Linking Non-peptide Ligand Binding Mode to Activity at the Human Cholecystokinin-2 Receptor*

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The CCK2R appears to be an excellent G protein-coupled receptor prototype on which a strategy of rational design of ligand can be tested. With this in mind, we have recently constructed and experimentally validated models of the active and the inactive CCK2R and delineated activation mechanism of this receptor (14).

In the present work we wanted to answer the important question previously raised in the course of CCK2R antagonist design, why two high affinity high selectivity very similar non-peptide ligands of the CCK2R display differential pharmacological activity, namely antagonism and partial agonism at the CCK2R (15). The two ligands have the same chemical core, but they differ by the absence (JB93,182) or presence (JB93,242) of a methyl group on the indole nitrogen (Fig. 1). We have used laboratory experiments to demonstrate that JB93,182 is a partial inverse agonist and JB93,242 is a partial agonist at human CCK2R and a combination of in silico and site-directed mutagenesis studies to show that these opposing activities are due to different anchoring modes of the two compounds to a residue of helix II (Thr-2.61) in the inactive state of the CCK2R. The binding mode of the inverse agonist allows the molecule to interact with a key amino acid for CCK2R activation (Trp-6.48), preventing CCK2R activation is favored. This study on the molecular mechanism of ligand action opens the possibility of target-based optimization of G protein-coupled receptor non-peptide ligands.

G protein-coupled receptors (GPCRs) are membrane-embedded proteins having seven transmembrane domains, responsible for communication between the cell and its environment (1). GPCRs represent, therefore, a major focus in functional genomics programs and drug development research (2).

GPCRs naturally exist in different conformations which correspond to both active and inactive states. Depending on their propensity to bind preferentially with one of these receptor conformations or to identically interact with both, synthetic ligands can behave as agonists, inverse agonists, or neutral antagonists. To date, three three-dimensional GPCR structures have been precisely determined by x-ray crystallography, namely that of rhodopsin and β2- and β1-adrenergic receptors (3–6). In this context, refinement of modeled GPCR structures in both active and inactive conformations and progress in the understanding of the precise mechanisms, which govern their activation, are of paramount importance for drug design strategies (7).

The cholecystokinin-2 (CCK-2) receptor (CCK2R) belongs to family A of rhodopsin-like GPCRs. It binds both cholecystokinin and gastrin with a similar, high affinity (8, 9). CCK2R mediates a wide spectrum of CCK- and gastrin-induced biological effects in the central nervous system and in periphery including anxiety, pain perception, and gastric acid secretion as well as controlling growth and differentiation of the gastric mucosa. The CCK2R and/or a constitutively active variant of this receptor may contribute to human diseases (10, 11). This has recently generated considerable interest in the identification, among the large panel of synthetic ligands, of CCK2R antagonists having an inverse agonist activity (12, 13).

The CCK2R appears to be an excellent G protein-coupled receptor prototype on which a strategy of rational design of ligand can be tested. With this in mind, we have recently constructed and experimentally validated models of the active and the inactive CCK2R and delineated activation mechanism of this receptor (14).

In the present work we wanted to answer the important question previously raised in the course of CCK2R antagonist design, why two high affinity high selectivity very similar non-peptide ligands of the CCK2R display differential pharmacological activity, namely antagonism and partial agonism at the CCK2R (15). The two ligands have the same chemical core, but they differ by the absence (JB93,182) or presence (JB93,242) of a methyl group on the indole nitrogen (Fig. 1). We have used laboratory experiments to demonstrate that JB93,182 is a partial inverse agonist and JB93,242 is a partial agonist at human CCK2R and a combination of in silico and site-directed mutagenesis studies to show that these opposing activities are due to different anchoring modes of the two compounds to a residue of helix II (Thr-2.61) in the inactive state of the CCK2R. The binding mode of the inverse agonist allows the molecule to interact with a key amino acid for CCK2R activation (Trp-6.48), preventing

The abbreviations used are: GPCR, G protein-coupled receptor; CCK2R, CCK2 receptor; TMD, targeted molecular dynamics; Ins-P, inositol phosphates; MD, molecular dynamics; DMEM, Dulbecco’s modified Eagle’s medium; WT, wild type.

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4 Amino acid numbering according to standardized Ballesteros-Weinstein numbering www.gpcr.org/7tm. Amino acid abbreviation is followed by two numbers: the first number corresponds to transmembrane helix of the receptor; the second number corresponds to amino acid position in the helix relative to the most-conserved amino acid to which the arbitrary number 50 was given.
its rotation, whereas the partial agonist bound deeper in the binding pocket, close to helix V, allowing rotation of helix VI. Such an understanding the molecular mechanism of ligand action opens the possibility of target-based optimization of GPCR non-peptide ligands.

EXPERIMENTAL PROCEDURES

Building, Refinement, and Molecular Dynamics of CCK2R-Ligand Complexes—The previous model of CCK2R* (14) was used as starting point to dock JB93,242 and JB93,182. The starting three-dimensional conformation of the ligands was obtained using Corina software (27). Electrostatic potential-derived charges were obtained with the restrained electric potential methodology (16) using a 6-31G* basis set as implemented in the Gaussian 98 program (28). The Lammackian genetic algorithm77 implemented in Auto-Dock 3.0.2 was used to generate docked conformations of JB93,242 and JB93,182 in a putative pocket within the transmembrane helices of CCK2R by randomly changing the overall orientation of the molecules as well as torsion angles. Default settings were used, except for the number of runs, population size, and maximum number of energy evaluations, which were fixed at 100, 100, and 250,000, respectively. Rapid inter- and intermolecular energy evaluation of each configuration was achieved by having the receptor atomic affinity potentials for carbon, oxygen, and hydrogen atoms precalculated in a three-dimensional grid with a spacing of 0.375 Å. The selected docking solutions were refined by atomic affinity potentials for carbon, oxygen, and hydrogen atoms. The selected docking solutions were then studied by MDs in an explicit lipid bilayer under the same conditions as those described above.

Targeted Molecular Dynamics Simulations—This technique has been used to explore in silico CCK2R* to CCK2R° conversion using as initial and target structures a representative snapshot of the inactive and active CCK2R, respectively. The targeted molecular dynamics (TMD) approach used the standard implementation recently incorporated into AMBER (Version 8.0) which allows the solvent molecules to move freely and follow the dynamics of the protein. A restraint was defined in terms of a mass-weighted root mean square superposition to the final reference structure (target, CCK2R*) and applied in the force field as an extra harmonic potential energy term of the form $E = 0.5 \times kr \times N (r.m.s.d. - t.r.m.s.d.)^2$, where $E$ is the biasing potential energy in kcal/mol during the TMD simulation, $kr$ is the force constant, $N$ is the number of atoms, r.m.s.d. is the root mean square deviation from the target structure, and t.r.m.s.d. is the desired r.m.s.d. value. The initial value of the desired root mean square deviation (r.m.s.d.) value (t.r.m.s.d.) was set as the r.m.s.d. between the initial and target structures (CCK2R* and CCK2R°, respectively), and during the simulation, t.r.m.s.d. was gradually decreased to zero. Only the heavy atoms of the protein were considered in the root mean square definition. A force constant of 0.25 kcal/mol Å over 0.5 ns appeared sufficient to find a low energy path leading from the simulated structure to the target structure.

Site-directed Mutagenesis of CCK2R and Transfection of COS-7 Cells—All mutant receptor cDNAs were constructed by oligonucleotide-directed mutagenesis (QuikChange™ site-directed mutagenesis kit, Stratagene, France) using human CCK2R cDNAs cloned in the pRFNeo vector as template. The presence of desired and the absence of undesired mutations were confirmed by automated sequencing of the complete CCK2R coding sequence (Applied Biosystem). COS-7 cells (1.5 × 106) were plated onto 10-cm culture dishes and grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% fetal calf serum in a 5% CO₂ atmosphere at 37 °C. After overnight incubation, cells were transfected with 1 μg/plate (except when mentioned) of pRFNeo vectors containing the cDNA for the wild-type or mutated CCK2 receptors using a modified DEAE-dextran method. Cells were transferred to 24-well plates 24 h after transfection at a density of 150,000 cells/well for inositol phosphate determination and 5–10,000 cells/well for binding assays.

Inositol Phosphate Production Determination—Approximately 24 h after transfer to 24-well plates and after overnight incubation in DMEM containing 2 μCi/ml of myo-[2-3H]inositol (specific activity, 10–25 Ci/ml, PerkinElmer Life Sciences), transfected cells were washed with DMEM and incubated for 30 min in 1 ml/well DMEM containing 20 mM LiCl at 37 °C. Cells were washed with inositol phosphates (Ins-P) buffer at pH 7.45: phosphate-buffered saline containing 135 mM NaCl, 20
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mm HEPES, 2 mm CaCl₂, 1.2 mm MgSO₄, 1 mm EGTA, 10 mm LiCl, 11.1 mm glucose, and 0.5% bovine serum albumin. Cells were then incubated for 60 min at 37 °C in 0.3-ml Ins-P buffer alone or with ligands. Reactions were stopped by adding 1 ml of methanol/HCl to each well, and contents were transferred to a column (Dowex AG 1-X8, formate form, Bio-Rad) for the determination of Ins-P. Columns were washed twice with 3 ml of distilled water and twice with 2 ml of 5 mm sodium tetraborate, 60 mm sodium formate. The content of each column was eluted by the addition of 2 ml of 1 m ammonium formate, 100 mm formic acid. Radioactivity of 1 ml of the eluted fraction was directly counted in a gamma counter (Auto-Gamma, Packard Instrument Co., Downers Grove, IL). Values for Ins-P accumulation were normalized to a constant number of expressed receptors at the cell surface (1 pmol) as determined in binding assays and were expressed as -fold number of the basal value achieved with the wild-type CCK2R. Mock cells were transfected with a pRFNeo non-coding vector. Efficacy was regarded as Ins-P production in the presence of a supramaximal concentration (1 μM) of a compound, whereas potency corresponded to concentration of that compound producing 50% of the maximal Ins-P production.

Receptor Binding Assay—Approximately 24 h after transfer of transfected cells to 24-well plates, the cells were washed with phosphate-buffered saline, pH 7.4, containing 0.1% bovine serum albumin and incubated for 60 min at 37 °C in 0.3 ml DMEM containing 0.1% bovine serum albumin with an appropriate concentration of high performance liquid chromatography-purified 125I-labeled BH-(Thr,Nle)-CCK9 (specific activity, 1600–2000 Ci/mmol.) in the presence or the absence of unlabeled CCK9. 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 0.1 N NaOH. The radioactivity was directly counted in a gamma counter (Auto-Gamma, Packard). Receptor density (Bmax) and Kᵦ for competitors were calculated using homologous 125I-labeled BH-(Thr,Nle)-CCK9 competition binding experiments using Ligand software (Kell, Cambridge, UK). Kᵦ for competitors were calculated using the non-linear curve fitting software GraphPad Prism (San Diego, CA).

RESULTS

Pharmacological Properties of JB93,182 and JB93,242—The effects of JB93,182 and 93,242 on CCK-stimulated production of Ins-P in COS-7 cells expressing the CCK2R were first analyzed. As illustrated in Fig. 2A, dose-response curves differed according to the concentration of CCK used to stimulate the cells. Indeed, JB93,182 inhibited accumulation of Ins-P after stimulation by 0.18, 0.5, and 10 nm CCK with IC₅₀ which were 35.1 ± 10.8, 70.3 ± 14.1, and 943 ± 110 nm, respectively, whereas JB93,242 enhanced (155%) production of Ins-P observed in the presence of 0.18 nm CCK but partially inhibited Ins-P production after stimulation by 0.5 or 10 nm (inhibitory effects, 18 and 60%, respectively).

Furthermore, when added alone to cells expressing the wild-type CCK2R, the two non-peptide ligands displayed opposite effects on CCK2R-related basal Ins-P production (Fig. 2B). JB93,182 acted as a partial inverse agonist that dose-dependently inhibited basal Ins-P production (IC₅₀ 2.8 ± 0.7 nm) to a maximum of 50% of the initial value (p < 0.001), whereas JB93,242 acted as a partial agonist stimulating Ins-P production (EC₅₀ 1.8 ± 0.2 nm) to a maximum representing 50% of the maximum achieved with 1 μM CCK (p < 0.001). Such a partial agonist activity of JB93,242 likely explains why this compound did not inhibit production of Ins-P in response to low concentrations of CCK, whereas it clearly decreased Ins-P production in response to 10 nm CCK (Fig. 2A, right panel). Interestingly, JB93,182 inhibited JB93,242-stimulated Ins-P production (IC₅₀ 14.6 ± 2.1 nm), confirming that the two molecules exert their effects through the CCK2R (Fig. 2C).

FIGURE 2. Pharmacological properties of JB93,182 (partial inverse agonist) and JB93,242 (partial agonist). A, inhibition of CCK-stimulated Ins-P by JB93,182. JB93,182 or JB93,242 were applied to COS-7 cells expressing CCK2R stimulated by 0.18 (left panel) or 0.5 nm (central panel) or 10 nm CCK (right panel), B, effects of JB93,182, JB93,242, or CCK on Ins-P basal activity of CCK2R. JB93,182, JB93,242, or CCK alone were applied to COS-7 cells transfected with 1 μg/plate plasmid encoding wild-type CCK2R. C, inhibition of JB93,242-stimulated Ins-P by JB93,182. COS-7 cells expressing the CCK2R were stimulated by 2 nm JB93,242 without or with increasing concentrations of JB93,182. D, inhibition of CCK binding to COS-7 cells expressing CCK2R by JB93,182 and JB93,242. Labeled CCK alone (60 pm) or in the presence of increasing concentrations of JB93,182 or JB93,242 was incubated with COS-7 cells expressing the wild-type CCK2R. E, effect of CCK2R expression levels on CCK2R basal activity and on pharmacological activity of CCK2R ligands. Basal activity of CCK2R and effects of JB93,182, JB93,242, or CCK to stimulated production of Ins-P were measured on COS-7 cells transfected with different amounts of plasmid encoding the CCK2R. F, pharmacological activity of CCK2R ligands on WT and constitutively active CCK2R. Efficacy of JB93,182, JB93,242, or CCK (1 μm) was measured on COS-7 cells expressing the WT-CCK2R or constitutively active mutants, E3.49A and LS.43A. In A, results of Ins-P production were expressed as percent of stimulation over basal value in presence of CCK alone. In B and F, values for Ins-P accumulation were normalized to a constant number of expressed receptors (1 pmol) and were expressed as -fold number of the basal value achieved with the wild-type CCK2R. Value 0 corresponded to Ins-Ps in cells were cells transfected with a pRFNeo non-coding vector. Routinely, the amount of inositol phosphates produced by 100,000 cells expressing the WT-CCK2R represented an average of 400–500 cpm, whereas that produced in Mock cells represented only 200–300 cpm.

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Competition binding experiments with radioiodinated CCK indicated that the two non-peptide ligands bound with a similar high affinity to the human wild-type CCK2R (Fig. 2D). Inhibition constants were: JB93,182, $K_i = 2.6 \pm 0.1 \text{ nM}$; JB93,242, $K_i = 2.4 \pm 0.2 \text{ nM}$; the Hill number was close to the unity in both cases (not shown).

Agonist activity of JB93,242 was detected and was significant in COS-7 cells transfected with amounts of plasmid as low as 0.1 $\mu$g/plate, whereas inverse agonism of JB93,182 required transfection of 1.0 $\mu$g of plasmid/plate, resulting in CCK2R expression levels ranging from 5 to 7 pmol/10^6 cells and basal values for Ins-P productions that were related to these CCK2R expression levels (13) (Fig. 2E). Inverse agonist activity of JB93,182 seen on wild-type CCK2R was confirmed by its inhibitory effect on constitutively active E3.49A mutant previously shown as oncogenic (17) and on the robust constitutively active L5.43A mutant recently identified (14) (Fig. 2F). Conversely, JB93,242 acted as a partial agonist on the two mutated CCK2R. All the effects observed at maximal concentrations of non-peptide ligands were dose-dependent (not illustrated).

In Silico Docking of JB93,242 and JB 93,182 to the Modeled CCK2R°—To identify binding site of the two compounds, in a first step automated docking of JB93,242 to a modeled CCK2R° was performed. The docking program provided 7 clusters for JB93,242, the most populated one ranking first in the score list (36 occurrences and $-17.09 \text{ kcal/mol}$). This orientation was selected as representative of the mode of binding based on the following considerations; overlapping with the bioactive part of CCK, namely the C-terminal tetrapeptide (Trp-Met-Asp-Phe-amide), and mimicking some of the structural determinants important for binding and activity of CCK (18–20). As shown in Fig. 3, in the selected solution the indole moiety overlapped the tryptophan of CCK, the phenylalanine ring partially overlapped the methionine and phenylalanine of CCK, and the dicarboxyphenyl overlapped Asp of CCK in a manner so that one of the carboxylate groups faced the guanidinium group of Arg-6.58. Automated docking of JB93,182 in CCK2R° provided 7 clusters. Among them, the second in the score and the most populated (64 occurrences and $-15.77 \text{ kcal/mol}$) corresponded to that obtained with JB93,242.

Comparison of the Refined CCK2R-Ligand Complexes Obtained from Molecular Dynamics Simulations—We anticipated that the way the two ligands precisely interact with the CCK2R° or the CCK2R° may provide explanation of their distinct activities. For this purpose, all complexes obtained from automated docking were submitted to 1 ns of molecular dynamics simula-

![FIGURE 3. Superposition of the selected docking solution of JB93,242 non-peptide ligand with CCK taken from CCK2R°-CCK] complex. JB93,242 and CCK are displayed as sticks, and the carbon atoms are colored in cyan and pink, respectively.](image)

![FIGURE 4. Serpentine representation of the CCK2R showing amino acids involved in the binding site of CCK, JB93183 or JB93,242 and/or in activation.](image)

![FIGURE 5. Schematic representation of the proposed binding sites for the non-peptide ligands in the CCK2R°. For simplicity, only the carbon traces for CCK2R are shown. Some of the protein residues relevant to the binding site have been labeled, and their side chains are shown as sticks. Left, binding site for JB93,242. Carbons of ligand and protein are colored in cyan and blue, respectively. Right, binding site for JB93,182. Carbons atoms of ligand and protein are colored in pale pink and magenta, respectively. Relevant hydrogen bonds are shown as dotted lines.](image)
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tion in presence of lipids. By doing so, the overall architecture of the CCK2R was preserved for the whole length of simulation for all the complexes (not illustrated).

Analysis of the CCK2R°-JB93,182 and CCK2R°-JB93,242 complexes revealed that the final location of JB93,182 and JB93,242 in the binding pocket and interacting residues was different at the end of the simulation period (Figs. 4 and 5). In the CCK2R°-JB93,182 complex, the ligand was maintained in its initial docking location, and the indole group of JB93,182 was located in an area formed by Tyr-1.39, Thr-2.61, and Tyr-7.43. It established π-π interactions with Tyr-7.43, hydrophobic interactions with the methyl group of Thr-2.61, and it donated a hydrogen bond to the hydroxyl oxygen of Tyr-1.39 or to the hydroxyl oxygen of Tyr-7.43. The dicarboxyphenyl ring was situated close to the second extracellular loop and to the extracellular ends of helices III, IV, and VI, and the two carboxylate groups interacted with Arg-6.58 and Tyr-4.60. Finally, the phenyl ring of JB93,182 was located in another sub-pocket constituted by residues from helices III, VI, and VII and established van der Waals contacts with Met-3.32 and Val-3.36, Trp-6.48, Val-6.51, and Tyr-6.52, and His-7.39. Importantly, Trp-6.48, a crucial residue for CCK2R° to CCK2R* conversion displayed a clear stacking interaction with the phenyl ring of JB93,182.

In contrast to JB93,182, the partial agonist JB93,242 slightly moved within the binding cavity of the CCK2R° during the molecular dynamics simulation. At its final location the indole moiety of JB93,242 established aromatic interactions with Tyr-7.43 and His-7.39, and the methyl of the ligand interacted with Thr-2.61 methyl. As a consequence of ligand displacement, the distance between the indole moiety of JB93,242 and helix II of the CCK2R° was different from that observed in the simulated CCK2R°-JB93,182 complex (distance between the indole nitrogen of the ligand and the Cα carbon of Thr-2.61 = 6.93 Å in complex with JB93,242 versus 5.25 Å in the complex with JB93,182). Additionally, the phenylalanine ring of JB93,242 was located in a region closer to residues Met-4.57, Tyr-4.60, Leu-5.46, and Leu-5.42 (Figs. 6 and 7).

Therefore, after molecular dynamics simulation, JB93,182 and JB93,242 occupied slightly different binding sites within the binding cavity of the CCK2R° (Fig. 7). This was due to different anchoring modes of the two ligands to Thr-2.61. Interactions bond between Thr-2.61 and JB93,182 maintained the ligand in a position so that its phenyl ring was in a clear stacking interaction with Trp-6.48 of the CCK2R°. This likely contributed to the stabilization of this key residue within the binding pocket and, as a consequence, prevented the rotation of helix VI which is required for CCK2R° to CCK2R* conversion. Conversely, in the presence of JB93,242, which did not display interaction with Trp-6.48, CCK2R activation could occur.

The position occupied by JB93,242 in the CCK2R° after molecular dynamics simulations was similar to that in the CCK2R° (Fig. 6). The binding site of JB93,242 in the CCK2R° was located between helices II, III, IV, V, and VI. The pocket was formed by Val-3.36, Met-4.57, Tyr-4.60, Leu-5.46, Val-6.51, and Tyr-6.52 interacting with the phenyl ring of the ligand. Tyr-4.60 established aromatic interactions with one side of the ligand dicarboxyphenyl ring, whereas the other side faced to Arg-6.58. This latter residue also paired through hydrogen bonding interactions with the indole carbonyl. The two carboxylate groups were involved in hydrogen bonding interactions with Arg-215 and Lys-128. Finally the indole group of the ligand was located in the same hydrophobic/aromatic area formed by Thr-2.61, Met-3.32, His-7.39, and Tyr-7.43. It is worthy of note that although the phenyl ring of the non-peptide ligand was sited in the same cavity as that of CCK-Phe, they did not totally overlap, the Phe of CCK being more deeply inserted than the phenyl ring of JB93,242. An attempt to dock the inverse agonist...
The effect of CCK2R mutations was determined as described under “Experimental Procedures” using radiolabeled CCK. Mutation factors (F) were calculated as Kᵢ (mutated CCK2R)/Kᵢ (WT-CCK2R). Results represent the means ± S.E. of 3–8 independent experiments performed in duplicate on separately transfected COS-7 cell batches. In the last column Fmut182 versus Fmut242 were compared. Differential effects of mutations on the affinity of JB93182 and JB93242 were observed for the two ligands. The same consistency was observed with different effects of W6.48A mutation on the binding affinity of JB93,182 and JB93,242, respectively, whereas the T2.61S mutation decreased by 10.1- and 14.1-fold the affinity of the partial agonist JB93,242. Conversely W6.48A mutations diminished by 7.8- and 3.1-fold that of the inverse agonist JB93,182. Conversely W6.48A mutations diminished by 7.8- and 3.1-fold that of the inverse agonist JB93,182. The Y4.60A mutation caused 6.3- and 9.9-fold decreases. The Y4.60A mutation of residues Val-3.36, Met-4.57, Tyr-4.60, Trp-6.48, Leu-5.42, Tyr-6.52, His-7.39, and Tyr-7.43, NS, not significant.

**Table 2**

| Receptor          | JB93,182 | JB93,242 | Fmut182 vs. Fmut242 |
|-------------------|----------|----------|---------------------|
| Kᵢ nM            | Fmut182  | Fmut242  |                     |
| WT                | 2.60 ± 0.08 | 1.0      | 2.5 ± 0.2           | 1.0       | NS         |
| Y1.39F            | 5.8 ± 0.03 | 2.3      | 4.0 ± 0.2           | 1.6       | NS         |
| T2.61A            | 26.4 ± 0.02 | 10.1     | 34.2 ± 1.4          | 14.1      | NS         |
| T2.61S            | 16.3 ± 0.04 | 6.3      | 24.3 ± 5.9          | 9.9       | NS         |
| T2.61F            | 63.6 ± 21.0 | 24.5     | 103 ± 41.0          | 42        | NS         |
| V3.36A            | 4.90 ± 0.3 | 1.9      | 0.95 ± 0.12         | 0.4       | NS         |
| M4.57A            | 30.60 ± 0.05 | 11.8     | 15.1 ± 2.0          | 6.2       | NS         |
| Y4.60A            | 8.0 ± 1.6  | 3.1      | 30.9 ± 3.2          | 12.6      | NS         |
| H207A             | 0.86 ± 0.20 | 0.3      | 1.7 ± 0.5           | 0.7       | NS         |
| L5.42A            | 2.90 ± 0.02 | 1.1      | 0.9 ± 0.11          | 0.4       | NS         |
| L5.43A            | 0.00 ± 0.04 | 0.3      | 1.0 ± 0.11          | 0.4       | NS         |
| T6.48A            | 20.2 ± 4.3 | 7.8      | 3.9 ± 0.3           | 1.6       | NS         |
| V6.51A            | 3.40 ± 0.09 | 1.3      | 2.5 ± 0.4           | 1.0       | NS         |
| Y6.52A            | 0.38 ± 0.05 | 0.1      | 0.77 ± 0.04         | 0.3       | NS         |
| N5.55A            | 2.7 ± 0.6  | 1.0      | 1.5 ± 0.33          | 0.6       | NS         |
| R6.58A            | 29.7 ± 10.6 | 11.4     | 37.8 ± 13.7         | 15.4      | NS         |
| H7.39A            | 230 ± 2.0 | 8.8      | 6.2 ± 0.4           | 2.5       | NS         |
| Y7.43A            | 290 ± 0.3 | 11.1     | 185 ± 3.1           | 7.5       | NS         |

* Significance at 0.01 < p < 0.05 comparing Kᵢ on CCK2R mutants versus wild-type CCK2R or Fmut182 versus Fmut242.

J39,182 in the CCK2R* resulted in an identical ligand location (not shown).

**Experimental Validation of JB93,182 and JB93,242 Binding Sites**—To verify in silico docking of the two non-peptide ligands into the CCK2R, competition binding experiments were carried out with CCK2R mutants. Interactions suggested in silico between non-peptide ligands and residues Thr-2.61, Val-3.36, Met-4.57, Tyr-4.60, Trp-6.48, Arg-6.58, and Tyr-7.43 of the CCK2R were confirmed by the effects caused by the mutation of these amino acids on the affinity of the ligands (Table 1). T2.61A mutation decreased by 10.1- and 14.1-fold the affinity of JB93,182 and JB93,242, respectively, whereas the T2.61S mutation caused 6.3- and 9.9-fold decreases. The Y4.60A mutation reduced by 12.6-fold the affinity of the partial agonist JB93,242 and only by 3.1-fold that of the inverse agonist JB93,182. Conversely W6.48A mutations diminished by 7.8-fold the affinity of JB93,182 and only by 1.6-fold the affinity of JB93,242. These different shifts in affinity fit with the view deduced from both Ins-P production and in silico docking data showing that JB93,182 display a better propensity to stabilize the CCK2R*, whereas JB93,242 displays a higher propensity to stabilize the CCK2R*. Indeed, in the JB93,242-CCK2R* complex, the carboxyphenyl ring of the ligand is in stacking interaction with that of Tyr-4.60, whereas in the JB93,182-CCK2R* complex such an interaction is not present. In the same manner, docking and molecular simulations showed that Trp-6.48 interacts with JB93,182 but not JB93,242, which agrees with the different effects of W6.48A mutation on the binding affinity of the two ligands. The same consistency was observed with mutant H7.39A. Pharmacological features of all mutants studied are reported in Table 2.
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FIGURE 8. Efficacy of non-peptide ligands JB93,182 and JB93,242 to stimulate production of inositol phosphates in cells expressing mutants of the CCK2R. In A and B experiments validate that the anchoring mode of non-peptide ligands to Thr-2.61 of the CCK2R is responsible for their distinct pharmacological features. Ins-P production was measured on non-stimulated cells (basal) or on stimulated cells with 1 μM CCK or 1 μM non-peptide ligands to Thr-2.61 of the CCK2R. Histograms in A show dependence of JB93,182 and JB93,242 activities on the type of CCK2R residues at positions 2.61 and 6.48. Histograms in B show efficacy of JB93,262 (see Fig. 1 for structure) on WT-CCK2R and CCK2R mutated at position 2.61. Histograms in C show efficacy of JB93,182 and JB93,242 on mutants of the CCK2R listed in Table 1. For expression of results, values for Ins-P accumulation were normalized to a constant number of expressed receptors (1 pmol) and were then expressed as fold number of the basal value achieved with the wild-type CCK2R. Value 0 corresponded to Ins-Ps in cells whereas cells transfected with a pRFNeo non-coding vector. All effects were dose-dependent (not illustrated).

We anticipated that this in silico method should be able to account for distinct activities of JB93,182 and JB93,242 based on the better ability of the molecules to stabilize the CCK2R* or the CCK2R°, respectively. We, therefore, studied CCK2R* to CCK2R° conversion in the presence of the two ligands.

In the presence of the partial agonist JB93,242, the conversion was similar to that studied in its absence, the rotation of Trp-6.48 being a consequence of a total tilting and rotation of helix VI and coordinated motion of helix V (14). But interestingly, in the presence of JB93,242, a force constant of 0.25 kcal mol⁻¹ was sufficient to complete CCK2R° to CCK2R* conversion. As a criterion for the tilting and rotation of helix VI, the distance between the Cα carbons of Val-6.51 and Met-3.32 was monitored, and torsion angles of Trp-6.48 (χ1 and χ2) were measured to account for the perpendicular to parallel transition of Trp-6.48 with respect to the membrane plane. Fig. 9 shows that after 244 ps of simulation in the presence of JB93,242, the Cα distance between Val-6.51 and Met-3.32 suddenly reached the value corresponding to that seen in the active conformation (8.5–9.0 Å). As a consequence, at this time Val-6.51 occupied the initial position of Trp-6.48 of helix VI, which adopted by the end of the dynamics (500 ps), at 420 ps, the Trp-6.48 achieved the desired location outside the binding pocket. This parallel disposition was maintained until the value corresponding to that seen in the active conformation (8.5–9.0 Å). As a consequence, at this time Val-6.51 occupied the initial position of Trp-6.48 of helix VI, which adopted by means of an inward movement a parallel disposition corresponding to the first change in the χ2 torsion angle (Fig. 9B). In this parallel position Trp-6.48 still faced helix III within the binding pocket. This parallel disposition was maintained until the end of the dynamics (500 ps), but at 420 ps, the Trp-6.48 achieved the desired location outside the binding pocket at the same time as the χ1 torsion angle reached the t value (Fig. 9B).

In the case of the inverse agonist JB93,182, the conformational changes were delayed. The stacking interactions in the CCK2R° between Trp-6.48 and the phenyl ring of JB93,182 ligands on a constitutively active W6.48A CCK2R mutant. As expected and shown on Fig. 8A, both ligands stimulated Ins-P production in cells expressing W6.48A mutant.

Finally, efficacy of JB93,182 and JB93,242 was tested on all mutants of the CCK2R shown in Table 1. Results on Fig. 8C indicate that the pharmacological features of the two non-peptide ligands, namely inverse agonism or partial agonism, were not conserved on all mutants. For example, JB93,182 remained an inverse agonist on constitutively active mutants V3.36A and M4.57A but was a neutral agonist on constitutively active mutant L5.42A. Efficacy of the partial agonist JB93,242, which reached 50% that of CCK on the wild-type CCK2R, was enhanced on mutants Met-4.57, L5.42A, and Y7.43A. Conversely, agonist activity of JB93,242 was diminished on mutant R6.58A and almost abolished on mutant Tyr-6.52. It is worthy to note that residues Val-3.36, Met-4.57, Tyr-4.60, Leu-5.42, Tyr-6.52, Arg-6.58, and Tyr-7.43 are involved in the binding pocket of the two non-peptide ligands, as supported by in silico docking and binding data, and were shown to participate to stabilization of either active or inactive conformation of the CCK2R (Table 1 and Ref. 14).

Targeted Molecular Dynamics Simulations Confirmed Distinct Activities of JB93,182 and JB93,242—We have previously validated the use of TMD to study at atomic levels how the inactive to active conversion of CCK2R proceeds, and we have shown that application of a force constant of 0.50 kcal mol⁻¹ over 500 ps was sufficient to achieve it in a lipid environment. We anticipated that this in silico method should be able to account for distinct activities of JB93,182 and JB93,242 based on the better ability of the molecules to stabilize the CCK2R* or the CCK2R°, respectively. We, therefore, studied CCK2R* to CCK2R° conversion in the presence of the two ligands.

To verify whether different positioning of JB93,182 and JB93,242 relative to Trp-6.48 (as the result and different anchoring modes to Thr-2.61) accounted for inverse agonist and partial agonist activities, respectively, we measured the activity of the two

tide ligands. We first exchanged Thr-2.61 for a bulky residue, namely Phe. On mutant T2.61F, JB93,182 and JB93,242 showed weak and strong partial agonist activities, respectively. To further assess that the distance between Thr-2.61 side chain and the indole of the non-peptide ligands was critical for CCK2R activation, we tested JB93,262 in which the hydrogen of the indole has been substituted by a butyl group. As shown on Fig. 8B, this compound behaved as a full agonist, stimulating Ins-P production through the wild-type CCK2R, T2.61A, and T2.61S mutants with efficacies similar to that of CCK. However, this butyl-substituted compound only slightly stimulated the T2.61F mutant. Such results support the view that an increase of steric hindrance to a certain level in the contact region between position 2.61 in the CCK2R and the non-peptide ligands enhanced activity of the CCK2R.
Molecular Basis of Partial Agonism and Inverse Agonism

In the current work we explain the different pharmacological behaviors of JB93,182 and JB93,242 by considering the binding mode of the two compounds in the validated modeled structure of the CCK2R binding pocket in inactive and active conformations together with the mechanism of CCK2R activation that we have recently delineated (14). Indeed, data from in silico and laboratory experiments strongly supported that the indole nitrogen of JB93,182 donated a hydrogen bond to Thr-2.61. This hydrogen bonding interaction has an anchoring role so that the phenyl ring of the ligand stacked over Trp-6.48 of the CCK2R. This last stacking aromatic interaction maintains the CCK2R in the CCK2R* conformation by preventing rotation of helix VI, a motion that is required for CCK2R* to CCK2R conversion (14). Methyl substitution in the partial agonist JB93,242 not only introduced a steric repulsion, as previously proposed (15), but also eliminated the hydrogen bonding capability of the indole nitrogen with Thr-2.61, causing displacement of the ligand in the CCK2R* binding pocket. Interestingly, JB93,242 finally adopted a location in the CCK2R* similar to that found in the active conformation (CCK2R*). The displacement of JB93,242 placed its phenyl moiety close to helix V. In this location the phenyl ring of the ligand could not interact with Trp-6.48. The type of movement of the partial agonist in the CCK2R is in line with the motion of the β-ionone ring toward the transmembrane domain V of rhodopsin during 11-cis to all-trans isomerization of the retinal, which produces activation of the receptor (23). It has been proposed that the translation eliminates the interaction with Trp-6.48 and allows an outward movement of this side chain to adopt the parallel disposition. Conversely, it was shown that interaction between retinal, the natural inverse agonist of rhodopsin, and Trp-265 contributes to maintenance of rhodopsin in the inactive state in the dark, whereas disruption of this interaction under light activation allows Trp-265 and helix VI to rotate and adopt the active conformation (24). Our proposal that inverse agonist JB93,182 maintains CCK2R in its inactive state through interaction with Trp-6.48 agrees with the data on retinal and rhodopsin. As another example, midazolam, an inverse agonist at the thyrotropin-releasing hormone receptor type 1, was reported to exert its pharmacological effect through a direct interaction with Trp-279 in the TRH-R1, which is equivalent to Trp-6.48 in the CCK2R (25).

The design of JB93,182 and JB93,242 was based on data from fluorescence structural analysis of gastrin carried out in a water environment and from molecular modeling studies. It was found that the distance between the two aromatic rings in the C-terminal tetrapeptide of gastrin (Trp and Phe) was of 5–7 Å, a distance that could be attained if the peptide backbone adopted the conformation of a 3_10 helix. It was further postulated that the designed ligands should mimic the two aromatic rings of gastrin, which are crucial for the binding affinity of the tetrapeptide. This view agrees with our data indicating that Trp

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and Phe of CCK and interacting residues in the CCK2R are involved in CCK2R activation (14, 18). However, in our modeled CCK2R-CCK complex, CCK did not adopt a \(3_{10}\) helix conformation, and the distance between Trp and Phe was twice that observed in water (26). In CCK2R-bound JB93,242, the distance between the centroids of the two aromatic moieties, the indole and the phenyl ring, is of about 9.5 Å. As can be seen in Fig. 3, none of the two aromatic moieties totally overlapped CCK-Trp and CCK-Phe. Interestingly, the N-methyl group in JB93,242 and the butyl group in JB93,262 overlapped the indole of CCK-Trp and possibly mimicked it. The fact that the \(N\)-butyl substituent relative to \(N\)-methyl improved overlap between the aliphatic chain and CCK-Trp is in perfect agreement with the increased efficacy of the JB93,262 relative to JB93,242.

To conclude, although no actual structure of CCK2R is available, we have succeeded in explaining at an atomic level the mechanism of inverse agonism and partial agonism of two high affinity, high selectivity, very similar non-peptide ligands of the mechanism of inverse agonism and partial agonism of two high affinity, high selectivity, very similar non-peptide ligands of the CCK2R that differ by the absence or presence of a methyl group on the indole moiety. We believe that the success of our approach is due to the "symbiosis" between in silico and laboratory experiments.

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