0026-895X/03/6305-973–982$7.00
MOLECULAR PHARMACOLOGY
Copyright © 2003 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 63:973–982, 2003

Vol. 63, No. 5
2018/1057196
Printed in U.S.A.

Identification of Tyrosine 189 and Asparagine 358 of the Cholecystokinin 2 Receptor in Direct Interaction with the Crucial C-Terminal Amide of Cholecystokinin by Molecular Modeling, Site-Directed Mutagenesis, and Structure/Affinity Studies

CÉLINE GALÉS, MARC POIROT, JULIEN TAILLEFER, BERNARD MAIGRET, JEAN MARTINEZ, LUIS MORODER, CHANTAL ESCRIEUT, LUCIEN PRADAYROL, DANIEL FOURMY, and SANDRINE SILVENTE-POIROT

Institut National de la Santé et de la Recherche Médicale (INSERM) U 531, Institut Louis Bugnard, CHU Rangueil, Toulouse, France (C.G., J.T., C.E., L.P., D.F.); INSERM U 563, Département Innovation Thérapeutique et Oncologie Moléculaire, Institut Claudius Regaud, Toulouse, France (M.P.); Laboratoire de Chimie Théorique, Université de Nancy, Vandoeuvre, Nancy, France (B.M.); Centre National de la Recherche Scientifique–Unité de Recherche Associée 1845, Faculté de Pharmacie, Montpellier, France (J.M.); and Max Planck Institute für Biochemie, Martinsried, Germany (L.M.).

Received July 22, 2002; accepted January 17, 2003

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

The cholecystokinin (CCK) receptors CCK1R and CCK2R exert important central and peripheral functions by binding the neuropeptide cholecystokinin. Because these receptors are potential therapeutic targets, great interest has been devoted to the identification of efficient ligands that selectively activate or inhibit these receptors. A complete mapping of the CCK binding site in these receptors would help to design new CCK ligands and to optimize their properties. In this view, a molecular model of the CCK2R occupied by CCK was built to identify CCK2R residues that interact with CCK functional groups. No such study has yet been reported for the CCK2R. Docking of CCK in the receptor was performed by taking into account our previous mutagenesis data and by using, as constraint, the direct interaction that we demonstrated between His207 in the CCK2R and Asp8 of CCK (*Mol Pharmacol* **54:**364–371, 1998; *J Biol Chem* **274:**23191–23197, 1999). Two residues that had not been revealed in our previous mutagenesis studies, Tyr189 (Y4.60) and Asn358 (N6.55), were identified in interaction via hydrogen bonds with the C-terminal amide of CCK, a crucial functional group of the peptide. Mutagenesis of Tyr189 (Y4.60) and Asn358 (N6.55) as well as structure-affinity studies with modified CCK analogs validated these interactions and the involvement of both residues in the CCK binding site. These results indicate that the present molecular model is an important tool to identify direct contact points between CCK and the CCK2R and to rapidly progress in mapping of the CCK2R binding site. Moreover, comparison of the present CCK2R.CCK molecular model with that of CCK1R.CCK, which we have previously published and validated, clearly argues that the positioning of CCK in these receptors is different.

Cholecystokinin (CCK) acts as a hormone and a neurotransmitter throughout the gastrointestinal tract and the nervous system by interacting with two pharmacologically distinct receptors, CCK1 and CCK2 (formerly named CCKA and CCKB). These two receptors belong to the superfamily of seven-transmembrane receptors coupled to G proteins and share approximately 50% sequence homology. CCK, acting through these receptors, regulates important functions (Silvente-Poirot et al., 1993; Wank, 1998; Noble et al., 1999). In the gastrointestinal system, CCK has been implicated in modulating pancreatic secretions and growth, motility, gastric emptying, and acid secretion. In the nervous system, CCK regulates memory, satiety, anxiety, and pain perception. Cholecystokinin receptors therefore represent important pharmacological targets and great interest has been devoted to the identification of efficient and selective CCK1 and CCK2 ligands by academic institutes as well as pharmacological companies (de Tullio et al., 2000). 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 α-amidation of the C-terminal phenylalanine.

This work was supported in part by a grant from the Association pour la recherche sur le cancer (ARC 5481).

ABBREVIATIONS: CCK, cholecystokinin; CCK1R, cholecystokinin 1 receptor; CCK2R, cholecystokinin 2 receptor; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; TM. transmembrane; IP, inositol phosphate; PLC, phospholipase C; GPCR, G protein-coupled receptor.

Although tyrosine sulfation is essential to confer CCK high affinity for the CCK1R, this modification increases affinity toward the CCK2R only slightly. In contrast, the amide moiety, which is found in numerous other polypeptides of the brain and gut, is required for CCK biological activity and high affinity in both receptors (Morley et al., 1965; Jensen et al., 1982; Galas et al., 1988; Gigoux et al., 1999a). The C-terminal sulfated and amidated octapeptide Asp-Tyr(SO_3H)-Met-Gly-Trp-Met-Asp-Phe-NH_2 retains the full spectrum of biological activity and binds CCK1R and CCK2R with similar high affinity. The C-terminal tetrapeptide of CCK corresponds to the minimal fragment endowed with biological activity and still binds with nanomolar affinity to the CCK2R but displays very low affinity for the CCK1R (Jensen et al., 1982; Knight et al., 1984; Gigoux et al., 1999a; Silvente-Poirot et al., 1999). Despite the fact that sulfated and amidated CCK presents a similar high affinity for both receptors, our previous studies using mutagenesis and molecular modeling techniques brought several pieces of evidence for a different anchoring of CCK in the CCK1 and CCK2 receptors. Indeed, complementary substitutions in the ligand and in the receptor allowed us to demonstrate that two amino acids in the second extracellular loop of the CCK1R, Met195 and Arg197, interact with the tyrosine residue of CCK (Tyr3) whereas His207, located in the same loop in the CCK2R, interacts with Asp8, the penultimate residue of CCK (Gigoux et al., 1998, 1999b; Silvente-Poirot et al., 1999). In contrast, in the CCK1R, Asp8 of CCK was found in interaction with Arg336 (RL6.58), further confirming that different interactions are involved between CCK and the two subtypes (Gigoux et al., 1999a). In light of our results, a complete mapping of the CCK binding site in both receptors and their molecular modeling seems to be a promising way to optimize CCK ligands. In addition, the knowledge of the agonist binding site would provide a better understanding of the basic mechanisms involved in receptor activation and in the selectivity of the biological response. Indeed, the binding of agonists represents the key step that induces conformational changes in the receptor and subsequently the transduction of biological signal (Gether et al., 1995; Bukusoglu and Jenness, 1996). To further progress in the characterization of the CCK binding site in the CCK2R, we built a three-dimensional molecular model of this receptor occupied by CCK. This approach has been successfully used with the CCK1R and has led to the identification of several residues of the CCK binding site (Gigoux et al., 1999a, 1998, 1999b, Escrieut et al., 2002). To dock CCK into the CCK2 receptor, we took into account our previous mutagenesis data that identified important residues for CCK binding at the top of TM1 and in the first and second extracellular loops of the CCK2R and used as constraint the direct interaction that we demonstrated between His207 located in the second extracellular loop of the CCK2R and Asp8 of CCK. This approach allowed us to identify two new residues, Asn358 (N6.55) located in TM6 and Tyr189 (Y4.60) located in TM4, that interact with the C-terminal amide of CCK. The present study was undertaken to determine whether the theoretical interactions between Asn358 (N6.55) and Tyr189 (Y4.60) and the crucial C-terminal amide of CCK could be experimentally validated by site-directed mutagenesis and structure/affinity studies.

Materials and Methods

Materials. the sulfated C-terminal nonapeptide of CCK [Thr^{28},Nle^{31}]-CCK25-33 was synthesized as described previously and referred to as CCK-9 (Moroder et al., 1981). ($PheCH_3$)^9-CCK and (phenylethylamide)^9-CCK compounds were synthesized as described by Galas et al. (1988) and Orosco et al. (1990). (Ala)^3-CCK was synthesized as described by Silvente-Poirot et al. (1999). ^{125}INa (2000 Ci/mmol) was from Amersham Biosciences (Les Ulis, France). (Thr^{28},Nle^{31})-CCK25-33 was conjugated with Bolton-Hunter reagent, purified, and radioiodinated as described previously (Fourmy et al., 1989); it is referred to as ^{125}I-BH-CCK-9.

Construction of Mutant Receptor cDNAs. Mutant receptor cDNAs were constructed by oligonucleotide-directed mutagenesis (QuikChange site-directed mutagenesis kit; Stratagene, Montigny-le-Bretonneux, France) using the rat CCK2R cDNAs as template. Mutations were confirmed by DNA sequencing using an automated sequencer (Applied Biosystems, Foster City, CA).

Transfection of Wild-Type and Mutant Receptor cDNAs into Mammalian Cells. COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum. Two micrograms of the CCK2R and mutant receptor cDNAs subcloned in pCDL-SRα were transiently transfected into COS-7 cells using the DEAE/Dextran method as described previously (Silvente-Poirot and Wank, 1996). Twenty-four hours after transfection, the transfected cells were transferred to 24-well culture plates and seeded at a density of approximately 1×10^5 cells/well and assayed for ^{125}I-BH-CCK-9 binding or inositol phosphate hydrolysis.

Binding of ^{125}I-BH-CCK-9 to Transfected COS-7 Cells. Twenty-four hours 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 (BSA) and incubated in DMEM containing 0.1% BSA for 60 min at 37°C with either 50 pM (WT-CCK2R), 300 pM [N358A (N6.55A) and Y189F (Y4.60F) mutants], and 500 pM [Y189A (Y4.60A) mutant] of ^{125}I-BH-CCK-9 with or without increasing concentrations of unlabeled peptides. Cell-associated ^{125}I-BH-CCK-9 was separated from free radioligand by washing twice with phosphate-buffered saline containing 2% BSA. Cell associated ^{125}I-BH-CCK-9 was collected with 0.5 ml of 0.1 N NaOH added to each well and radioactivity detected in a gamma counter. Nonspecific binding (determined in presence of 1 μM CCK-9) was always less than 10% of total binding. Binding assays were performed in duplicate in at least three separate experiments. Binding data were determined using the nonlinear, least-squares, curve-fitting computer programs Ligand (Munson and Rodbard, 1980) and GraphPad Prism (San Diego, CA). K_i values were calculated as $K_i = IC_{50}/(1 + [\text{labeled ligand}]/K_d \text{ of labeled ligand})$.

Measurement of Total Inositol Phosphates Accumulation. Twenty-four hours after COS-7 cell transfection, the transfected cells were transferred to 24-well culture plates and incubated overnight in DMEM with 2 μCi/well of [*myo*-2-^{3}H]inositol (18.6 Ci/mmol; PerkinElmer, Boston, MA). After aspiration of the medium containing the [*myo*-^{3}H]inositol, the cells were incubated at 37°C for 20 min with 1 ml of DMEM containing 20 mM LiCl. The cells were washed with IP buffer, pH 7.45 (20 mM HEPES, 135 mM NaCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM EGTA, 10 mM LiCl, 11.1 mM glucose, and 0.5% BSA) and then incubated for 1 h at 37°C with IP buffer containing the indicated concentrations of peptides. The reaction was stopped with 1 ml of methanol/HCl added to each well and the content was transferred to an AG 1-X8 (formate form) column (Bio-Rad Laboratories, S.A., Marnes La Coquette, France). 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 columns with 2 ml of 1 M ammonium formate/100 mM formic acid. [*myo*-^{3}H]Inositol phosphate β-radioactivity was detected in a liquid scintillation counter (PerkinElmer). EC_{50} was calculated using GraphPad Prism.

Locus Numbering Scheme. The positions of amino acids in the CCK2R and in the CCK1R are identified by their original numbering and by a consensus numbering (indicated in parentheses) as described previously to facilitate the comparison among different GPCRs (Ballesteros and Weinstein, 1995). Briefly, in the consensus numbering, transmembrane amino acids are identified by a transmembrane number followed by the position relative to the most conserved residue in that helix, which is assigned the number 50.

Molecular Modeling of the CCK2R.CCK Complex. The present CCK2R model started from a previous model of this receptor built several years ago and described by Jagerschmidt et al. (1996). This CCK2R model was itself obtained from an AT1a receptor model detailed by Joseph et al. (1995) and was constructed using the transmembrane (TM) helical positions found in bacteriorhodopsin structure (Henderson et al., 1990). This starting CCK2R model was then successively modified to take into account new data coming from improvements of inactive rhodopsin three-dimensional structures (Unger et al., 1997; Krebs et al., 1998; Palczewski et al., 2000). Relative positioning of the TM transmembrane helices, in particular TM3 and TM5, was modified to reproduce the projection structure of bovine rhodopsin. The repositioning of these helices was considered crucial because of the differences observed between the bacteriorhodopsin and rhodopsin projection maps. We used the "in/out" concept associated to the VISEUR program to rotate and translate the helices in the appropriate way (Campagne et al., 1999).

Finally, the extracellular and intracellular loops connecting the transmembrane helices were built using the "homology" loops module, which uses a library of loop templates from the Protein Data Bank (http://www.rcsb.org/pdb/). These loops were then added to the preliminary 7-helix bundle and the structural model was optimized by the use of simulated annealing procedures (a cooling procedure going from 1000 to 300 K in steps of 100 K was used). This annealing procedure was applied only to the loops connecting the helices, the backbone of which was kept fixed. After minimizing the final annealing conformation, removing all the constraints, the entire system was finally relaxed and submitted to 1-ns molecular dynamics runs with possible translational and rotational movements of individual TM helices taken into account (the helices were conserved by imposing the conservation of their backbone-backbone hydrogen bonds).

To dock CCK-9 into this CCK2 receptor model, we took into account our previous mutagenesis data that identified important residues for CCK binding at the top of TM1 and in the first and second extracellular loops of the CCK2R (Silvente-Poirot et al., 1998). In addition, the previously identified contact point between residue His207 located in the second extracellular loop and the carboxylate side chain of CCK-Asp^8 served as a first constraint to place CCK-9 within the CCK2R (Silvente-Poirot et al., 1999). This constraint was kept active, and the resulting complex was submitted to annealing molecular dynamics calculations, first keeping all the transmembrane helices fixed. Starting the simulation from the energy minimum obtained this way, another round of annealing was performed, considering now all the degrees of freedom of the whole system.

The CCK2R.CCK complex model obtained in this way and used in the present study was found to respect most TM arrangements found in the recent inactive rhodopsin X-ray structure (Palczewski et al., 2000), in particular those for TM1, TM3, TM5, and TM6, while presenting significant differences for TM2, TM4 and TM7. The root-mean-square deviation value obtained on the Cα carbons of the CCK2R TM helices was 6 Å. A similar root-mean-square deviation of 6 Å was found between the TM Cα carbon arrangement of the activated-rhodopsin three-dimensional model recently proposed (Choi et al., 2002) and the inactive rhodopsin structure (Palczewski et al., 2000). We found that our model is in good agreement with the TM positioning of this activated-rhodopsin model concerning TM3 to TM7 but in disagreement concerning the TM1 and TM2 helices. Insight II (discover, homology, biopolymer) from Accelrys (San Diego, CA) was used for all the calculations.

Results and Discussion

Because no experimental information about the three-dimensional structure of the cholecystokinin 2 receptor was yet available, a theoretical molecular model of the CCK2R occupied by CCK-9 [NH_2-Arg^1-Asp^2-Tyr(SO_3H)^3-Thr^4-Gly^5-Trp^6-Nle^7-Asp^8-Phe^9-NH_2] was built to complete our previous studies on the CCK binding site and to identify new residues in interaction with CCK. The orientation of the seven-TM domains of the unoccupied CCK2R and their relative positions were built according to the crystal structure of rhodopsin in the inactive state, as described under *Materials and Methods*. Molecular dynamics-based docking of CCK-9 in this model was performed based on our previous mutagenesis data, which identified several residues involved in CCK high-affinity binding (Silvente-Poirot et al., 1998, 1999). Therefore, the present model probably reflects the active state of the receptor occupied by CCK. The N-terminal part of CCK was oriented toward Arg57 (R1.35) (located at the top of TM1). This residue was identified as important for CCK binding by mutagenesis and was shown to be part of the CCK2R sequence covalently linked to a photoreactive CCK probe containing a *p*-benzoylbenzoyl moiety at its N terminus (Silvente-Poirot et al., 1998; Anders et al., 1999). In addition, the direct interaction that we demonstrated between Asp8 of CCK-9 and His207 of the CCK2R, localized in the second extracellular loop of the receptor, was used as constraint to dock CCK-9 in the three-dimensional model (Silvente-Poirot et al., 1999). We focused our interest on the C-terminal amidated tetrapeptide of CCK that bears the crucial residues of CCK as reported previously (Knight et al., 1984). In particular, this approach allowed us to identify several residues surrounding the C-terminal amide of CCK that had not been revealed in our previous mutagenesis studies. A view of the CCK-occupied CCK2R three-dimensional model is presented in Fig. 1A. Hydrogen bonds (distance <2 Å) were found to link the oxygen atom of the carboxamide functional group of Asn358 (N6.55) with a proton of the NH_2 group of the C-terminal amide of CCK and the proton of the hydroxyl group of Tyr189 (Y4.60) with the oxygen of the C=O group of the C-terminal amide of CCK (Fig. 1B). In addition, a stacking interaction (distance 5 Å) was observed between the aromatic rings of Tyr189 (Y4.60) and Phe9 of CCK (Fig. 1B). The hydroxyl group of Tyr192 (Y4.63) was found at the vicinity of the NH_2 of the C-terminal amide of CCK but at a distance higher (distance 2.5 Å) to be involved in a hydrogen bond (Fig. 1B). Because of the importance of the C-terminal amide function for CCK binding toward the CCK2R, we first determined the validity of these interactions.

To evaluate the contribution of these amino acids to CCK affinity, Tyr189 (Y4.60) and Tyr192 (Y4.63) were mutated to Phe and Asn358 (N6.55) was mutated to Ala. These mutations were performed to eliminate the potential hydrogen bonds between these residues and the C-terminal amide of CCK. Competition binding experiments between ^{125}I-BH-CCK-9 and CCK-9 were conducted on COS-7 cells transfected with the Y189F (Y4.60F), Y192F (Y4.63F), and N358A (N6.55A) mutant receptors. Analysis of CCK competition binding revealed that the Y189F (Y4.60F) and N358A (N6.55A) mutants bound CCK with 30- and 19-fold decreased affinities, respectively (K_i, 39.3 $\pm$ 5.6 and 24.2 $\pm$ 5.3 nM) compared with the WT-CCK2R (K_i, 1.3 $\pm$ 0.3 nM). The anal-

ysis of the maximal binding capacities of both mutants demonstrated that they displayed a cell surface expression near that of the WT-CCK2R, indicating that the Y189F (Y4.60F) and N358A (N6.55A) mutations did not introduce a gross conformational change in the receptor [B_{max} Y189F (Y4.60F), 1.2 ± 0.2 pmol/10^6cells; B_{max} N358A (N6.55A), 1.3 ± 0.3 pmol/10^6cells; B_{max} WT-CCK2R, 1.5 ± 0.5 pmol/10^6cells). These data suggest that residues Tyr189 (Y4.60) and Asn358 (N6.55) are important for CCK binding. It must be noted that the effects of Y189F (Y4.60F) and N358A (N6.55A) mutations on CCK affinity are in the same range. A 19- to 30-fold decrease in affinity represents a loss of 2 to 3 kcal/mol of binding energy and is consistent with the disruption of a hydrogen bond as predicted from the molecular modeling. In contrast the Y192F mutant bound CCK with an affinity similar to that of the WT-CCK2R and its expression was not affected (K_i, 1.3 ± 0.4 nM; B_{max}, 1.4 ± 0.4 pmol/10^6 cells). The fact that the mutation of residue Tyr192 has no effect on CCK affinity is consistent with the higher distance found between the hydroxyl of Tyr192 and the NH_2 of the C-terminal amide of CCK.

We next determined whether substitutions in the C-terminal amide of CCK could correlate with the effects of Y189F (Y4.60F) and N358A (N6.55A) mutations in the receptor. The contribution of the NH_2 and C=O groups of the amide to CCK affinity were evaluated by testing the affinities of compounds modified at the C terminus for the WT-CCK2R. The contribution of the NH_2 of the amide to CCK affinity was measured by using an analog of CCK in which the NH_2 was substituted with a methyl to give a methyl-ketone, the $(PheCH_3)^9$-CCK compound (Fig. 2). This peptide is an isosteric analog on the C terminus of CCK. As shown in Fig. 2 and Table 1, the WT-CCK2R bound $(PheCH_3)^9$-CCK with a 13-fold decrease in affinity compared with CCK-9, indicating that the NH_2 of the amide is important for CCK affinity. The modification in the peptide and the N358A (N6.55A) mutation in the receptor produce a similar effect, because the affinity of $(PheCH_3)^9$-CCK for the WT-CCK2R (K_i, 17.4 ± 3.2 nM) correlate with that of CCK for the N358A (N6.55A) mutant (K_i, 24.2 ± 5.3 nM). These results are in accordance with the fact that the same bond is disrupted and, hence, that Asn358 (N6.55) could interact with the NH_2 of the amide. To determine the contribution of the C=O group of the C-terminal amide to CCK affinity, we used an analog of CCK having the C-terminal amidated phenylalanine (Phe^9–NH_2) substituted with a phenylethylamide, the $(phenylethylamide)^9$-CCK compound (Fig. 2). In fact, the isosteric substitution of the C=O group to eliminate its hydrogen bond donor properties was not possible, keeping the amidic properties of the NH_2. Replacement of the carbonyl by a methylene group will have two main consequences: 1) it will transform the hybridization state of the carbon from sp2 (planar) to sp3 (tetrahedral), thus changing the spatial orientation of its substituents; 2) the NH_2 will become a primary amine that will be cationic at physiological pH and will introduce a positive charge, whereas the amidic NH_2 is neutral. Thus, a modified compound lacking the C-terminal amide was used to indirectly evaluate the substitution of the C=O group and to determine the effect of complete removal of the C-terminal amide on CCK binding. As shown in Fig. 2 and Table 1, $(phenylethylamide)^9$-CCK displayed a 262-fold decreased affinity for the WT-CCK2R compared with CCK, confirming that the C-terminal amide is crucial for conferring high affinity to CCK. The above experiment indicates that the substitution of the NH_2 group of the amide

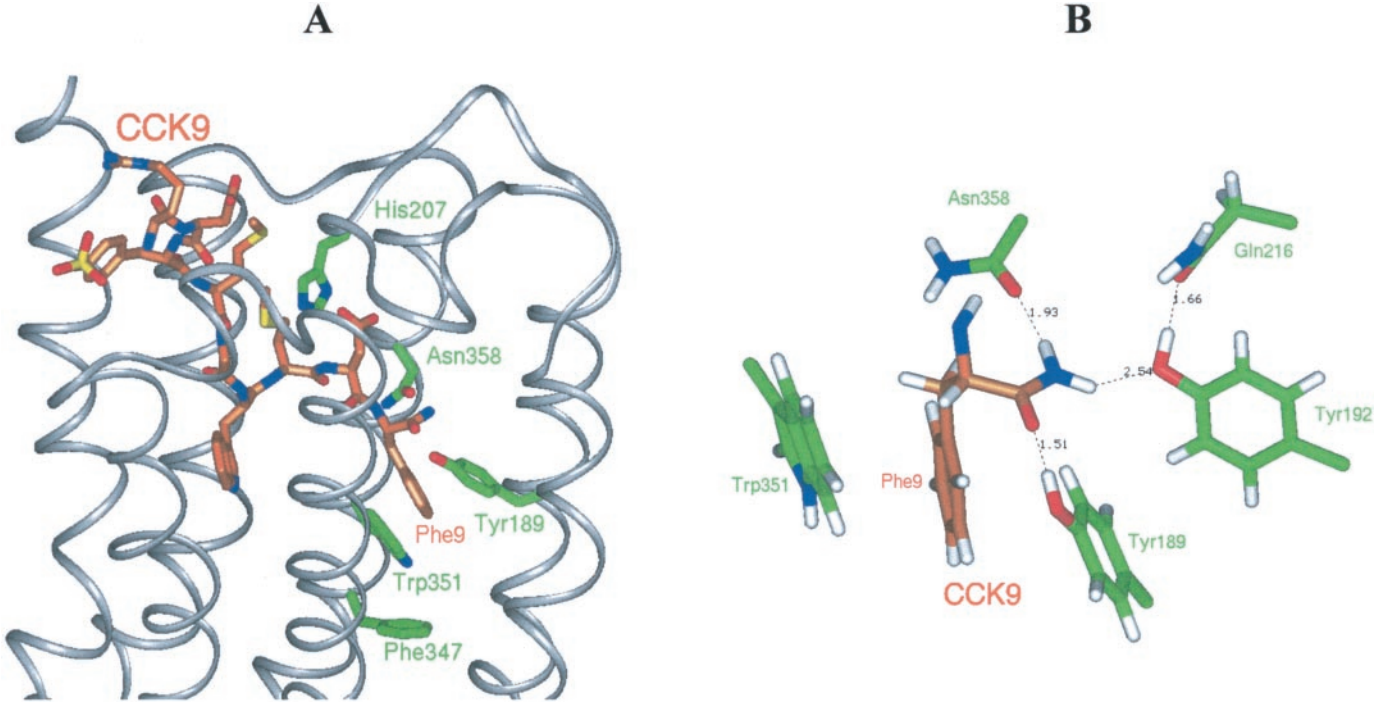

Fig. 1. Side view of the three-dimensional model of the CCK-9.CCK2R complex. The model was built as described under *Materials and Methods*. A, the side chains of important residues in proximity to the C-terminal phenylalanine amide are highlighted and labeled. CCK2R helices are in gray, oxygen atoms are in red, and nitrogen atoms are in dark blue. B, detailed view of amino acid side chains in interaction with the C-terminal phenylalanine amide of CCK. Distances between chemical function are represented by dotted lines. According to Ballesteros and Weinstein (1995) nomenclature, N358 is N6.55, Y189 is Y4.60, W351 is 6.48, F347 is 6.44, Tyr192 is Y4.63, and Q216 is 5.36.

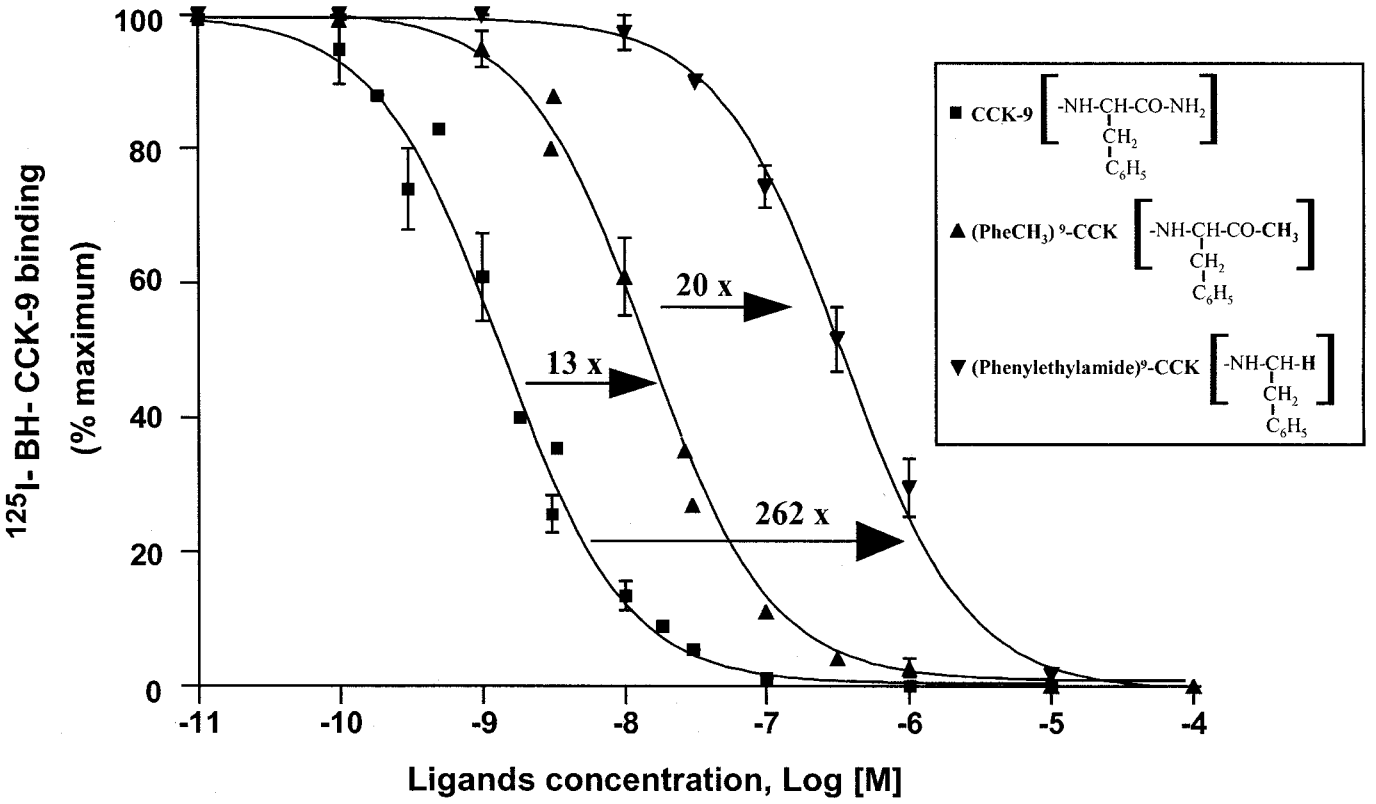

Fig. 2. Competition of ^{125}I-BH-CCK-9 binding by CCK-9, $(PheCH_3)^9$-CCK, and (phenylethylamide)^9-CCK on the WT-CCK2R. Binding is expressed as the percentage of specifically bound ^{125}I-BH-CCK-9. Results are expressed as mean ± S.E. of five separate experiments performed in duplicate. Inset, structure of the C terminus of CCK-9 and modified compounds.

results in a 13-fold decrease CCK affinity; therefore, the 262-fold lower affinity measured when the amide is completely removed suggests that the C=O group is also important for CCK affinity and contributes for ~20-fold to CCK affinity. These results indicate that the contribution of the C=O group correlates with the effect of the Y189F (Y4.60F) mutation on CCK affinity and is in accordance with a potential interaction of the C=O group of the amide with the hydroxyl of Tyr189 (Y4.60).

The $(PheCH_3)^9$-CCK and (phenylethylamide)^9-CCK compounds were then tested for their affinities toward the N358A (N6.55A) and Y189F (Y4.60F) mutants to determine to what extent these mutants were sensitive to changes in the C-terminal amide of CCK. As shown in Table 1 and Fig. 3a, the N358A (N6.55A) mutant bound $(PheCH_3)^9$-CCK with an affinity similar to that of CCK-9 (K_i, 26.1 ± 7.8 nM versus 24.2 ± 5.3 nM), indicating that the mutant is no further affected by the substitution of the NH_2 of the C-terminal amide of CCK. In contrast, the WT-CCK2R that bound $(PheCH_3)^9$-CCK with a 13-fold decreased affinity seems sensitive to this substitution (Table 1 and Fig. 3a). This result is consistent with the fact that the mutation of Asn358 (N6.55) to Ala has already disrupted the hydrogen bond between the oxygen atom of the carboxamide of Asn358 (N6.55) and the proton of the NH_2 group of the C-terminal amide of CCK. This result strongly argues for a direct interaction between Asn358 (N6.55) and the C-terminal NH_2 group of CCK.

We then tested the effect of (phenylethylamide)^9-CCK on the N358A (N6.55A) mutant. Because the N358A (N6.55A) mutant is not affected by the substitution of the NH_2 group of the C-terminal amide of CCK, as determined in the previous experiment, the affinity of (phenylethylamide)^9-CCK for the N358A (N6.55A) mutant should only reflect the loss of the C=O group. As shown in Table 1 and Fig. 3b, the N358A (N6.55A) mutant bound (phenylethylamide)^9-CCK with a 22-fold decreased affinity in comparison with CCK. This effect is

TABLE 1

Affinities of CCK-9 and modified peptides for the WT-CCK2R, Y189F (Y4.60F) mutant, and N358A (N6.55A) mutant

K_i values were calculated from competition curves of ^{125}I-BH-CCK-9 binding by the indicated ligand as described under *Materials and Methods*. The factor F_{CCK-9} represents the effect of the mutation on the affinity of the ligand tested relative to CCK-9. Results are expressed as mean ± S.E. of three to five separate experiments performed in duplicate.

	WT- CCK2R		Y189F Mutant		N358A Mutant	
	K_i	F_{CCK-9}	K_i	F_{CCK-9}	K_i	F_{CCK-9}
	nM		*nM*		*nM*	
CCK-9	1.3 ± 0.3	1	39.3 ± 5.6	1	24.2 ± 5.3	1
$(PheCH_3)^9$-CCK	17.4 ± 3.2	13	252 ± 42	6	26.1 ± 7.8	1
(Phenylethylamide)^9-CCK	341 ± 53	262	816 ± 157	21	543 ± 154	22
$(Ala)^3$-CCK	18.1 ± 6.1	14	382 ± 15	10	233 ± 55	10

consistent with the contribution of the C=O group to CCK affinity, which was estimated to be 20-fold by binding of $(PheCH_3)^9$-CCK and (phenylethylamide)^9-CCK to the WT-CCK2R. Together, these results favor an interaction of Asn358 (N6.55) with the NH_2 group of the C-terminal amide and confirm that the C-terminal C=O group interacts with a residue other than Asn358 (N6.55).

The same compounds were then tested for their affinities toward the Y189F (Y4.60F) mutant. We hypothesized that if the proton of the hydroxyl group of Tyr189 (Y4.60) interacted with the oxygen of the C=O group of the C-terminal amide of CCK, $(PheCH_3)^9$-CCK should have an affinity for the Y189F (Y4.60F) mutant equivalent to that of (phenylethylamide)^9-CCK for the WT-CCK2R. Indeed, the Y189F (Y4.60F) mutation and the substitution of the NH_2 in $(PheCH_3)^9$-CCK should result in the disruption of the two hydrogen bonds linking the C-terminal amide to residues Tyr189 (Y4.60) and Asn358 (N6.55). In accordance with this hypothesis, we found that $(PheCH_3)^9$-CCK presented an affinity for the Y189F (Y4.60F) mutant similar to that of (phenylethylamide)^9-CCK for the WT-CCK2R (K_i, 252 ± 42 nM versus 341 ± 53 nM) (Table 1 and Fig. 3c). Therefore, this result is consistent with the interaction of the hydroxyl group of Tyr189 (Y4.60) with the C=O group of the C-terminal amide of CCK.

We then tested (Phenylethylamide)^9-CCK binding to the Y189F (Y4.60F) mutant. If the hydroxyl of Tyr189 (Y4.60) was in interaction with the C=O of the C-terminal amide of CCK, the Y189F (Y4.60F) mutant should only be sensitive to the substitution of the NH_2, because the hydrogen bond between the hydroxyl of Tyr189 (Y4.60) and the C=O of the amide should be already disrupted by the Y189F (Y4.60F) mutation. In line with this hypothesis, the affinity of the Y189F (Y4.60F) mutant for (phenylethylamide)^9-CCK was only 21-fold lower than its affinity for CCK, whereas the affinity of the WT-CCK2R for (phenylethylamide)^9-CCK was decreased 262-fold (Table 1 and Fig. 3d).

In addition, the fact that the Y189F (Y4.60F) mutant bound (phenylethylamide)^9-CCK with an affinity near that of $(PheCH_3)^9$-CCK (K_i, 816 ± 157 nM versus 252 ± 42 nM, Table 1), further argues that the Y189F (Y4.60F) mutant is insensitive to the substitution of the C=O group. Together, these data provide compelling evidence that the hydroxyl group of Tyr189 (Y4.60) interacts with the C=O group of the C-terminal amide of CCK.

To further demonstrate that the effects of mutating Asn358 (N6.55) and Tyr189 (Y4.60) on CCK affinity were specific to their interactions with the C-terminal amide of CCK, we tested a CCK peptide modified at another position. We used a compound in which the sulfated tyrosine at position 3 was exchanged for an alanine [(Ala)^3-CCK compound].

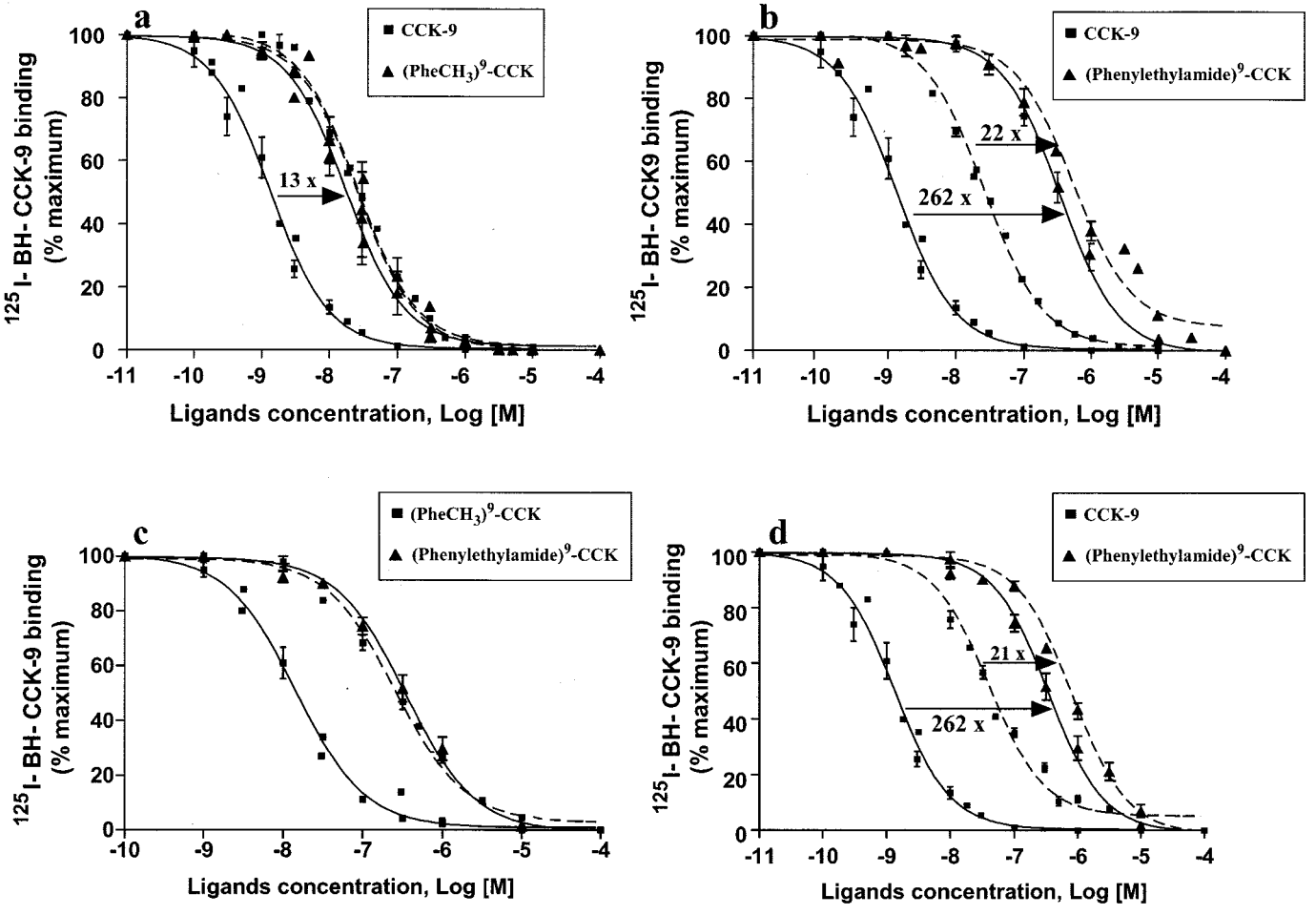

Fig. 3. Competition of ^{125}I-BH-CCK-9 binding by CCK-9, $(PheCH_3)^9$-CCK, and (phenylethylamide)^9-CCK on the WT-CCK2R (solid line), N358A (N6.55A) mutant (broken line, a and b) and Y189F (Y4.60F) mutant (broken line, c and d). Binding is expressed as the percentage of specifically bound ^{125}I-BH-CCK-9. Results are expressed as mean ± S.E. of five separate experiments performed in duplicate.

We previously determined that this residue contributes to CCK affinity to an extent similar to that of the C=O and NH_2 groups of the C-terminal amide of CCK (Silvente-Poirot et al., 1999). As reported in Table 1, this peptide had an additive effect on the N358A (N6.55A) mutation. An additional decrease of 10-fold in the affinity of this peptide was measured for the N358A (N6.55A) mutant compared with CCK, which was similar to that observed with the WT-CCK2R (14-fold decrease in affinity). This additive effect indicates that the N358A (N6.55A) mutant is sensitive to the modification of this peptide, unlike what we observed with $(PheCH_3)^9$-CCK. $(Ala)^3$-CCK was then tested on the Y189F (Y4.60F) mutant. The effect of this peptide on the WT-CCK2R and on the Y189F (Y4.60F) mutant was similar. Compared with CCK, 14- and 10-fold decreased affinities were measured, respectively, indicating that the WT-CCK2R and the Y189F (Y4.60F) mutant are similarly sensitive to the modification of this peptide (Table 1). Together, these results indicate that the effects of mutating Tyr189 (Y4.60) or Asn358 (N6.55), as well as substituting the sulfated tyrosine, are fully additive, as would be expected for independent effects, and thus further confirm the specificity of the interactions between residues Tyr189 (Y4.60) and Asn 358 (N6.55) and the C-terminal amide of CCK.

We then tested the effects of both mutations on PLC activation by measuring total inositol phosphates (IP) production. PLC activation, one of the main signaling pathways activated by the CCK2R, has been well described in COS-7 cells (Jagerschmidt et al., 1995; Silvente-Poirot et al., 1998, 1999). The potencies and efficacies of CCK and modified CCK analogs to stimulate IP production through the wild-type and mutated receptors expressed in COS-7 cells are reported in Table 2. The potencies of the different ligands tested correlate well with their affinities for the WT-CCK2R or the mutant receptors. The N358A (N6.55A) and Y189F (Y4.60F) mutants displayed efficacies for CCK-stimulated increase of IP production near that of the WT-CCK2R, which reached a 13-fold increase above basal in IP production (Fig. 4a). These results indicate that each mutation induces a small effect on maximal IP production. In the same way, $(PheCH_3)^9$-CCK induced a significant response through the WT-CCK2R (E_{max}, 85%) or the N358A (N6.55A) mutant (E_{max}, 70%), indicating that the maintenance of a single hydrogen bond between the C-terminal amide of CCK and the CCK2R is sufficient for a significant biological response (Fig. 4b). In contrast, a more pronounced effect was observed when the two hydrogen bonds between Tyr189 (Y4.60) and Asn358 (N6.55) and the C-terminal amide were disrupted. Indeed, the maximal IP production was affected more dramatically, as observed with $(phenylethylamide)^9$-CCK on the WT-CCK2R, the N358A (N6.55A) mutant, and the Y189F (Y4.60F) mutant (E_{max}, 40, 41, and 20% respectively; Fig. 4c) and with $(PheCH_3)^9$-CCK on the Y189F (Y4.60F) mutant (E_{max}, 48%) (Fig. 4b). These results confirm that the C-terminal amide of CCK is important for full-peptide biological activity, in particular for phosphoinositide turnover, and show that Asn358 (N6.55) and Tyr189 (Y4.60) contribute equally to the efficacy of this biological response in addition to their role in the CCK binding site. The influence of the C-terminal amide seems to vary according to the biological activity considered because phenylethylamide tetrapeptide derivatives of CCK or gastrin have been shown to display an antagonistic effect on gastric acid secretion (Galleyrand et al., 1992). Interestingly, mutagenesis studies in the TM6 domain of the CCK2R have identified two highly conserved residues in G protein coupled-receptors, Trp351 (W6.48) and Phe347 (F6.44), to be important for inositol phosphates production (Jagerschmidt et al., 1998). Substitution of Trp351 (W6.48) with alanine induced a 34% decrease in IP production and a slight decrease in CCK affinity. Similar mutation of Phe347 (F6.44) produced a mutant that displayed no change in CCK affinity but that was inactive in IP stimulation, suggesting a complete loss of the transduction process. Interestingly, as illustrated in the CCK2R model (Fig. 1A), a stacking interaction is observed between the indole group of Trp351 (W6.48) and the phenyl ring of Phe9 of CCK (distance, 6.8 Å), which is in interaction with the phenolic group of Tyr189 (Y4.60). Phe347 (F6.44) is located one helical turn above Trp351 (W6.48) and its side chain also forms a stacking interaction with the indole group of Trp351 (W6.48) (distance 6.7 Å). Because ligand-regulated conformational changes in receptor were shown to underlie activation of GPCRs (Gether, 2000), it could be hypothesized that upon CCK binding, these residues would participate in CCK2R activation by inducing conformational changes in the TM6 domain. Consistent with an interaction of Tyr189 (Y4.60) with Phe9 of CCK, we found that removing the aromatic ring of Tyr189 (Y4.60) by mutating Tyr189 to alanine (Y4.60A) induced an additional decrease of 28-fold in CCK affinity compared with the Y189F (Y4.60F) mutant without effect on mutant expression (K_i, 1095 ± 230 nM; B_{max}, 1.2 ± 0.1 pmol/10^6 cells versus K_i, 39.3 ± 5.6 nM; B_{max}, 1.2 ± 0.2 pmol/10^6 cells, respectively). This decrease in CCK affinity is compatible with the loss of the stacking interaction between the aromatic rings of Phe9 and Tyr189 (Y4.60). In addition, the Y189A (Y4.60A) mutant was found to be unable to pro-

TABLE 2

Potencies and efficacies of CCK-9 and modified peptides to stimulate inositol phosphates production on the WT-CCK2R, N358A (N6.55A), and Tyr189F (Y4.60F) mutant

Potencies (EC_{50}) of the indicated ligands were calculated from total IP production dose-response curves. Results are expressed as mean ± S.E. of three to five separate experiments performed in duplicate. The efficacies (E_{max}) of the peptides tested to stimulate IP production are expressed as the percentage of the maximal increase obtained using 10^{-6} M CCK-9 on the WT-CCK2R.

	CCK-9		$(PheCH_3)^9$-CCK		$(Phenylethylamide)^9$-CCK	
	EC_{50}	E_{max}	EC_{50}	E_{max}	EC_{50}	E_{max}
	nM	%	*nM*	%	*nM*	%
WT-CCK2R	0.69 ± 0.97	100	11.2 ± 2.9	85	111 ± 22	40
N358A mutant	20.9 ± 7.4	90	38.2 ± 7.5	70	450 ± 73	41
Y189F mutant	41.9 ± 6.3	90	320 ± 33	48	N.A.	20

N.A., not applicable.

duce IP after CCK stimulation even when stimulated with 10^{-4} M CCK (data not shown), suggesting that the interaction between the aromatic rings of Tyr189 (Y4.60) and Phe9 is important for receptor activation and might help to position the side chain of Phe9 in contact with helix 6 and Trp351 (W6.48). Interestingly, several mutations within this domain in the CCK2R were shown to result in the conversion of nonpeptide antagonists to agonists, highlighting the critical role of this domain in ligand binding and receptor activation (Blaker et al., 1998). In GPCR, the specific regions at which the ligand binds to the receptor and induces receptor activation vary importantly depending on the subfamily of receptors and on the chemical structure of the ligand. Even agonists acting at the same receptor may not necessarily share an overlapping binding site (Ulloa-Aguirre et al., 1999; Gether, 2000; Gershengorn and Osman, 2001). Three major subfamilies (A, B, and C) have been established based on highly conserved residues (Gether, 2000). Cholecystokinin receptors are members of the largest and best-studied subfamily, A, which includes rhodopsin and adrenergic receptors. Thus, receptors for small ligands, such as retinal chromophore, biogenic amines, nucleosides, or nucleotides, bind the agonist through a pocket involving transmembrane residues of TMs 3, 5, 6, and 7; TMs 3, 6, and 7 are more important for receptor activation. For receptors for peptides, such as angiotensin, there is evidence that both TM (2 to 7) and extracellular domains contribute to the binding pocket. In these receptors, a concerted participation of these domains in receptor activation has been reported. For moderate-size peptides, binding occurs in the extracellular loops and the N-terminal segment, whereas for large ligands, such as glycoprotein hormones, the high-affinity binding site is mostly located within the N-terminal segment. In these receptors, activation involves extracellular loops and TM domains. For metabotropic glutamate, GABA, and calcium-sensing receptors, the ligand binds exclusively in the large amino-terminal domain of the receptor, whereas the core regions of these receptors are involved in receptor activation. Despite the fact that structure-function studies in GPCRs argue for multiple domains involved in ligand binding and activation, different approaches using biophysical, biochemical, and mutagenesis techniques provide evidence that the activation mechanisms are similar in many aspects among at least subfamily A. Thus, it seems that agonist binding provokes the breaking of intramolecular constraints that stabilize the inactive state of the receptor and triggers conformational changes in loops and TM domains. These conformational switches allow the binding of G proteins and other regulatory proteins to the cytoplasmic regions of the receptor (Gether, 2000). So, the disruption of the contraints formed in the ground state of the receptors could be the initiating event leading to receptor activation. The transmembrane domain 6 has been shown to have a central stabilizing role in different receptors. Relative movements of TM3 and TM6 domains were demonstrated to play critical roles in rhodopsin and β-2 adrenergic receptor activation (Farrens et al., 1996; Lin and Sakmar, 1996; Gether et al., 1997) and were also predicted from the crystal structure of rhodopsin receptor (Palczewski et al., 2000). However, this does not exclude the idea that movements of other domains may contribute to receptor activation. Indeed, movements in TM7 of rhodopsin were reported to occur in response to photoactivation using spectroscopic studies (Altenbach et al., 1999). This domain has been showed to be specifically involved in G(q) protein activation in the CCK2R (Gales et al., 2000).

In conclusion, this study presents strong evidence to support the interaction between Tyr189 (Y4.60) and Asn358 (N6.55) and the C-terminal amide of CCK and consequently validate the three-dimensional model of the CCK.CCK2R complex that we have constructed. Together, our results indicate that the upper half part of the TM helices 4 and 6 in addition to the second extracellular loop of the CCK2R are involved in CCK binding pocket. A similar approach has been successfully used with the CCK1R and has led to the identification of several residues involved in the CCK binding site and CCK1R activation (Gigoux et al., 1998, 1999a,b; Escrieut et al., 2002). However, the present results differ from those obtained with the CCK1R, for which we showed that a single residue, Asn353 (N6.55), the homologous residue of Asn358 (N6.55), interacts via two hydrogen bonds with the C-terminal amide of CCK (Gigoux et al., 1999a). A schematic overview of the CCK binding site in the CCK1R and CCK2R showing the different interactions demonstrated between CCK and each receptor is presented in Fig. 5. Clearly, our previous investigations on the CCK1 and CCK2 receptors and the present study argue that the positioning of CCK in

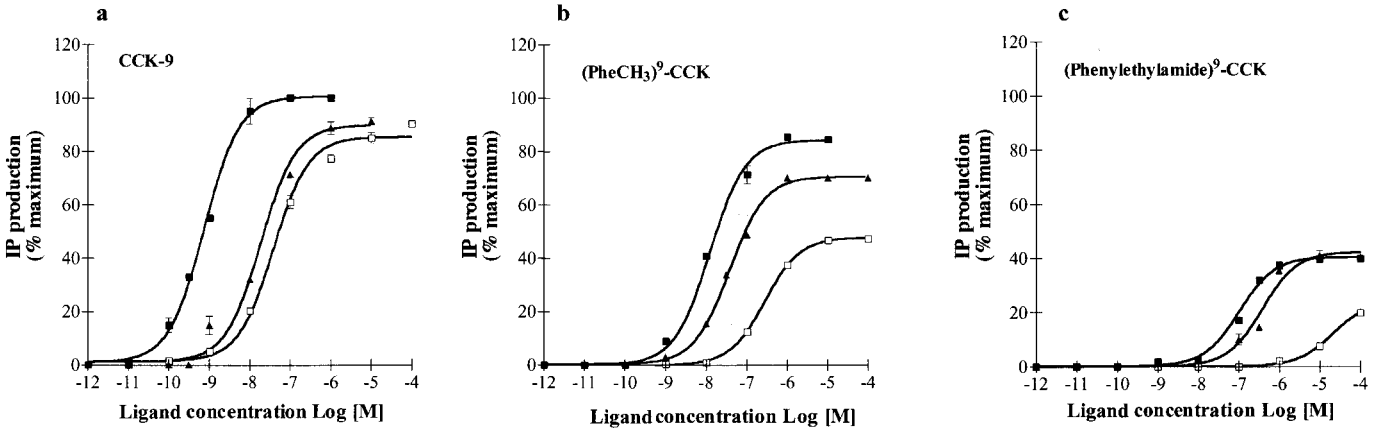

Fig. 4. Stimulation of total IP production by CCK-9, $(PheCH_3)^9$-CCK, and (phenylethylamide)^9-CCK on the WT-CCK2R (■), Y189F (Y4.60F) mutant (□), and N358A (N6.55A) mutant (▲). IP production is expressed as the percentage of the maximal increase obtained using 10^{-6} M CCK-9 on the WT-CCK2R. Results are expressed as mean ± S.E. of three to five separate experiments performed in duplicate.

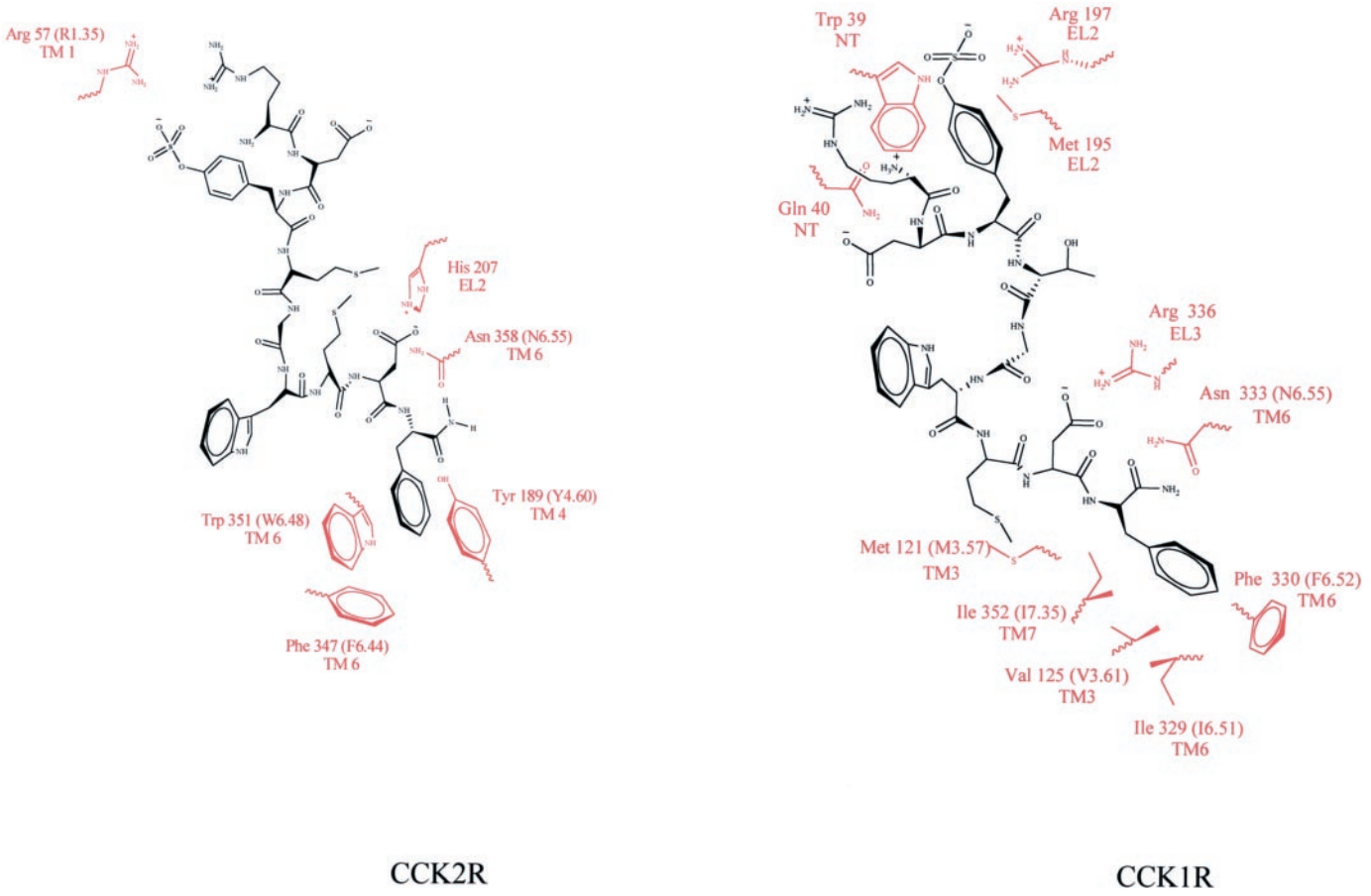

Fig. 5. Two-dimensional representation of the CCK-9 binding site in the CCK2R and CCK1R. Residues in the CCK2R and CCK1R (in red) reported to be in interaction with CCK-9 (in black) were indicated. For the CCK1R: Trp39 and Gln40 were shown in interaction with the N-terminal moiety of CCK-9 (Kennedy et al., 1997), Met195 with the Tyr3 aromatic ring (Gigoux et al., 1998), Arg197 with the Tyr3 sulfate group (Gigoux et al., 1999b; Ding et al., 2002), Arg336 (R6.58) and Asn333 (N6.55) with the Asp8 and C-terminal amide, respectively (Gigoux et al., 1999a), and several residues forming two hydrophobic pockets were identified in interaction with Met7 [Val125 (V3.61), Met121 (M3.57) and Ile352 (I7.35)] and Phe9 [Phe330 (F6.52), Val125 (V3.61) and Ile329 (I6.51)] (Escrieut et al., 2002). For the CCK2R: His207 was shown in interaction with Asp8 of CCK-9 (Silvente-Poirot et al., 1999). R57 (R1.35) was identified as important for CCK binding (Silvente-Poirot and Wank, 1996) and was shown to be part of the CCK2R sequence covalently linked to an N-terminal photoreactive CCK probe (Anders et al., 1999), and the current study shows interaction of Tyr189 (Y4.60) and Asn358 (N6.55) with the C-terminal amide of CCK. NT, N terminal; EL, extracellular loop.

these receptors is different. Thus, Asp8 of CCK has been shown to interact with a nonconserved residue of the extracellular loop 2 in the CCK2R, His207, whereas in the CCK1R, we have demonstrated that Asp8 interacts with a conserved residue, Arg336, located in the third extracellular loop. It seems that the second extracellular loop of the CCK2R enters deeper into the cavity formed by the seven helices to interact with the C-terminal part of CCK than the second extracellular loop of the CCK1R, which was shown to interact via two nonconserved residues, Met195 and Arg197, with the N-terminal Tyr^3 of CCK (Gigoux et al., 1998, 1999b; Ding et al., 2002). Moreover, although the C-terminal amide of CCK in the CCK2R has been shown in the current study to interact with both Tyr189 (Y4.60) and Asn358 (N6.55), in the CCK1R, this function interacts exclusively with Asn 333 (N6.55). Indeed, mutation of the conserved Tyr189 (Y4.60) to Phe in the CCK1R was shown to have no effect on CCK binding (data not shown).

In addition, our results highlight that residues Tyr189 (Y4.60) and Asn358 (N6.55) are required for the full activation of PLC, because an important loss in maximal IP production is observed when the two hydrogen bonds between these residues and the C-terminal amide of CCK are disrupted (loss of 60 to 80%) and when Tyr189 (Y4.60) is mutated to Ala (total loss). However, when a single hydrogen bond is disrupted, a significant maximal IP response is measured (70 to 90%), suggesting that the maintenance of a single hydrogen bond between the C-terminal amide of CCK and one of these residues is enough to stabilize the CCK2R in an active conformation and to ensure substantial IP production. Thus, the present work represents an important step toward the complete identification of CCK binding site in the CCK2R and the understanding of the molecular mechanisms that govern the transduction and specificity of the biological response. Such knowledge seems now essential to optimize CCK ligands and improve their selectivity of action. The present three-dimensional model of the CCK2R.CCK complex, as well as the CCK1R.CCK molecular model that we have previously published and validated, represent important tools that will allow to work in this direction.

Acknowledgments

We thank Riad Quanbar for careful reading of the manuscript.

References

Altenbach C, Cai K, Khorana HG, and Hubbell WL (1999) Structural features and light-dependent changes in the sequence 306–322 extending from helix VII to the

palmitoylation sites in rhodopsin: a site-directed spin-labeling study. *Biochemistry* **38:**7931–7937.
Anders J, Bluggel M, Meyer HE, Kuhne R, ter Laak AM, Kojro E, and Fahrenholz F (1999) Direct identification of the agonist binding site in the human brain cholecystokinin B receptor. *Biochemistry* **38:**6043–6055.
Ballesteros JA and Weinstein H (1995) Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. *Methods Neurosci* **25:**366–428.
Blaker M, Ren Y, Gordon MC, Hsu JE, Beinborn M, and Kopin AS (1998) Mutations within the cholecystokinin-B/Gastrin receptor ligand 'Pocket' interconvert the functions of nonpeptide agonists and antagonists. *Mol Pharmacol* **54:**857–863.
Bukusoglu G and Jenness DD (1996) Agonist-specific conformational changes in the yeast alpha-factor pheromone receptor. *Mol Cell Biol* **16:**4818–4823.
Campagne F, Jestin R, Reversat JL, Bernassau JM, and Maigret B (1999) Visualisation and integration of G protein-coupled receptor related information help the modelling: description and applications of the Viseur program. *J Comput Aided Mol Des* **13:**625–643.
Choi G, Landin J, Galan JF, Birge RR, Albert AD, Yeagle PL. Structural studies of metarhodopsin II, the activated form of the G-protein coupled receptor, rhodopsin. *Biochemistry* **41:**7318–7324, 2002.
de Tullio P, Delarge J, and Pirotte B (2000) Therapeutic and chemical developments of cholecystokinin receptor ligands. *Expert Opin Investig Drugs* **9:**129–146.
Ding X, Pinon D, Furse K, Lybrand T, and Miller LJ (2002) Refinement of the conformation of a critical region of charge-charge interaction between cholecystokinin and its receptor. *Mol Pharmacol* **61:**1041–1052.
Escrieut C, Gigoux V, Archer E, Verrier S, Maigret B, Behrendt R, Moroder L, Bignon E, Silvente-Poirot S, Pradayrol L, et al. (2002) 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 Met121 in the activation process. *J Biol Chem* **277:**7546–7555.
Farrens DL, Altenbach C, Yang K, Hubbell WL, and Khorana HG (1996) Requirement of rigid-body motion of transmembrane helices for light activation of rhodopsin. *Science (Wash DC)* **274:**768–770.
Fourmy D, Lopez P, Poirot S, Jimenez J, Dufresne M, Moroder L, Powers SP, and Vaysse N (1989) A new probe for affinity labelling pancreatic cholecystokinin receptor with minor modification of its structure. *Eur J Biochem* **185:**397–403.
Galas MC, Lignon MF, Rodriguez M, Mendre C, Fulcrand P, Laur J, and Martinez J (1988) Structure-activity relationship studies on cholecystokinin: analogues with partial agonist activity. *Am J Physiol* **254:**G176–G182.
Gales C, Kowalski-Chauvel A, Dufour MN, Seva C, Moroder L, Pradayrol L, Vaysse N, Fourmy D, and Silvente-Poirot S (2000) Mutation of Asn-391 within the conserved NPXXY motif of the cholecystokinin B receptor abolishes Gq protein activation without affecting its association with the receptor. *J Biol Chem* **275:**17321–17327.
Galleyrand JC, Fulcrand P, Bali JP, Rodriguez M, Magous R, Laur J, and Martinez J (1992) Biological effects of human gastrin I and II chemically modified at the C-terminal tetrapeptide amide. *Peptides* **13:**519–525.
Gershengorn MC and Osman R (2001) Minireview: insights into G protein-coupled receptor function using molecular models. *Endocrinology* **142:**2–10.
Gether U (2000) Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocr Rev* **21:**90–113.
Gether U, Lin S, Ghanouni P, Ballesteros JA, Weinstein H, and Kobilka BK (1997) Agonists induce conformational changes in transmembrane domains III and VI of the beta2 adrenoceptor. *EMBO J* **16:**6737–6747.
Gether U, Lin S, and Kobilka BK (1995) Fluorescent labeling of purified beta 2 adrenergic receptor. Evidence for ligand-specific conformational changes. *J Biol Chem* **270:**28268–28275.
Gigoux V, Escrieut C, Fehrentz JA, Silvente-Poirot S, Maigret B, Moroder L, Gully D, Martinez J, Vaysse N, and Fourmy D (1999a) Arginine 336 and asparagine 333 of the human cholecystokinin-A receptor binding site interact with the penultimate aspartic acid and the C-terminal amide of cholecystokinin. *J Biol Chem* **274:**20457–20464.
Gigoux V, Escrieut C, Silvente-Poirot S, Maigret B, Gouilleux L, Fehrentz JA, Gully D, Moroder L, Vaysse N, and Fourmy D (1998) Met-195 of the cholecystokinin-A receptor interacts with the sulfated tyrosine of cholecystokinin and is crucial for receptor transition to high affinity state. *J Biol Chem* **273:**14380–14386.
Gigoux V, Maigret B, Escrieut C, Silvente-Poirot S, Bouisson M, Fehrentz JA, Moroder L, Gully D, Martinez J, Vaysse N, et al. (1999b) Arginine 197 of the cholecystokinin-A receptor binding site interacts with the sulfate of the peptide agonist cholecystokinin. *Protein Sci* **8:**2347–2354.
Henderson R, Baldwin JM, Ceska TA, Zemlin F, Beckmann E, and Downing KH (1990) Model for the structure of bacteriorhodopsin based on high-resolution electron cryo-microscopy. *J Mol Biol* **213:**899–929.
Jagerschmidt A, Guillaume N, Goudreau N, Maigret B, and Roques BP (1995) Mutation of Asp100 in the second transmembrane domain of the cholecystokinin B receptor increases antagonist binding and reduces signal transduction. *Mol Pharmacol* **48:**783–789.
Jagerschmidt A, Guillaume N, Roques BP, and Noble F (1998) Binding sites and transduction process of the cholecystokinin B receptor: involvement of highly conserved aromatic residues of the transmembrane domains evidenced by site-directed mutagenesis. *Mol Pharmacol* **53:**878–885.
Jagerschmidt A, Guillaume-Rousselet N, Vikland ML, Goudreau N, Maigret B, and Roques BP (1996) His381 of the rat CCKB receptor is essential for CCKB versus CCKA receptor antagonist selectivity. *Eur J Pharmacol* **296:**97–106.
Jensen RT, Lemp GF, and Gardner JD (1982) Interactions of COOH-terminal fragments of cholecystokinin with receptors on dispersed acini from guinea pig pancreas. *J Biol Chem* **257:**5554–5559.
Joseph MP, Maigret B, Bonnafous JC, Marie J, and Scheraga HA (1995) A computer modeling postulated mechanism for angiotensin II receptor activation. *J Protein Chem* **14:**381–398.
Kennedy K, Gigoux V, Escrieut C, Maigret B, Martinez J, Moroder L, Frehel D, Gully D, Vaysse N, and Fourmy D (1997) Identification of two amino acids of the human cholecystokinin-A receptor that interact with the N-terminal moiety of cholecystokinin. *J Biol Chem* **272:**2920–2926.
Knight M, Tamminga CA, Steardo L, Beck ME, Barone P, and Chase TN (1984) Cholecystokinin-octapeptide fragments: binding to brain cholecystokinin receptors. *Eur J Pharmacol* **105:**49–55.
Krebs A, Villa C, Edwards PC, and Schertler GF (1998) Characterization of an improved two-dimensional p22121 crystal from bovine rhodopsin. *J Mol Biol* **282:**991–1003.
Lin SW and Sakmar TP (1996) Specific tryptophan UV-absorbance changes are probes of the transition of rhodopsin to its active state. *Biochemistry* **35:**11149–11159.
Morley JS, Tracy HJ, and Gregory RA (1965) Structure-function relationships in the active C-terminal tetrapeptide sequence of gastrin. *Nature (Lond)* **207:**1356–1359.
Moroder L, Wilschowitz L, Gemeiner M, Gohring W, Knof S, Scharf R, Thamm P, Gardner JD, Solomon TE, and Wunsch E (1981) Cholecystokinin-pancreozymin synthesis. Synthesis of [28-threonine, 31- norleucine]- and [28-threonine, 31-leucine]cholecystokinin-pancreozymin- (25–33)-nonapeptide]. *Hoppe Seylers Z Physiol Chem* **362:**929–942.
Munson PJ and Rodbard D (1980) Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* **107:**220–239.
Noble F, Wank SA, Crawley JN, Bradwejn J, Seroogy KB, Hamon M, and Roques BP (1999) International Union of Pharmacology. XXI. Structure, distribution and functions of cholecystokinin receptors. *Pharmacol Rev* **51:**745–781.
Orosco M, Gourch A, Rodriguez M, Martinez J, Jacquot C, and Cohen Y (1990) Comparative activity of two cholecystokinin analogues with partial agonist activity: effects on food intake and brain monoamines. *Peptides* **11:**873–877.
Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp RE, et al. (2000) Aug 4) Crystal structure of rhodopsin: a G protein-coupled receptor. *Science (Wash DC)* **289:**739–745.
Silvente-Poirot S, Dufresne M, Vaysse N, and Fourmy D (1993) The peripheral cholecystokinin receptors. *Eur J Biochem* **215:**513–529.
Silvente-Poirot S, Escrieut C, Gales C, Fehrentz JA, Escherich A, Wank SA, Martinez J, Moroder L, Maigret B, Bouisson M, et al. (1999) Evidence for a direct interaction between the penultimate C-terminal aspartic acid of cholecystokinin and histidine 207 located in the second extracellular loop of the cholecystokinin B receptor. *J Biol Chem* **274:**23191–23197.
Silvente-Poirot S, Escrieut C, and Wank SA (1998) Role of the extracellular domains of the cholecystokinin receptor in agonist binding. *Mol Pharmacol* **54:**364–371.
Silvente-Poirot S and Wank SA (1996) A segment of five amino acids in the second extracellular loop of the cholecystokinin-B receptor is essential for selectivity of the peptide agonist gastrin. *J Biol Chem* **271:**14698–14706.
Ulloa-Aguirre A, Stanislaus D, Janovick JA, and Conn PM (1999) Structure-activity relationships of G protein-coupled receptors. *Arch Med Res* **30:**420–435.
Unger VM, Hargrave PA, Baldwin JM, and Schertler GF (1997) Arrangement of rhodopsin transmembrane alpha-helices. *Nature (Lond)* **389:**203–206.
Wank SA (1998) G protein-coupled receptors in gastrointestinal physiology. I. CCK receptors: an exemplary family. *Am J Physiol* **274:**G607–G613.

Address correspondence to: Sandrine Silvente-Poirot, INSERM U563, Département Innovation Thérapeutique et Oncologie Moléculaire, Institut Claudius Regaud, 20-24 rue du Pont Saint Pierre, 31052 Toulouse Cedex, France. Email: poirot_s@icr.fnclcc.fr