate anxiety/panic attacks and dopamine release, implicated in the pathogenesis of dopaminergic related movement and behavioral disorders in humans. In the gut, CCK2Rs regulate acid and histamine secretions, gastrointestinal motility, as well as growth in the gastric mucosa (1, 2). The important physiological functions mediated by CCK receptors, and therefore their possible implication in associated disorders, have generated considerable interest in the identification of ligands that selectively activate or block CCK1R and CCK2R (6). To date, a large panel of such molecules has been designed covering a wide range of functional activities, such as full, partial, and inverse agonists, as well as antagonists (6). Pharmacological characterization of selective synthetic CCK2R ligands among different species pointed out an interspecies genetic polymorphism that does not alter endogenous hormone-induced signaling but markedly affects both the affinity and activity of synthetic ligands (7). This is the case for L-365,260 and L-364,718, two reference non-peptidic CCK2R antagonists that present reversal in the affinity rank order on canine or human recombinant CCK2R despite a 93% identity in their protein sequence (8). Furthermore, efficacy of PD-135,158, a CCK2R partial agonist, is 60% of CCK-induced maximal production of inositol phosphates on mouse CCK2R but only 20% on human CCK2R, despite 90% identity (9). These interspecies differences toward synthetic ligands were shown to be caused by sequence variations within the binding site of the CCK2R (7). Such data pointed out the high importance of in vivo and in vitro tests with human protein targets.

The aims of the present study, using site-directed mutagenesis and molecular modeling, were to delineate the binding site for CCK on the human CCK2R and to investigate whether insensitivity of endogenous ligands to interspecies polymorphism between rat and human CCK2R can be explained by differences in their positioning into the CCK2R binding site or whether they are related to distinct susceptibilities to mutations. For that purpose, we took advantage of our previous data, which demonstrated an interaction between His167 of rat...
CCK2R and Asp^9 of CCK, between the Tyr^149 and Asn^358 residues of rat CCK2R and the carboxyl-terminal amide of CCK (10), as well as the contribution of Arg^257 and Phe^269 residues in CCK high affinity binding (11, 12). These key amino acids of the CCK2R binding site were mutated in the human CCK2R, and the mutant receptors were characterized for CCK affinity, as well as efficacy and potency, to stimulate inositol phosphates accumulation. By doing so, we demonstrated that, although resembling that of rat CCK2R, the human CCK2R binding site also displays important distinct structural features that are confirmed by susceptibility to point mutations and docking, followed by dynamic simulations.

**EXPERIMENTAL PROCEDURES**

**Materials**—Sulfated [Thr^{28},Nle^{31}]CCK25–33 (H-Arg-Asp-Tyr(SO_3H)-Thr-Gly-Try-Nle-Asp-Phe-NH_2, CCK2S), was synthesized as described previously (13). ^125I-Sodium (2000 Ci/mmol) was obtained from Amersham Biosciences. Sulfated [Thr^{28},Nle^{31}]CCK25–33 was conjugated with Bolton-Hunter reagent, purified, and radioiodinated as described previously (14) and is referred to as ^125I[CCK2S].

**Site-directed Mutagenesis and Transfection of COS-7 cells**—All mutants were constructed by the QuikChangeTM site-directed mutagenesis (Stratagene) using the human CCK2R cDNAs cloned into pRFENeo vector as a template. The presence of the desired and the absence of undesired mutations were confirmed by automated sequencing of the complete CCK2R coding sequence (Applied Biosystems).

**COS-7 cells** (1.5 × 10^6) were plated onto 10-cm culture dishes and grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% ammonium formate/60 mM sodium formate. The columns were washed twice with 3 ml of distilled water and twice with the content was transferred to a column (Dowex AG 1-X8, formate form, Bio-Rad, Hercules, CA) for the determination of inositol phosphates. The cells were then incubated for 60 min at 37 °C in 0.3 ml of inositol phosphate buffer, pH 7.45, phosphate-buffered saline containing 135 mM NaCl, 20 mM HEPES, 2 mM CaCl_2, 1.2 mM MgSO_4, 1 mM EGTA, 10 mM LiCl, 11.1 mM glucose, and 0.5% bovine serum albumin.

**Bioassays**—Approximately 24 h after the transfer of the transfected cells to 24-well plates, the cells were washed with phosphate-buffered saline, pH 7.4, containing 0.1% bovine serum albumin and then incubated for 60 min at 37 °C in 0.3 ml of DMEM containing 0.1% bovine serum albumin, with either 50 pM (wild-type CCK2R, R356K), 100 pm (R57A, F120A, Y189F), 250 pm (H207A, N356A, Y189A, R356D, 500 pm) or 500 pm (R356H) ^125I[CCK9S] in the presence or in the absence of unlabeled CCK9S. The cells were washed twice with cold phosphate-buffered saline, pH 7.4, containing 2% bovine serum albumin, and cell-associated radioligand was collected by cell lysis with NaOH 0.1 N. The radioactivity was directly counted in a γ counter (Autoγ, Packard, Downers Grove, IL). Receptor density and K_D were calculated from the dose-response agonist competition binding experiments using Ligand software (Kell, Cambridge, UK). K_D values were calculated using the non-linear curve fitting software GraphPad Prism (San Diego, CA).

**Inositol Phosphate Assay**—Approximately 24 h after the transfer to 24-well plates and following overnight incubation in DMEM containing 2 μCi/ml myo-[2-^3H]inositol (Amersham Biosciences), the transfected cells were washed with DMEM and then incubated for 30 min in 1 ml/well DMEM containing 20 μM LiCl at 37 °C. The cells were washed with inositol phosphate buffer, pH 7.45, phosphate-buffered saline containing 135 mM NaCl, 20 mM HEPES, 2 mM CaCl_2, 1.2 mM MgSO_4, 1 μM EGTA, 10 mM LiCl, 11.1 mM glucose, and 0.5% bovine serum albumin. The cells were then incubated for 60 min at 37 °C in 0.3 ml of inositol phosphate buffer with or without increasing CCK9S concentration. The reaction was stopped by adding 1 ml of mannitol/HCl to each well, and the content was transferred to a column (Dowex AG 1-X8, formate form, Bio-Rad, Hercules, CA) for the determination of inositol phosphates.

**Molecular Modeling of Human CCK2R-CCK Complex and Dynamic Simulations**—The human CCK2R model was built by homology modeling from the rat CCK2R-CCK complex model described by Gales et al. (11) using Insight II modules (Homology, Discover 3, Biopolymer, Accelrys, San Diego, CA). Alignment of the human CCK2R sequence on the rat CCK2R sequence was performed using the alignment procedure based on this alignment. The resulting model was then assigned to those of the rat CCK2R three-dimensional model, based on this alignment. The resulting model was then submitted to energy minimization by the steepest descent (until convergence to 2 kcal/mol/Å) and conjugated gradient (until convergence to 0.01 kcal/ mol/Å) to remove steric clashes. During minimization, C-a trace was tethered using a quadratic potential. This was performed using the Discover 3 module with the consistent force field. The non-bond cut-off method and the dielectric constant were set up to cell multipole and were distance-dependent, respectively (e = r). The minimized model of human CCK2 receptor was used to perform manual docking of CCK.

**RESULTS**

**Effects of R57A, F120A, H207A, Y189F, Y189A, and N353A Mutations in the Human CCK2R on CCK Affinity and Potency**—Approximately 24 h after the transfer of Human R57A, F120A, H207A, Y189A, Y189F, and N353A mutants were constructed and transiently expressed in COS-7 cells. Scatchard analysis of ^125I[CCK9S] binding to the wild-type human CCK2R indicated that CCK9S binds to high affinity sites with K_D values of 0.75 ± 0.03 nM (B_max 3.2 ± 0.3 pmol/10^6 cells) (Tables I and II). Mutation of Arg^257 and Phe^269 in alanine or mutation of Tyr^189 in phenylalanine slightly reduced, between 4- and 6-fold, CCK9S affinity with K_D values of 3.4 ± 0.4 nM (B_max 0.85 ± 0.09 pmol/10^6 cells), 3.1 ± 0.4 nM (B_max 0.79 ± 0.08 pmol/10^6 cells), and 4.3 ± 0.4 nM (B_max 1.2 ± 0.2 pmol/10^6 cells), respectively (Tables I and II). Y189A, H207A, and N353A mutants displayed between 25- and 73-fold decreased affinity for CCK9S with respective K_D values of 55 ± 8 nM (B_max 2.7 ± 0.5 pmol/10^6 cells), 19 ± 2 nM (B_max 1.1 ± 0.1 pmol/10^6 cells), and 20 ± 2 nM (B_max 0.8 ± 0.1 pmol/10^6 cells) (Tables I and II). In agreement with binding data, the potency of CCK9S to stimulate inositol phosphate accumulation was reduced for all mutants (E_max) and directly correlated with the loss of affinity for CCK9. The mutants displayed CCK9S efficacy (E_max, Table I) comparable with that of wild-type CCK2R, except for the Y189A mutant, which presented a 44% decrease of CCK9S maximal effect despite similar receptor density (Table I). Comparison of these results with those from our previous studies performed in exactly the same experimental conditions on rat CCK2R mutants reveals that, except for N353A mutation (the human corresponding mutant of rat N358A), all of the mutants carried out on the human CCK2R less markedly affected CCK affinity and activity, supporting the view that the conserved residues are differentially involved in the binding sites of the two CCK2Rs (Table II, F_max). Effects of R566A, R566H, R566K, and R567D Mutations in Human CCK2R on CCK Affinity and Potency—Our previous studies (15) on the binding site of the human CCK1R demonstrates that Arg^326, located at the top of TM 6, highly contributes to the binding of CCK. Because the homologous amino acid...
in the human CCK2R is Arg<sup>356</sup>, we determined whether this residue could also be involved in the binding site of the human CCK2R. For this purpose, R356A, R356H, R356K, and R356D mutants were constructed and analyzed. These mutants were designed to remove partially or completely the positive charges of this arginine (R356A, R356H, and R356K mutants) or to introduce a negative charge at that position (R356D mutant). Mutation of Arg<sup>356</sup> in lysine did not modify CCK9S affinity (K<sub>i</sub> = 0.50 ± 0.05 nM, B<sub>max</sub> = 3.9 ± 0.4 pmol/10<sup>6</sup> cells) nor the potency and efficacy of CCK9S to stimulate inositol phosphate accumulation (EC<sub>50</sub> = 0.54 ± 0.06 nM). Replacement of Arg<sup>356</sup> by a histidine or an alanine reduced 10- and 76-fold, respectively, CCK9S affinity, whereas R356D displayed a 932-fold lower affinity relative to the high affinity component of the wild-type human CCK2R (Table I and Fig. 1). In agreement with binding data, R356H, R356A, and R356D mutants displayed reduced CCK9S potencies to stimulate inositol phosphate accumulation but unchanged efficacy (Table I and Fig. 1). These results indicate that positive charges of the amino acid side chain at position 356 of the CCK2R are important for high affinity binding of CCK.

**Structural Study of Human CCK2R Binding Site Using Docking and Dynamic Simulations—**Results from the current site-directed mutagenesis study support the view that homologous residues in the human CCK2R binding sites are differentially involved in the binding site of CCK compared with rat CCK2R, which we previously characterized. Because human and rat CCK2R present non-conserved amino acids in extracellular domains and transmembrane helices (Fig. 2), in a first attempt to explain distinct involvement of His<sup>356</sup>, we exchanged the neighboring amino acid, namely Val<sup>206</sup>, for the corresponding amino acid of the rat sequence, Met. However, the resulting CCK2R mutant (V206M/H207A) did not display an altered pharmacological profile, as compared with the human H207A mutant (not shown). We also considered the possibility that His<sup>356</sup>, which can exist in different tautomeric forms, could be differently protonated in human and rat CCK2R. However, pH calculations using the Poisson-Boltzmann equation and the generalized Born approach (embodied in the website chekhov.cs.vt.edu/completion/index.php) yielded similar values (pH, for rat 3.3 and 4.2, and for human 3.5 and 4.6) thus ruling out this hypothesis. This led us to hypothesize that non-conserved amino acids, including those in transmembrane helices, likely act collectively to position differently CCK in the binding site of the human CCK2R relative to the rat CCK2R. We therefore performed in silico experiments by building a three-dimensional structure of the human CCK2R-CCK complex based on the coordinates of the rat CCK2R-CCK complex that we previously experimentally validated (11). Thereafter, we applied molecular dynamics to assess whether variations in the CCK2R sequence could result in a distinct location of CCK that could explain pharmacological results. A simplified view of the obtained human CCK2R-CCK complex is presented in Fig. 3, A and C. The docking, followed by dynamic simula-
tions, revealed that Arg₁ and Asp² of CCK form internal ionic bonds; sulfated Tyr³ of CCK is in ionic interaction with Arg⁵⁷ and makes a hydrogen bond with Tyr⁶¹. Thr⁴ forms a hydrogen bond with Arg³⁵⁶. Trp⁶ forms both a hydrogen bond with His³⁷⁶ and aryl sulfur interactions with Met⁶⁷ and Cys¹⁰⁷ and hydrophobic interactions with Phe¹²⁰ and Phe¹¹⁰. Nle⁷ lies in an hydrophobic pocket composed of Pro³⁷¹, Trp³⁵⁵, and Leu³⁶⁷. The carboxyl group of Asp⁸ interacts with Arg³⁵⁶ and Ser¹³¹. The phenyl group of Phe⁹ is sited in an aromatic pocket made of Tyr¹⁸⁹, Tyr¹⁹², Tyr³⁵⁰, Trp³⁴⁶, Phe³⁴², and Phe²²⁷; and the carbonyl group of CCK amide forms hydrogen bound with the hydroxyl group of Tyr¹⁸⁹, whereas —NH₂ of the CCK amide interacts with Asp³⁵³ and Thr³⁵³ (Fig. 3A). According to these results, the binding site of the human CCK2R resembles that of the rat CCK2R. However, and importantly, CCK drifts deeper in the human CCK2R, representing a difference of ~1.5 Å, and the C-terminal part of CCK between Trp⁶ and Phe⁹ displays a deviation of the backbone of ~2.5 Å, as compared with rat CCK2R-CCK complex, which was subjected to the same procedure of dynamic simulations (see Fig. 3C for comparison). In addition, the phenyl group of Phe⁹ has an affected orientation, as compared with the rat complex, where it approximated Tyr¹⁸⁹. In the human complex, Phe⁹ seems more inserted in a cavity composed of Tyr¹⁸⁹, Tyr¹⁹², Tyr³⁵⁰, Trp³⁴⁶, Phe³⁴², and Phe²²⁷ (Fig. 3, A and B). Moreover, Asp⁸ forms stable ionic interactions mostly with Arg³⁵⁶ but less with His, which dominated in the rat complex.

**DISCUSSION**

Despite the high degree of homology between species homologs of the CCK2 receptor (ranging from 84 to 93%), pharmacological studies have demonstrated that interspecies polymorphism can significantly alter both the affinity and activity of synthetic ligands (7). Other examples describing polymorphism-induced alteration can be found in the literature for neurokinin, serotonin, and VIP receptors (16–18), but in those cases, both endogenous and synthetic ligands are affected. However, in the case of CCK2R, comparison of CCK or gastrin affinity and activity in cells that express canine, murine, or human recombinant CCK2R revealed similar profiles. It is suggested that the apparent insensitivity of endogenous ligand to receptor polymorphism might be explained by evolutionary selection, which resulted in the elimination of receptor variants that compromised endogenous ligand function (7). In this context, the aim of the present study was to delineate the binding site for CCK on the human CCK2R and to investigate whether...
insensitivity of endogenous ligands to interspecies polymorphism between rat and human CCK2R can be explained by differences in their location into the CCK2R binding site or whether there are related to distinct susceptibilities to mutations.

Using site-directed mutagenesis and pharmacological studies, we found that some mutations were less unfavorable when performed on the human CCK2R than on the rat receptor. The magnitude of the differences between rat and human mutants varied depending on the amino acid studied. Mutation of Arg57 located at the top of the first transmembrane domain in alanine reduced 5-fold the CCK affinity in the human receptor versus 21-fold in rat receptor (12). Similarly, Kopin et al. (19) have shown that the human R57Q mutant displayed a 6-fold decreased affinity for CCK, confirming the slight contribution of Arg57 in the human CCK2R for high affinity binding of CCK. Moreover, photoaffinity labeling experiments, using a CCK ligand having its photosensitive moiety attached to its N terminal end of CCK (orange), identified amino acids at the top of helix I, which agrees with current results supporting that the sulfate of CCK slightly interacts with Arg57 (20). Surprisingly, Phe120, located in the first extracellular loop and which is essential for the high affinity binding of CCK to the rat CCK2R, does not appear to be as essential in human CCK2R. Indeed, mutation F120A in the rat receptor resulted in complete loss of radioligand binding (12), whereas in the current study, the human F120A mutant

![Modeled structure of the CCK2R binding site for CCK.](image)
only displayed a 4-fold lower affinity as compared with wild-type CCK2R. Similar results were obtained with the H207A mutant. Our previous results with the rat receptor clearly demonstrated a direct interaction between His207, located in the second extracellular loop, and penultimate aspartic acid of CCK (10). Here, the contribution of His207 in the human CCK2R binding pocket is quantitatively different. Indeed, the human H207A mutant displayed a 25-fold lower CCK affinity versus 456-fold in the rat (10). Taken together, all of the results thus suggest that the contribution of extracellular residues of human CCK2R is less important than in the rat CCK2R, and most of the residues involved in high affinity binding of CCK are probably located in transmembrane domains.

We next evaluated, in human CCK2R, the contribution of Tyr189 and Asn353, the corresponding amino acids of rat Tyr189 and Asn358 known to make direct interaction with the C-terminal amide of CCK (11). Human N353A and rat N358A mutants displayed a similar pharmacological profile with respect to CCK affinity and activity, suggesting that, as in the rat receptor, Asn353 located at the top of the sixth transmembrane domain directly interacts with the C-terminal amide of CCK. In contrast, the contribution of Tyr189 is different in human and rat receptors. Mutation of Tyr189, located at the top of the fourth transmembrane domain, in phenylalanine or alanine reduced by 6- and 73-fold CCK affinity in the human receptor versus 30- and 842-fold in the rat receptor, respectively. In addition, the human Y189A mutant displayed a reduced CCK potency (109-fold) and efficacy (44%) to stimulate inositol phosphatase accumulation, whereas the rat Y189A mutant was found to be unable to produce inositol phosphates (11). Even if human Y189A mutant is less affected than rat Y189A mutant, our results are compatible with an involvement of the Tyr189 aromatic ring in CCK2R activation (reduced potency and efficacy). We also evaluated the contribution of Arg356, located at the top of the sixth transmembrane domain. Mutation of Arg356 in lysine did not modify CCK affinity, whereas its mutation in histidine or alanine reduced by 9- and 76-fold CCK affinity, respectively, suggesting that the presence of positive charges is important for high affinity binding of CCK. The R356D mutant displays an even lower affinity for CCK (413-fold reduction) that likely reflects electrostatic repulsion between CCK and R356D mutant. Interestingly, we previously demonstrated that, in human CCK1R, the corresponding amino acid (Arg336) interacts with the penultimate aspartic acid of CCK (15).

Detailed analysis of the human- and rat-modeled CCK2R-CCK complexes allows us to propose structure-based explanations for the different mutational data obtained in human and rat CCK2R. According to dynamic simulations, CCK drifts deeper in the human CCK2R than in the rat CCK2R, leading the peptidic backbone corresponding to the C-terminal region of CCK (which is the most important for recognition by the CCK2R) to site $>2.5$ Å deeper in the receptor binding pocket. This can influence the contributions of several extracellular key residues to the binding of CCK, especially because these belong to flexible extracellular loops 1 and 2. For example, Phe120 is less important for CCK recognition in human CCK2R than in rat CCK2R. This is likely the result of an increase of the collective contribution of other residues, such as His376, Met67, Cys107, and Phe110, to the binding site of the human CCK2R. This view fully agrees with previous mutagenesis data showing that exchange of His$^{376}$ for a leucine decreased the affinity of CCK8 for the human CCK2R by 30-fold, whereas mutation of the corresponding amino acid in the rat CCK2R did not affect CCK8 recognition (19, 21).

Thereafter, we demonstrated that different contributions of His$^{207}$ in human versus rat CCK2R were likely not due to different protonated states. Alternatively, the deepest location of the CCK molecule within the binding site of the human CCK2R compared with rat CCK2R could favor interactions of the Asp side chain of CCK with Arg9$^{56}$, to the detriment of His207. Furthermore, there is a Val at position 130 in human CCK2 and an Ile in the rat receptor. Due to the possible hindrance of amino acid side chains, one can suggest that the Val residue favors interaction with Arg$^{56}$, whereas Ile can favor interaction with His$^{207}$.

Concerning the lower contribution of Tyr189 in the human CCK2R binding site (as compared with rat CCK2R), in both cases, two types of interactions are seen in the modeled structure: a hydrogen bond with the carbonyl group of Phe9 of CCK and $\pi-\pi$ electronic interactions between the two rings. However, the relative positions of the two aromatic rings (Tyr189 and Phe9 of CCK) are sufficiently different in the human versus the rat complexes to explain the mutagenesis results. In fact, according to dynamic simulation experiments, the relative position of Tyr189 and Phe9 of CCK, and therefore the location of CCK, within the binding pocket appears to be at least partially governed by the presence of Met234 (TM V) in the human CCK2R instead of an isoleucine in the rat receptor, and conversely, the presence of Met194 (TM IV) in the rat CCK2R instead of a valine in human receptor. In the modeled human CCK2R, Met234 forms a sulfur-$\pi$ interaction with Phe342, which in turn can influence the aromatic network comprising residues Phe342, Trp346, Phe227, and Tyr350 (Fig. 3, A and B). In the rat CCK2R, it is Met194 that can influence this aromatic network but in an opposite way. Interactions between methionine and aromatic residues, such as Phe and Tyr, are recognized to play an important role in the three-dimensional structure and function of many proteins. For instance, in the CCK1R, a Met residue located in the second extracellular loop was demonstrated to play a key role for correct positioning of Tyr(316)H of CCK toward its interacting partner, Arg197 (22, 23). In this receptor, another methionine in helix III was demonstrated to govern receptor coupling to G protein by influencing the hydrophobic pocket, which positions Phe of CCK relative to an aromatic residue of helix VI (24). The physicochemical basis of interactions between a sulfur atom and aromatic ring has been investigated and was claimed to explain why, in the protein data base, ~50% of the sulfur atoms are contacting aromatic rings (25).

Analysis of data from studies of other groups reinforces our current findings, showing that conserved residues in the CCK2R among different species contribute differently in CCK high affinity and CCK2R activation. First, although residue Trp546 of the human CCK2R belongs to the aromatic network (Fig. 3, A and B), its exchange for an alanine did not affect pharmacological properties of the receptor (26). In contrast, the corresponding mutation in the rat receptor caused a 34% decrease of inositol phosphate accumulation and a 10-fold decrease of both CCK affinity and potency (27). This is consistent with dynamic simulation data predicting that the C-terminal phenylalanine of CCK makes stacking ring-ring interactions with Tyr189 and Trp351 (corresponding to human Trp546) located in the sixth transmembrane domain. This is also in line with the key role of Tyr189 in the activation of the rat CCK2R that we documented using site-directed mutagenesis (11). Second, the key role of Phe342 (TM VI) in the aromatic network and the fact that in the human CCK2R this residue interacts with Met234 (TM V) must be compared with findings that the corresponding residue in the rat sequence (Phe542) is crucial for activation of phospholipase-C by the CCK2R (27, 28). Third, the works of Kopin and colleagues (19, 26) points out the importance of Tyr61 (junction between TM I and N-terminal...
region), Met\textsuperscript{186} (TM IV), and Thr\textsuperscript{193} for CCK binding to the human CCK2R. Their data are fully compatible with the refined human CCK2R-CCK complex, which shows hydrogen bonds involving Tyr\textsuperscript{61} and Tyr of CCK as well as Thr\textsuperscript{193} and the amide of CCK (Fig. 3A). On the other hand, the role of Met\textsuperscript{186} is likely indirect and linked to the aromatic network within the binding pocket. Using NMR nuclear Overhauser enhancement transfer, Mierke and co-worker (29) proposed interactions between Trp and Met of CCK with Pro\textsuperscript{222} from the human CCK2R. Their data are fully compatible with the re-
fined structure, Nle, which mimics Met of CCK, approx-
imates Pro\textsuperscript{222}. It is interesting to note that there is a good overlapping between the binding site for the C-terminal of CCK and the suggested binding site of non-peptide ligands, as indicated by the delete/rescue approach in which deleterious mu-
tations within the receptor were rescued by subsequent modification of the ligand (30).

To conclude, delineation of the CCK binding site of the hu-
man CCK2R, using site-directed mutagenesis and docking fol-
lowed by dynamic simulations, allowed us to demonstrate that, al-
though roughly resembling that of the rat CCK2R, this bind-
ing site also displays important distinct structural features that are confirmed by susceptibility to point mutations. These new important data, together with our experience with the CCK1R (31), will be used to understand how some of the so-
called non-peptide antagonists present partial agonist activi-
ties (7). This goal is of timely importance due to the fact that CCK2R is a biological target of therapeutic interest (6).

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