Molecular Mechanism Underlying Partial and Full Agonism Mediated by the Human Cholecystokinin-1 Receptor*

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The cholecystokinin-1 receptor (CCK1R) is a G protein-coupled receptor (GPCR) that regulates important physiological functions. As for other GPCRs, the molecular basis of full and partial agonism is still far from clearly understood. In the present report, using both laboratory experiments and molecular modeling approaches, we have investigated the partial agonism mechanism of JMV 180, on the human CCK1R. We first predicted that efficacy of the CCK1R to activate phospholipase C is dependent on the correct orientation of the C-terminal end of peptidic ligands toward residue Phe³³⁰ of helix VI. We have previously reported that a single mutation of Met¹²¹ (helix III) markedly reduced the receptor-mediated inositol phosphate production upon stimulation by CCK. Computational simulations predicted that residue 121 affected orientation of the C-terminal end of CCK, thus suggesting that the molecular complex with a reduced inositol phosphate production observed with the mutated CCK1R resembles that resulting from binding of JMV 180 to the WT-CCK1R. Pharmacological, biochemical, and functional characterizations of the two receptor-ligand complexes with decreased abilities to signal were carried out in different cell types. We found that they presented the same features, such as total dependence of inositol phosphate production to Goα expression, single affinity of binding sites, insensitivity of binding to non-hydrolyzable GTP, absence of GTPγ[S³⁵] binding following agonist stimulation, similarity of dose-response curves for amylase secretion, and incapacity to induce acute pancreatitis in pancreatic acini. We concluded that helices VI and III of the CCK1R are functionally linked through the CCK1R agonist binding site and that positioning of the C-terminal end of peptide agonists toward Phe³³⁰ of helix VI is responsible for extent of phospholipase C activation through Goα coupling. Given the potential therapeutic interest of partial agonists such as JMV 180, our structural data will serve for target structure-based design of new CCK1R ligands.

* This work was supported by the Association pour la Recherche sur le Cancer (Grants 4439 and 3282) and by the Ligue contre le Cancer.
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spect to CCK1R-mediated stimulation of inositol phosphate production, JMV 180 is a partial agonist, but the molecular mechanism, at the receptor level, underlying this partial agonism remains unknown.

In the recent period, we have devoted much effort toward mapping of the human CCK1R binding site for the full agonist CCK, because we reasoned that knowledge of receptor binding site is required for the understanding of ligand action. In the current work, we investigated the molecular mechanism of JMV 180 partial agonism on the CCK1R using a multidisciplinary approach. We found that helices VI and III of the CCK1R are functionally linked through the CCK1R agonist binding site, and positioning of the C-terminal ends of peptidic agonists toward Phe330 of helix VI is responsible for the extent of phospholipase C activation through Goαi coupling.

**EXPERIMENTAL PROCEDURES**

**Materials**—The C-terminal nonapeptide analogue (Nle)-CCK-9, (Met)-CCK-9, JMV 172, and JMV 180 were synthesized as described previously (20, 21). [125I]NaI, [125I]-Bolton-Hunter reagent, N-succinimidyl [2,3-3H]propionate, and [3H]-inositol (5 µCi/ml) were from American Radiolabeled Chemicals. Labeled peptides were purified and prepared as described previously (22). CCKAR−/− mice were generated as described previously (23). Collagenase (grade) was purchased from Worthington. Dulbecco's modified Eagle's medium (DMEM) was used for cell culture, and RPMI 1640 medium (containing 1% BSA at 37 °C) was used for enzymatic digestion. NaOH was from Biochemicals (Freehold, NJ). InfinityTM amylase reagent was from Boehringer-Mannheim. Recombinant human CCK1R cDNAs were cloned into the shuttle vector pcDNA3.1(+) vector as template. Mutations were confirmed by automated sequencing of both cDNA and genomic DNA (Pharmacia-LKB Genecode). 

**Site-directed Mutagenesis and Transfection of COS-7 Cells**—Mutant receptor cDNAs were constructed by oligonucleotide-directed mutagenesis (QuickChangeTM site-directed mutagenesis kit, Stratagene) using the human CCK1R cDNAs cloned into pRFNeo vector as template. Mutations were confirmed by automated sequencing of both cDNA strands. COS-7 cells (1.6 × 10^6) were plated onto 10-cm culture dishes and grown in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum. After overnight incubation, cells were transiently transfected with 0.1–0.5 µg/plate of pRFNeo vectors containing the cDNA for the wild-type or mutated CCK1R, using a modified DEAE-dextran method. Cells were transfected to 24-well plates at a density of 20,000–80,000 cells/well 24 h after transfection, on the transfected mutant and experiment to be performed.

**Computer Modeling**—The model of the CCK1R was built as described previously (24). A starting conformation of the JMV 180 molecule was first built and refined by 10,000 steps of energy minimizations using Insight II software (Accelrys, San Diego, CA). The consistent valence forcefield was used in the Discover Accelrys molecular mechanics and molecular dynamics program. This starting conformation was next used for a simulated annealing conformational sampling using molecular dynamics in vacuo. The dielectric constant used here was a distance-dependent one to stimulate roughly the electrostatic shielding due to the solvent. The simulated annealing method used consisted of 100 loops of slow cooling, each one leading to a low energy conformer. Each loop starts by fixing the temperature to 1,000 K, followed by 5,000 molecular dynamics steps of 1 fs each. The temperature was then decreased by steps of 100 K; by decreasing the temperature this way every 5,000 molecular dynamics steps, after 40,000 steps the temperature of the system correspond to 300 K. The final conformation obtained at the end of this process was energy-refined and after storage was used to start a new simulation at 1,000 K with a slow cooling as described above. This procedure produced 100 minimized and different conformations of JMV 180. All these 100 conformers were used for the docking procedure using the GOLD algorithm (25). The "chem score" scoring function was used. GOLD ranked the conformational samples in such a way that we decided to examine only the 10 top solutions. From these 10 solutions, only 2 were found in accordance with pharmacological results (see "Results"). The two resulting complexes were submitted to annealing molecular dynamics calculations followed by energy minimization, the backbone of the receptor being first fixed. Then, all the receptor-ligand complex was submitted to energy minimization. For JMV 172 and (Met)-CCK, models of the peptide and of the complexes were obtained as described here for JMV 180. The whole modeling was carried out with Accelrys software Insight II modules of a Silicon Graphics Onyx station.

**Analysis of Amylase Secretion and Pancreatic Acinar Cells Morphology**—Infected acini were washed and resuspended in KREBS buffer containing 1% BSA at −1 mg/ml. Aliquots of 2 ml were incubated with an increasing concentration of CCK or JMV 180 for amylose assay and with a high concentration of CCK (10−8 m) or JMV 180 (3 × 10−5 m) for morphology studies, at 37 °C for 30 min, under gentle shearing. Incubation was terminated by centrifugation of aliquots for 1 min at 400 rpm. The concentration of amylase in the medium was measured using InfintyTM amylase reagent (Sigma Diagnostics®). Results were expressed as percentage of maximal amylase release. For morphological studies, acinar pellets were fixed 20 h in 100% alcohol, 40% formaldehyde, and 17 m acetic acid (75/25/25), embedded in paraffin, hematoxylin and eosin stained, and visualized by optic microscopy.

**RESULTS**

JMV 180, a High Affinity Agonist of the CCK1R with a Low Intrinsic Activity—In COS-7 cells expressing the human CCK1R receptor, (Nle)-CCK and (Met)-CCK (Fig. 1) indistinguishably stimulated production of inositol phosphates with a potency (ED50) of 0.42 ± 0.10 nm and a maximal stimulation of 15- to 25-fold the basal value (Fig. 2). In the same cells, JMV 180-induced production of inositol phosphates reached a max-
and Y360F. On the other hand, I352A mutant bound [3H]JMV Q40N, L50F, L53A, C94L, M121A, M121Q, M121V, V125A, nM) but an efficacy that represented 75% of that of CCK (Fig. 2). Partial agonism of JMV 180 in COS-7 cells was maintained from 2 to 6.8 pmol/10⁶ cells (not illustrated), indicating that the mutants in response to JMV 180. However, most of these residues previously shown to be part of CCK1R binding site, on molecular recognition of JMV 180. Amino acids of the CCK1R residues previously shown to be part of CCK1R binding site (Table I). A first series of CCK1R mutants bound [3H]JMV 180 with identical or very similar affinities to the wild-type CCK1R (WT-CCK1R) (0.2 < Fmut < 5, Table I); these were W39A, Q40N, L50F, L50A, C94L, M121A, M121Q, M121V, Y125A, F218A, W326A, W326F, I329A, I329F, F330A, N333A, L356A, and Y360F. On the other hand, I352A mutant bound [3H]JMV 180 with a 18-fold decreased affinity compared with the WT-CCK1R. The third set of mutants, F107A, M195L, R197M, and R336M, did not bind [3H]JMV 180. These mutants were previously shown to respond to CCK with strong drops of potency as the mutated residues are involved in CCK1R binding site (Table II) (26, 28–30). Owing to structural homology between JMV 180 and CCK, defective or decreased [3H]JMV 180 binding to mutants F107A, M195L, R197M, R336M, and I352A can be interpreted as an involvement of those residues in the binding site of JMV 180. Additional experiments to evaluate contribution of these residues to recognition of JMV 180 were performed, by measuring inositol phosphate production induced by the mutants in response to JMV 180. However, most of the mutants did not produce sufficient amounts of inositol phosphates to allow accurate analysis (not illustrated). As a partial agonist, JMV 180 was able to inhibit CCK1R stimulations by the full agonist CCK. We took advantage of this behavior to evaluate contribution of mutated amino acids to recognition of CCK1R, amino acids involved in binding and activity are marked. The amino acids of the receptor and CCK are color-coded to indicate specific interactions between the two molecules. Extracellular and intracellular loops (RCL and ICL) are indicated.

**Table I**

| CCK1R       | Kd (nM) | Fmax (pmol/10⁶ cells) | Bmax (pmol/10⁶ cells) |
|-------------|---------|-----------------------|-----------------------|
| WT          | 9.3 ± 1.4 | 1.0                   | 22.5 ± 2.8             |
| W39A        | 12.0 ± 0.2 | 1.3                   | 18.6 ± 0.1             |
| Q40N        | 18.4 ± 0.6 | 2.0                   | 14.3 ± 0.3             |
| L50F        | 7.5 ± 0.4  | 0.8                   | 23.7 ± 0.3             |
| L53A        | 10.9 ± 1.9 | 1.2                   | 17.4 ± 0.6             |
| C94L        | 9.4 ± 2.3  | 1.0                   | 7.4 ± 0.8              |
| F107A       | No binding | No binding           | No binding             |
| M121A       | 3.1 ± 0.1  | 0.3                   | 9.8 ± 3.4              |
| M121Q       | 16.1 ± 4.7 | 1.7                   | 13.1 ± 2.7             |
| M121V       | 6.7 ± 0.6  | 0.7                   | 19.5 ± 3.4             |
| V125A       | 7.6 ± 1.6  | 0.8                   | 9.2 ± 1.5              |
| M195L       | No binding | No binding           | No binding             |
| R197M       | No binding | No binding           | No binding             |
| F218A       | 4.1 ± 1.3  | 0.4                   | 2.7 ± 0.3              |
| W326A       | 40.6 ± 11.2 | 4.4                 | 12.4 ± 1.4             |
| W326F       | 17.4 ± 0.8  | 1.9                   | 14.4 ± 1.4             |
| I329A       | 33.7 ± 6.9 | 3.6                   | 23.9 ± 3.3             |
| I329F       | 8.3 ± 0.6  | 0.9                   | 20.1 ± 3.1             |
| F330A       | 2.3 ± 0.2  | 0.25                  | 7.2 ± 0.9              |
| N333A       | 9.5 ± 1.4  | 1.0                   | 25.4 ± 3.2             |
| R336M       | No binding | No binding           | No binding             |
| I352A       | 165.2 ± 40.2 | 17.8               | 25.0 ± 3.1             |
| L356A       | 11.2 ± 1.6 | 1.2                   | 17.9 ± 4              |
| Y360F       | 5.8 ± 1.5  | 0.6                   | 17.4 ± 4.7             |

[FIG. 2. Partial agonism of JMV 180 on inositol phosphates production induced by CCK1R. Inositol phosphates production was measured upon stimulation by (Nle)-CCK, (Met)-CCK, ns-CCK, and JMV 180, on COS-7 cells expressing the wild-type human CCK1R. Results are expressed as percentage of maximal inositol phosphate production obtained with (Nle)-CCK. Efficacies of non-sulfated CCK and of JMV 180 were 75% and 9.3% relative to (Nle)-CCK. ED50 values were as follows: (Nle)-CCK/(Met)-CCK, 0.42 ± 0.10 nM; JMV 180, 17.8 ± 6.3 nM; non-sulfated CCK, 215 ± 18 nM. Results are the mean ± S.E. from a least three individual experiments performed in duplicate.

Partial agonism of JMV 180 on inositol phosphates production induced by JMV 180 should be due to the inability of this compound to inhibit CCK-induced production of inositol phosphates to allow accurate analysis (not illustrated). As a partial agonist, JMV 180 was able to inhibit CCK1R stimulations by the full agonist CCK. We took advantage of this behavior to evaluate contribution of mutated amino acids to recognition of CCK1R. Amino acids of the CCK1R residues previously shown to be part of CCK1R binding site, on molecular recognition of JMV 180. Amino acids of the CCK1R binding site for CCK are shown on the serpentine representation of the CCK1R (Fig. 3). Binding studies with labeled JMV 180 allowed identification of three main categories of mutants (Table I). A first series of CCK1R mutants bound [3H]JMV 180 with identical or very similar affinities to the wild-type CCK1R (WT-CCK1R) (0.2 < Fmut < 5, Table I); these were W39A, Q40N, L50F, L50A, C94L, M121A, M121Q, M121V, Y125A, F218A, W326A, W326F, I329A, I329F, F330A, N333A, L356A, and Y360F. On the other hand, I352A mutant bound [3H]JMV 180 with a 18-fold decreased affinity compared with the WT-CCK1R. The third set of mutants, F107A, M195L, R197M, and R336M, did not bind [3H]JMV 180. These mutants were previously shown to respond to CCK with strong drops of potency as the mutated residues are involved in CCK1R binding site (Table II) (26, 28–30). Owing to structural homology between JMV 180 and CCK, defective or decreased [3H]JMV 180 binding to mutants F107A, M195L, R197M, R336M, and I352A can be interpreted as an involvement of those residues in the binding site of JMV 180. Additional experiments to evaluate contribution of these residues to recognition of JMV 180 were performed, by measuring inositol phosphate production induced by the mutants in response to JMV 180. However, most of the

[FIG. 3. Simplified representation of the CCK1R and of its agonist binding site. Using a serpentine representation of the human CCK1R, amino acids involved in binding and activity are marked. The amino acids of the receptor and CCK are color-coded to indicate specific interactions between the two molecules. Extracellular and intracellular loops (RCL and ICL) are indicated.]
binding sites of the two peptides and also reflects different chemical structures of the two ligands. All these results indicate that residues Phe107, Met195, Arg197, Arg336, and Ile352 are part of the CCK1R binding site for JMV 180.

Dynamic Docking of JMV 180 in the Modeled CCK1R—Modeled structures of JMV 180 molecule were obtained and docked within the receptor as described under “Experimental Procedures.” At the end of modeling and docking procedures, among the 10 best JMV 180/H18528CCK1R complexes obtained, two models positioned JMV 180 in accordance with site-directed mutagenesis results demonstrating importance of residues Phe107, Met195, Arg197, Arg336, and Ile352 for ligand recognition. In the two models, JMV 180 presented very similar positions, but the orientation of the C-terminal phenyl group was remarkably different, suggesting that this ligand may adopt two conformations within the receptor groove (Fig. 5, A and B). Trp of JMV 180 approximated Phe107 (distance: 2.87 Å); the Tyr aromatic ring was at 6.70 Å from Met195 sulfide, a distance that was ideal for quadrupole-quadrupole interactions (31); the sulfate moiety of JMV 180 and the penultimate Asp carboxylate residue of JMV180 paired with Arg197 and Arg336 guanidium side chains, respectively (distances: 2.36 and 1.90 Å). The comparison of CCK/H18528CCK1R and JMV 180/H18528CCK1R complexes (Fig. 5, A–C) revealed very similar positions of the two peptides in the receptor groove, except for their C-terminal moieties. Indeed, in the CCK/CCK1R complex, the phenyl side chain of the

![Image](image-url)

**FIG. 4.** Effects of CCK1R mutations on JMV 180 potency to inhibit CCK-stimulated production of inositol phosphates. COS-7 cells transiently transfected with the wild-type- or mutated CCK1R, were stimulated by a concentration of CCK fixed at the EC50 for each mutant. Inhibition of inositol phosphate production in the presence of increasing concentration of JMV 180 was then studied. Results are expressed as percentage of CCK-induced inositol phosphates production with each mutant. The mutation factors ($F_{\text{mut}}$) were calculated as IC50 (mutated receptor)/IC50 (wild-type-CCK1R). Results are the mean ± S.E. from at least three experiments performed in duplicate.

| CCK1R        | Stimulation of Inositol Phosphates by CCK | Inhibition of CCK-stimulated Inositol Phosphates by JMV 180 |
|--------------|-----------------------------------------|-----------------------------------------------------------|
|              | EC50 ($\mu$M) | $F_{\text{mut}}$ | Ref. | IC50 ($\mu$M) | $F_{\text{mut}}$ |
| F107A        | 3140 ± 232   | 4500 | 24 | 1104 ± 225 | 16 |
| M195L        | 25.5 ± 0.3   | 54  | 28 | 1414 ± 183 | 20 |
| R197M        | 1480 ± 292   | 3150| 30 | 8833 ± 1229| 127|
| W326A        | 5.7 ± 0.1    | 4.3 | 26 | 403 ± 144  | 6  |
| I329F        | 608 ± 148    | 468 | 26 | 27 ± 4.4   | 0.4|
| F330A        | 3.4 ± 0.7    | 2.6 | 26 | 16 ± 10   | 0.6|
| N333A        | 635 ± 171    | 1351| 29 | 164 ± 56  | 2.3|
| R336M        | 4370 ± 565   | 9300| 29 | 8654 ± 2395| 125|
| I352A        | 277 ± 12     | 213 | 26 | 656 ± 220 | 9.5|
| L356A        | 38 ± 18      | 29  | 26 | 31 ± 12  | 0.7|
| Y360F        | 42 ± 9       | 32  | 26 | 16.4 ± 4.8 | 0.5|

| *a* Inositol phosphate.
C-terminal Phe residue formed a T-shape with the phenyl side chain of Phe\(^{330}\) of the CCK1R, this residue being itself in interaction with Phe\(^{218}\) and involved in a hydrophobic network comprising Ile\(^{352}\), Leu\(^{356}\), Phe\(^{360}\) (TM VII), Val\(^{125}\) (TM III), Phe\(^{218}\) (TM V), Trp\(^{326}\), Ile\(^{329}\), and Phe\(^{330}\) (TM VI). In the JMV 180/CCK1R complexes, the phenyl ethyl ester moiety was either stretched toward Phe\(^{330}\), as seen in CCK/H18528 CCK1R complex, or folded so that the aromatic ring pointed in the direction of Met\(^{121}\) side chain within a hydrophobic pocket formed by residues Met\(^{121}\) (TM III), Ile\(^{352}\), Leu\(^{356}\), Phe\(^{360}\) (TM VII), Leu\(^{54}\), Leu\(^{53}\) (TM I), and Cys\(^{34}\) (TM II) of the CCK1R.

Experimental Evidences That Different Positions of C-terminal Ends of JMV 180 and CCK in the CCK1R Binding Site Are Responsible for Their Distinct Efficacies—We further experimentally verified if the C-terminal aromatic rings of JMV 180 and CCK were differently positioned in the CCK1R binding site and if these distinct positions were responsible for their different intrinsic activities. We first incorporated a bulky residue (Phe) at position 329 in the CCK1R, to determine whether this mutation could affect recognition of CCK and JMV 180. The I329F mutant, which did not bind radiolabeled CCK, responded to CCK stimulations with a 600-fold decreased potency. This result, which can be explained by steric hindrance of Phe\(^{329}\), is in accordance with the stretched position of the C-terminal aromatic ring of CCK (Fig. 6A). In contrast, I329F mutant bound \([^{3}H]\)JMV 180 with an affinity that was similar to that of the WT-CCK1R (\(k_d = 8.3 \pm 0.6\) nM, versus 9.3 \(\pm\) 1.4 nM, \(n = 3\)) (Fig. 6B) thus supporting a strong preference for a folded conformation of JMV 180 in the CCK1R binding site.

In a previous work, a folding of the C-terminal aromatic ring of CCK toward Met\(^{121}\) side chain was found in the modeled (M121V)-CCK1R-CCK complex. Moreover, this complex was experimentally shown to lack any biological activity (26). In contrast, only a stretched conformation of CCK was found in fully active CCK-CCK1R complex. Therefore, we hypothesized that the relative position of the C-terminal aromatic ring of ligands within the bottom of the receptor pocket could determine the extent of CCK1R activation. In line with this view,
JMV 180-CCK1R complex in which JMV 180 displays a folded position of its C-terminal end (Fig. 5A) should correspond to an inactive one, whereas JMV 180-CCK1R complex exhibiting a stretched conformation of JMV 180 should represent the active one (Fig. 5B). Accordingly, the lack of preference for a stretched positioning of the JMV 180 C-terminal aromatic ring could explain both the absence of effect of Phe299 mutation on JMV 180 recognition and its weak efficacy.

To experimentally verify that the stretched conformation of bound-JMV 180 corresponded to the active one, we searched, in Jean Martinez’s library of peptides, for a peptide having a C-terminal phenyl group constrained in a stretched position. JMV 172, which has two phenyl moieties at its C-terminal end, fulfilled such a criterion (Fig. 1). The JMV 172-CCK1R complex obtained from modeling studies suggested that this peptide should mimic both conformations of JMV 180 at the same time, because one C-terminal aromatic ring was folded and the other was stretched and located toward Phe330 (Fig. 7A). JMV 172 inhibited CCK binding to COS-7 cells expressing the CCK1R with a 500-fold lower potency than JMV 180 (Fig. 7B). Therefore, constraining the phenyl moiety of a CCK ligand toward Phe330 increased the level of agonist efficacy on phospholipase C activation.

To test the critical role of Phe330 in CCK1R activation, we exchanged Phe330 for an Ala. This mutation abolished inositol phosphate responses of CCK1R to both JMV 180 and JMV 172 and diminished that to CCK by 43% (not illustrated). Thus, although weakly involved in binding, Phe330 strongly contributed to CCK1R activation. These results represent an additional support for a crucial role of the T-shape interaction between the C-terminal phenyl moiety of peptide ligands and Phe330 of CCK1R in the activation process of phospholipase-C.

Both CCK and JMV 180 Stimulate Production of Inositol Phosphate through Gαq Coupling—Agonist-directed trafficking of coupling, a concept whereby different agonists of the same GPCR can functionally induce distinct G protein coupling, have been documented for a number of GPCRs (32). Moreover, several types of phospholipase C have been identified, including β1, β2, β3, and β4, which can be stimulated either by Gαq or Gαi dimers (33). In the case of CCK1R, stimulation of phospholipase C via a pertussis toxin-sensitive (PTX) G protein was reported for cat gallbladder muscle (34), although this receptor is recognized to predominantly couple to Gαq (16). We therefore examined the possibility that partial agonism of JMV 180 could result from CCK1R coupling to a G protein distinct from that involved in CCK stimulations. Pretreatment with PTX of COS-7 cells expressing the WT-CCK1R did not significantly affect inositol phosphate responses to both CCK and JMV 180. In control experiments with COS-7 cells expressing the α2-adrenergic receptor reported to weakly stimulate phospholipase C through PT-sensitive G proteins, a 75% decrease of inositol phosphate production was observed in presence of PTX (not illustrated). This first series of experiments, showing that PTX-sensitive G proteins are not involved in human CCK1R-mediated activation of phospholipase C in COS-7 cells strengthened the hypothesis that both CCK and JMV 180 stimulate phospholipase C through coupling to a PTX-insensitive G protein, presumably Gαq.

We then examined if JMV 180-induced production of inositol phosphates was abolished by triple mutation of amino acids Lys308-Lys309-Arg310 located at the C-terminal end of the third intracellular loop of the CCK1R (Fig. 3). Indeed, the triple-basic sequence Lys308-Lys309-Arg310, that is conserved in the CCK2R, has been shown to be without any importance on Gαq/PLA2 activation, but its mutation abolished coupling to Gαq/PLC (35). As illustrated on Fig. 8A, triple mutation K308M/K309R/R310L fully inhibited ability of the CCK1R to induce inositol phosphate production in response to JMV 180 in COS-7 cells. This triple basal residues mutation altered response of the WT-CCK1R to CCK by 60% (Fig. 8A). In control binding experiments, the triple mutant bound radiolabeled CCK to a single and low affinity class of binding sites (Kd = 15.3 ± 0.7 nM, Bmax: 0.99 ± 0.04 pmol/106 cells) and had its affinity for radiolabeled JMV 180, which remained unaffected (Kd = 8.2 ± 0.6 nM, Bmax: 2.3 ± 0.2 pmol/106 cells for the triple mutant versus 9.7 ± 0.6 nM, Bmax: 13.0 ± 0.8 pmol/106 for the WT-CCK1R).

As a second approach, we expressed CCK1R in EF-88 cells derived from αq11−/− mice. Attempts to transfect CCK1R cDNA-containing plasmid using different protocols were unsuccessful, confirming previous reports (36). This led us to use adenoviral infections. In absence of co-infection with adenovirus encoding mouse Gαq, EF-88 cells expressing CCK1R did not respond to both JMV 180 and to CCK. In contrast, EF-88 cells co-expressing Gaq and CCK1R responded to both CCK and JMV 180, and efficacy of JMV 180 represented 61.1 ± 0.5% of that of CCK (Fig. 8B).

At this step of the study, our results strongly support that the full agonist, CCK, and the partial agonist, JMV 180, stimulate production of inositol phosphates through coupling to the same G protein, most likely Gαq. Moreover, they indicate that positioning of C-terminal ends of the peptide ligands in the CCK1R binding site is responsible for their different efficacies to stimulate inositol phosphate production.
Partial Agonism of (Met)-CCK on the M121V-CCK1R Involves a Molecular Mechanism Similar to Partial Agonism of JMV 180 on the WT-CCK1R—

A set of previous results suggested that activation of the CCK1R, and positioning of the C-terminal phenyl moiety of the peptidic ligand, can be regulated by exchanging residues in the vicinity of Met121 of the receptor (26). In particular, although M121V-CCK1R mutant did not respond to (Nle)-CCK, partial stimulation was recovered if (Met)-CCK was used. This led us to consider that partial agonism of (Met)-CCK on the M121V-CCK1R could involve a molecular mechanism similar to that of JMV 180 on the WT-CCK1R.

We compared molecular and functional features of (Met)-CCKM121V-CCK1R complex with that of JMV180-CCK1R complex. Automated docking of (Met)-CCK into the binding site of M121V-CCK1R yielded two complexes in which the C-terminal aromatic ring of CCK Phe was either in a stretched or in a folded conformation (not shown), as found for JMV180-CCK1R complex (Fig. 5, A and B). The second feature of JMV180-CCK1R complex was its inability to activate phospholipase C via a PTX-sensitive G protein but its functional coupling to Goq. Experiments with PTX-treated cells expressing M121V-CCK1R indicated that inositol phosphate production in response to (Met)-CCK was not affected (not illustrated). Triple mutation of Lys308-Lys309-Arg310 in M121V-CCK1R completely inhibited partial stimulation by (Met)-CCK (Fig. 9A). So, integrity of Lys308-Lys309-Arg310 motif appeared as identically critical for activation of both M121V-CCK1R by (Met)-CCK and CCK1R by JMV 180. Furthermore, as for stimulation of inositol phosphate production by JMV 180 in COS-7 cells expressing the WT-CCK1R, stimulation of M121V-CCK1R by (Met)-CCK was totally dependent on re-expression of Goq in EF-88 cells (Fig. 9B).

A third characteristic of JMV180-CCK1R complex, which is in good accordance with its coupling to Goq and known to be little sensitive to GTP (37), was reflected by binding of [3H]JMV 180 to COS-7 cells expressing the WT-CCK1R. Indeed, a single affinity class of binding sites was found, confirming previous findings of another group, with a peptide analogous to JMV 180 (38). Furthermore, when performed on plasma membranes from the same cells, this binding of [3H]JMV 180 was not inhibited by the non-hydrolyzable analogue of GTP, GTPγ[S]. In agreement with this finding, JMV 180 did not stimulate binding of GTPγ[S] to membranes from COS-7 expressing the CCK1R. In the same way, COS-7 cells expressing the M121V-CCK1R mutant bound 125I-BH-(Met)-CCK with a single class of affinity sites (Kd: 23.6 ± 6.6 nM, Bmax: 6.9 ± 0.15 pmol/10^6 cells). This binding was not inhibited by GTPγ[S] and (Met)-CCK did not stimulate binding of...
of GTPγS (not illustrated). In contrast, binding of $^{125}$I-BH-(Met)-CCK to COS-7 cells expressing the WT-CCK1R presented usual characteristics of CCK binding, namely, two affinity sites ($K_d^1$: 0.47 ± 0.03 nM, $B_{max}$: 0.14 ± 0.02 pmol/10⁶ cells, $K_d^2$: 28.1 ± 4.0 nM, $B_{max}$: 2.6 ± 0.2 pmol/10⁶) and dependence of GTPγS. Furthermore, (Met)-CCK stimulated binding of GTPγS to the WT-CCK1R. These last results, indicating that JM180-CCK and (Met)-CCK-M121V-CCK1R complexes behave similarly with respect to action of GTP, further support that the two complexes are functionally dependent on the same $\alpha$-subunit of G protein, most likely Goα. On the other hand, binding properties of CCK-CCK1R complex is in accordance with previously reported CCK-induced coupling of the CCK1R to several G-protein subunits, including Goα and Goi (12).

Finally, two additional recognized features of JM180 are the shape of its dose-response curve for amylase secretion in rat pancreatic acini and its inability to induce acute pancreatitis in rats. We investigated whether responses of pancreatic acini expressing M121V-CCK1R to stimulation by (Met)-CCK were performed upon stimulation by (Met)-CCK (10⁻⁶ M). B, production of inositol phosphates was measured on EF-88 cells (deficient in Goαq; ) expressing (M121V)-CCK1R alone or co-expressing Goq. Assays were performed upon stimulation by (Met)-CCK (10⁻⁶ M), 48 h after infection with viruses (100 multiplicity of infection). Results are the mean ± S.E. of three separated experiments performed in duplicate.

**DISCUSSION**

The aim of the current work was to investigate the mechanism that, at the ligand binding site of a GPCR specimen of family I, the human CCK1R, is responsible for partial activation of phospholipase C, taken as an enzyme effector of this receptor.

Using both an in silico approach of molecular modeling, and pharmacological and biological analysis of CCK1R mutants, we provide evidence that the binding sites for the partial agonist, JM180, and for the full agonist, CCK, are overlapping. However, the two ligands have their C-terminal aromatic rings differently positioned. Indeed, all results supported that the Phe aromatic ring of CCK interacts with aromatic ring of Phe. The helix VI (stretched position). In contrast, in the modeled JM180-CCK1R complex, the phenyl ester moiety of JM180 appeared either in a stretched position toward Phe, or in a folded position within a hydrophobic pocket surrounding Met of helix III. Experimental results showing absence of hindrance effect of Ala toward Phe was in agreement with its position on JM180 binding, indicating that, in the majority of JM180-CCK1R complexes, JM180 is most likely in a folded conformation. Based on these results, we hypothesized that JM180 is a partial CCK agonist, because it does not show a preference for a stretched positioning of its C-terminal phenyl ethyl ester. According to binding data demonstrating a single class of affinity sites for JM180 on the CCK1R, the stretched and folded positions appear to generate complexes of equal affinity. Alternatively, the two complexes could be indiscernible by conventional binding experiments, due to the fact that complexes with JM180 in a folded position may represent the majority of total JM180-CCK1R complexes. The demonstration that JM172 stimulated inositol phosphate production more efficiently than JM180 despite its very low affinity for the CCK1R, provided a strong experimental proof that correct orientation of the C-terminal aromatic ring toward Phe is critical for agonist efficacy. In line with this last result, Phe on helix VI appeared to be essential for phospholipase C activation, especially by partial agonists. Results with JM172 also indicate that the CCK1R is still active when occupied by a peptide having at the same time one phenyl moiety in a folded position and the second one in a stretched conformation. Accordingly, the folded conformation of ligands, which is unable to activate the CCK1R, does not prevent its activation.
investigated mechanisms underlying partial agonism of JMV 180 and that whereby (Met)-CCK partially stimulated M121V-CCK1R mutant by comparing pharmacologically and functionally the (Met)-CCK-M121V-CCK1R complex with that of JMV 180-CCK1R complex for which much data exist in the literature. Strikingly, we found that the two types of complexes presented rigorously the same features such as total dependence of inositol phosphate production to G
\_q expression, single affinity of binding sites, insensitivity of binding to non-hydrolyzable GTP, absence of GTP-\gamma\[S\] binding following agonist stimulation, similarity of dose-response curves for amylase secretion from pancreatic acini, and incapacity to induce acute pancreatitis in pancreatic acini. Such experimental results obtained in different cell types do not provide a demonstration that the same molecular mechanism is the origin of partial activation of the two complexes. Nevertheless, they indicate that the proposed mechanism at the binding site level is highly probable and involves G\_q.

To explain the distinct locations of the ligand C-terminal aromatic ring within the bottom of the CCK1R binding site, one must take into account the relative importance of regions and amino acids, which compose the binding site of the CCK1R in relation to the structure of the ligands. In fact, the binding site of CCK on the CCK1R is composed of amino acids of the first and second extracellular loops (Phe\(^{107}\), Arg\(^{197}\), and Met\(^{195}\)) and of amino acids of the upper third of transmembrane helices that form a pocket surrounding the C-terminal tripeptide of CCK. This binding pocket clearly involves 5 amino acids of helix VI (Arg\(^{236}\), Asn\(^{333}\), Thr\(^{326}\), Ile\(^{329}\), and Phe\(^{330}\)), 3 amino acids of helix VII (Ile\(^{352}\), Leu\(^{356}\), and Phe\(^{360}\)), 2 amino acids of helix III (Val\(^{125}\) and Met\(^{121}\)), 1 amino acid of helix V (Phe\(^{218}\)), and amino acids Leu\(^{51}\) and Leu\(^{53}\) (helix I), and Cys\(^{84}\) (helix II). In this binding pocket, the region that energetically contributes the most to anchoring of CCK is helix VI. In particular, two hydrophilic residues, Arg\(^{236}\) and Asn\(^{333}\), form strong bonds with the penultimate Asp and the C-terminal amide of CCK, respectively (29). Each of these two contact points accounts for 1,000- to 10,000-fold in association constant of CCK (29). Unlike Arg\(^{236}\) and Asn\(^{333}\), Phe\(^{330}\) weakly contributes to CCK affinity but instead plays a role in the occurrence of different affinity states of the CCK1R and behaves as an activation switch (Ref. 26 and this study). In the JMV 180-CCK1R-mod-
eled complex, at the difference of CCK-CCK1R complex, the C-terminal end of JMV 180, which lacks the amide function, was not anchored to the Asn233 side chain. Furthermore, the phenyl ethyl ester of JMV 180 is more flexible than the aromatic ring of CCK Phe. These structural differences, together with the shape of the binding site consisting in two communicating hydrophobic pockets, allow C-terminal phenyl moiety of JMV 180 to move laterally from a stretched position to a folded position. In the case of (Met)-CCK-M121V-CCK1R complex, (Met)-CCK, at the difference of JMV 180, is bound through its C-terminal amide to Asn333 side chain. However, our data showed that the presence of Val residue at position 121 on helix III leads the aromatic ring of C-terminal Phe of CCK to adopt either a folded or a stretched conformation. Based on these modeling data, Met121 appears as a key residue important for CCK1R activation, as previously stated, most likely because it is a critical regulator of hydrophobic interactions, which can take place within the deepest part of CCK1R binding site. The fact that (Nle)-CCK did not activate M121V-CCK1R, whereas (Met)-CCK partially activated this mutant was previously interpreted as an indication that amino acid at position 121 on helix III and amino acid Met/Nle of CCK are so close that they are interchangeable to regulate physico-chemical properties of the binding cavity (26). Thus, helices III and VI of the CCK1R can be considered as functionally linked through the ligand binding site. It is worthy to note that in the three-dimensional model, Phe330, which is T-shape linked with the C-terminal end of JMV 180, which lacks the amide function, was only obtained with the CCK1R complex, the M121V-CCK1R complex, the JMV 180-CCK1R complex, and the CCK-CCK1R complex, whereas JMV 180 could not (15). However, available modeling techniques still remain unable to discriminate between such conformational states. Direct evidence of graded conformational changes induced by ligands of distinct efficacies were only obtained with the β2-adrenergic receptor in which a fluorescent probe was incorporated at the bottom of helix VI. In this GPCR, agonists of the β2-adrenoceptor having different intrinsic efficacy, such as full and partial agonists, were shown to induce and/or stabilize distinct conformational states of the receptor (44–46).

To conclude, in this study using both experimental and molecular modeling approaches, we demonstrate that positioning of the C-terminal end of peptidic agonists within the binding site of the CCK1R is responsible for the extent of phospholipase C activation through Goq coupling. Our findings may have general impact toward the understanding of the functioning of other peptide GPCRs. Moreover, given the potential therapeutic interest in partial agonists such as JMV 180, our structural data will serve for screening and for rational design of new CCK1R ligands.

Acknowledgments—We are grateful to Hubert Llulka and and Ghyslaine Portolan (IFR31, Centre Hospitalier Universitaire Rangueil) for providing EF-88 cells and Dr. Hervé Paris for giving the α2-adrenergic receptor-containing plasmid.

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