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Partial Agonism, Neutral Antagonism, and Inverse Agonism at the Human Wild-Type and Constitutively Active Cholecystokinin-2 Receptors

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ABSTRACT

Cholecystokinin receptor-2 (CCK2R) is a G protein receptor that regulates a number of physiological functions. Activation of CCK2R and/or expression of a constitutively active CCK2R variant may contribute to human diseases, including digestive cancers. Search for antagonists of the CCK2R has been an important challenge during the last few years, leading to discovery of a set of chemically distinct compounds. However, several early-discovered antagonists turned out to be partial agonists. In this context, we carried out pharmacological characterization of six CCK2R antagonists using COS-7 cells expressing the human CCK2R or a CCK2R mutant having a robust constitutive activity on inositol phosphates production, and we investigated the molecular mechanisms which, at a CCK2R binding site, account for these features. Results indicated that three compounds, 3*R*(+)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl)-*N*′-(3-methylphenyl)urea (L365,260), 4-{[2-[[3-(l*H*-indol-3-yl)-2-methyl-1-oxo-2-[[[1.7.7-trimethyl-bicyclo[2.2.1]hept-2-yl)-oxy]carbonyl]amino]propyl]amino]-1-phenylethyl]amino-4-oxo-[l*S*-la.2[*S**(*S**)]4*a*]}-butanoate *N*-methyl-D-glucamine (PD135,158), and (*R*)-1-naphthalenepropanoic acid, *b*-[2-[[2-(8-azaspiro-[4.5]dec-8-ylcarbonyl)-4,6-dimethylphenyl]amino]-2-oxoethyl] (CR2945), were partial agonists; one molecule, 1-[(*R*)-2,3-dihydro-1-(2,3-dihydro-1-(2-methylphenacyl)-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl)urea (YM022), was a neutral antagonist; and two compounds, *N*-(+)-[1-(adamant-1-ylmethyl)-2,4-dioxo-5-phenyl2,3,4,5-tetrahydro-1*H*-1,5-benzodiazepin-3-yl]-*N*′-phenylurea (GV150,013X) and ([(*N*-[methoxy-3 phenyl] *N*-[*N*-methyl *N*-phenyl carbamoylmethyl], carbomoyl-methyl)-3 ureido]-3-phenyl)2-propionic acid (RPR101,048), were inverse agonists. Furthermore, target- and pharmacophore-based docking of ligands followed by molecular dynamic simulation experiments resulted in consistent motion of aromatic residues belonging to a network presumably important for activation, thus providing the first structural explanations for the different pharmacological profiles of tested compounds. This study confirms that several referenced so-called antagonists are in fact partial agonists, and because of this undesired activity, we suggest that newly generated molecules should be preferred to efficiently block CCK2R-related physiological effects. Furthermore, data on the structural basis for the different pharmacological features of CCK2R ligands will serve to further clarify CCK2R mechanism of activation.

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The cholecystokinin (CCK)-2 receptor (CCK2R), formerly named CCKB/gastrin receptor, is a G protein-coupled receptor that presents seven transmembrane helices and binds cholecystokinin and gastrin, two structurally related neuropeptides, with a similar high affinity (Kopin et al., 1992;

ABBREVIATIONS: CCK, cholecystokinin; CCKR2, cholecystokinin receptor-2; BSA, bovine serum albumin; Ins-P, insositol phosphates; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; WT, wild type; TM, transmembrane; GPCR, G protein-coupled receptor; TRH, thyrotropin-releasing hormone; (Thr,Nle)-CCK9, sulfated [Thr28,Nle31]-CCK25-33; ^{125}I-BH-(Thr,Nle)-CCK9, sulfated [Thr28,Nle31]-CCK25-33 conjugated with Bolton-Hunter reagent, purified, and radioiodinated; L365,260, 3*R*(+)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl)-*N*′-(3-methylphenyl)urea; PD135,158, 4-{[2-[[3-(l*H*-indol-3-yl)-2-methyl-1-oxo-2-[[[1.7.7-trimethyl-bicyclo[2.2.1]hept-2-yl)oxy]-carbonyl]amino]propyl]amino]-1-phenylethyl]amino-4-oxo-[l*S*-la.2[*S**(*S**)]4*a*]}-butanoate *N*-methyl-D-glucamine; CR2945, (*R*)-1-naphthalene-propanoic acid, *b*-[2-[[2-(8-azaspiro[4.5]dec-8-ylcarbonyl)-4,6-dimethylphenyl]amino]-2-oxoethyl]; GV150,013X, *N*-(+)-[1-(adamant-1-ylmethyl)-2,4-dioxo-5-phenyl2,3,4,5-tetrahydro-1*H*-1,5-benzodiazepin-3-yl]-*N*′-phenylurea; RPR101,048, ([(*N*-[methoxy-3 phenyl] *N*-[*N*-methyl *N*-phenyl carbamoylmethyl], carbomoyl-methyl)-3 ureido]-3-phenyl)2-propionic acid; YM022, 1-[(*R*)-2,3-dihydro-1-(2,3-dihydro-1-(2-methylphenacyl)-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl)urea; L-740,093, *N*-[(3*R* or 3*S*)-5-(3-azabicyclo[3.2.2]nonan-3-yl]-2,3-dihydro-1-methyl-2-oxo-1*H*-1,4-benzodiazepin-3-yl]-*N*-(3-methylphenyl)urea.

Wank et al., 1992). CCK2R is expressed in the central nervous system and in the gut, in which it represents the predominant CCK receptor subtype (Silvente-Poirot et al., 1993; Noble et al., 1999). CCK2R mediates a wide spectrum of CCK- and gastrin-induced biological effects, including pain perception, anxiety, gastric acid secretion, growth, and differentiation of the gastric mucosa (Silvente-Poirot et al., 1993; Noble et al., 1999). The physiological functions mediated by CCK receptors and therefore their possible implication in associated disorders have generated considerable interest in the identification of ligands that selectively block CCK2R activation. So far, a large panel of chemically distinct antagonists of the CCK2R has been described and used to assess functions mediated by CCK2R in animals and humans (Herranz, 2003). Several of these compounds have reached a clinical evaluation step with indications such as anxiety and panic disorders, sleep disorders, drug dependence, pain, gastroesophageal reflux, and gastric secretion disorders (Herranz, 2003). Despite these advances, one major question that recently arose with some of the nonpeptide antagonists was whether these were pure antagonists rather than partial agonists. Indeed, some referenced so-called antagonists turned out to be endowed of some agonist activity in the stomach, pancreas, or on transfected cells with the cDNA encoding the CCK1R or the CCK2R (Blevins et al., 1994; Schmassmann et al., 1994; Blaker et al., 2000; Kopin et al., 2000). Such undesired agonist activity of CCK2R ligands may represent a risk factor for human patients, especially in the context of long-term use of the drugs. Indeed, aside from multiple peripheral and central effects linked to its short-term activation, CCK2R is endowed with oncogenic potential and/or growth-promoting activity (Dufresne et al., 2006). Further molecular investigations pointed out an interspecies genetic polymorphism regarding the CCK2R that markedly affect both affinity and activity of synthetic ligands without altering endogenous ligand-induced activity (Kopin et al., 1997, 2000; Blaker et al., 2000). These interspecies differences toward synthetic ligands were shown to be caused by sequence variations within the binding site of the CCK2R, thus pointing out the high importance of in vivo and in vitro tests with human protein targets before clinical evaluation of the molecules in humans (Kopin et al., 2000). Availability of synthetic ligands presenting distinct pharmacological profiles, together with data on the structure of the human CCK2R binding site such as those recently obtained (Langer et al., 2005), offers a unique opportunity to investigate the molecular basis for receptor activation or blocking and to further test the feasibility of "rational drug design or improvement".

Given this context, we decided to characterize pharmacological properties of several reference synthetic antagonists of the CCK2R. We used COS-7 cells as host cells for expression of the human wild-type and constitutively active CCK2R because this cell line offers the advantage to allow either high or low expression levels. Then, we docked the tested synthetic ligands into the CCK2R binding site using a procedure combining data from pharmacophore analysis on the basis of CCK2R-bound CCK conformation, flexible docking, and site-directed mutagenesis data. Results from this study show that three of the so-called antagonists of the CCK2R are, in fact, partial agonists, one is a neutral antagonist, and two are inverse agonists on both wild-type and constitutively active receptors. Docking and dynamic simulation experiments provided the first consistent structural explanations for the different pharmacological profiles of synthetic CCK2R ligands. In the near future, these new insights will serve to investigate the mechanism(s) whereby the human CCK2R is activated or inactivated by ligands and to optimize synthetic ligands.

Materials and Methods

Materials

Sulfated [Thr28,Nle31]-CCK25-33 [(Thr,Nle)-CCK9] was synthesized as described previously (Moroder et al., 1981). ^{125}I-Sodium (2000 Ci/mmol) and [*myo*-^{3}H]inositol (5 μCi/ml) were from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Sulfated [Thr28,Nle31]-CCK25-33 was conjugated with Bolton-Hunter reagent, purified, and radioiodinated as described previously (Fourmy et al., 1989) and is referred to as ^{125}I-BH-(Thr,Nle)-CCK9. The following synthetic ligands of the CCK2R were used (Fig. 1): L365,260 (Lotti and Chang, 1989), YM022 (Satoh et al., 1995), PD135,158 (Hughes et al., 1990), CR2945 (Revel et al., 1998), RPR101,048 (Bertrand et al., 1994), and GV150,013X (Ursini et al., 2000) were supplied by Merck (Whitehouse Station, NJ), Yamanouchi Pharmaceutical (Tokyo, Japan), Pfizer (Cambridge, UK), Rotta Research Laborotorium (Monza, Italy), SANOFI Aventis (Paris, France), GlaxoSmithKline (Verona, Italy), respectively.

Site-Directed Mutagenesis and Transfection of COS-7 Cells

All mutant receptor cDNAs were constructed by oligonucleotide-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit; Stratagene, Paris, France) using the human CCK2R cDNAs cloned into pRFNeo vector as template. The presence of the desired and the absence of undesired mutations were confirmed by automated sequencing of the complete CCK2R coding sequence (Applied Biosystems, Foster City, CA). These mutants were characterized previously for their ability to bind and to respond to CCK (Langer et al., 2005).

COS-7 cells (1.5×10^6) were plated onto 10-cm culture dishes and grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum in a 5% CO_2 atmosphere at 37°C. After overnight incubation, cells were transfected with 50 to 1000 ng/plate 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 at a density of 10,000 to 150,000 cells/well 24 h after transfection, depending on the transfected mutant and the experiment to be performed.

Receptor Binding Assay

Approximately 24 h after the transfer of transfected cells to 24-well plates, the cells were washed with phosphate-buffered saline (PBS), pH 7.4, containing 0.1% BSA and then incubated for 60 min at 37°C in 0.3 ml of DMEM containing 0.1% BSA with either 50 pM (WT-CCK2R) or 100 pM (Y189A, H207A, N353A, R356A) ^{125}I-BH-(Thr,Nle)-CCK9 in the presence or absence of unlabeled CCK or synthetic ligands. The cells were washed twice with cold PBS, pH 7.4, containing 2% BSA 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; PerkinElmer Life and Analytical Sciences, Boston, MA). Receptor density and K_d values were calculated from homologous ^{125}I-BH-(Thr,Nle)-CCK9 competition binding experiments using Ligand software (Kell, Cambridge, UK). K_i values were calculated using the nonlinear curve-fitting software Prism (GraphPad Software Inc., San Diego, CA).

Inositol Phosphate Assay

Approximately 24 h after the transfer to 24-well plates and after overnight incubation in DMEM containing 2 μCi/ml *myo*-[2-^{3}H]inositol (GE Healthcare), the transfected cells were washed with DMEM and then incubated for 30 min in 1 ml/well DMEM containing 20 mM LiCl at 37°C. The cells were washed with Ins-P buffer at pH 7.45 (PBS containing 135 mM NaCl, 20 mM HEPES, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM EGTA, 10 mM LiCl, 11.1 mM glucose, and 0.5% BSA). The cells were then incubated for 60 min at 37°C in 0.3 ml of Ins-P buffer alone or with CCK in the presence of increasing concentrations of synthetic ligands. The reaction was stopped by adding 1 ml of methanol/HCl to each well, and the content was transferred to a column (Dowex AG 1-X8 formate form; Bio-Rad, Hercules, CA) for the determination of Ins-P. The 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 evaluated using a liquid scintillation counter (PerkinElmer). EC_{50} values were calculated from dose-effect curves by nonlinear regression curve-fitting using GraphPad Prism.

Molecular Modeling

Modeling of CCK2R.CCK-4 Complex. CCK2R.CCK-4 (CCK-4: Boc-Trp-Met-Asp-Phe-$CONH_2$) complex model was built on the basis of the CCK2R.CCK9 complex model published previously (Langer et al., 2005) by deleting the first five residues, adding Boc-group and refining as described previously.

Flexible Docking of Synthetic Ligands. All ligands were docked into human CCK2 receptors using the docking program GOLD (Jones et al., 1997). The receptor models were taken from the optimized complexes of human CCK2R.CCK-4. The scoring function used to rank the dockings was Goldscore. GOLD default setting was used (100,000 Genetic Algorithm Operations, five islands). Flipping of ring-free corners, amide bonds, and planar nitrogen atoms was allowed. A maximum of 50 docking solutions were generated for each structure. For verification of obtained docking positions, pharmacophore analysis was carried out as an independent and complementary method as described below.

Pharmacophore Analysis of Synthetic Ligands. The pharmacophore analysis was carried out using the DISCOtech in SYBYL 7.0 (Tripos, St. Louis, MO). The conformation of CCK2R-bound CCK-4 was taken as a reference compound for mapping all synthetic ligands. The following assumptions were made 1) CCK-4 is a ligand having all features for affinity and efficacy, 2) there is an overlap between binding sites for agonists and antagonists, and 3) synthetic ligands must keep a major part of pharmacophore domains of the natural ligand. Structure-activity relationship study and alanine scanning of CCK-4 showed three hydrophobic points (leucine, tryptophan, and phenylalanine), two acceptors or one negative center (two oxygens of aspartic acid), and one donor point (amide group of CCK-4) as elements that are important for binding to and activation of CCK2R (Silvente-Poirot et al., 1999). The distance between these elements was taken from CCK2R-bound CCK-4 so that CCK-4 was considered to be a geometrical figure. The next step was the conformational search using a stochastic approach (maximum conformers was 100, and among them, 50 were selected with maximum diversity) for other all ligands. Each conformation of each ligand was further presented again by points of potential interest and distance between them. Then, all produced geometrical figures of synthetic ligands were compared with the CCK-4 figure to find the geometrical figure presenting the maximum of CCK-4 features and the minimum of the pair-wise distance tolerance value between them (the variation of this value was allowed from 0.25 to 10 by 0.25 Å).

Optimization. For each synthetic ligand, the position in the CCK2R was selected on the basis of best-fitting between data from flexible docking, pharmacophore study, and site-directed mutagenesis study. Then, the complexes were subjected to energy minimization by Steepest Descent (until convergence to 2 kcal/mol/Å) and conjugated Gradient (until convergence to 0.01 kcal/mol/Å) to remove steric clashes, using the Discovery module of Insight II. During minimization, C-α trace was tethered using a quadratic potential. This was performed using the Discover 3 model of the Insight II module with the CFF force field. The nonbond cutoff method and the

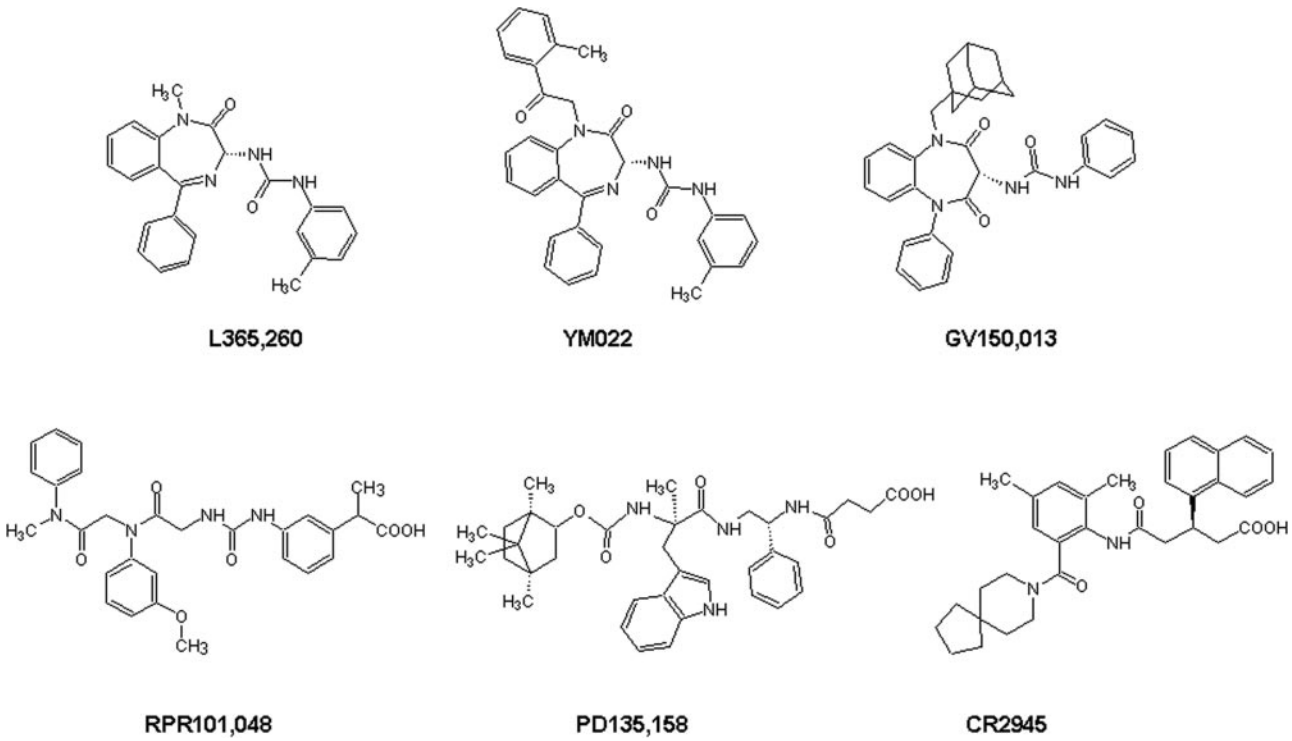

Fig. 1. Chemical structure of synthetic ligands of the CCK2R.

dielectric constant were set up to cell multipole and distance-dependent, respectively (e = r). Then, the complexes were further subjected to molecular dynamics in a three-step procedure. In the first step, the complex was heated from 10 K up to 500 K over 5000 fs; in the second step, it was cooled down to 300 K over 5000 fs and kept at 300 K until equilibration in the third. The tethering force on C-α trace was maintained during heating and cooling periods and was successively decreased by reducing the force constant value of the quadratic potential from 100 to 80, 50, 30, 10, and 0 every 1000 ps in the third step. The integration time step was set up to 1 fs, and the calculations were performed at constant volume and temperature. A snapshot of the system was saved every 100 fs. Once the system was equilibrated, the coordinates of 20 snapshots were averaged and submitted again to the mentioned minimization procedure with no C-α restraints. In the case of refinement based on results with CCK2R having mutated Asn351, a constrain was applied between Asn353 side chain and the closest atoms in nonpeptide ligand capable to form hydrogen bond during 2000 fs, and then the system was again unconstrained until equilibration.

Results

Binding Properties of Nonpeptide Ligands to the Human Wild-Type and E151A-CCK2R. We first determined binding affinities of synthetic ligands for the human wild-type and constitutively active CCK2R. As illustrated only with the wild-type CCK2R, all tested ligands dose-dependently and fully inhibited 125-I-BH-(Thr,Nle)-CCK-9 (Fig. 2). Inhibition constants for the wild-type CCK2R were the following (K_i ± S.E.M.): L365,260, 12.4 ± 6.0 nM; YM022, 0.38 ± 0.05 nM; GV150,013X, 2.1 ± 0.4 nM; PD135,158, 1.6 ± 0.2 nM; CR2945, 14.3 ± 4.2 nM; RPR101,048, 4.2 ± 0.4 nM; and CCK, 0.52 ± 0.05 nM. Inhibition constants for E151A-CCK2R were the following (K_i ± S.E.M.): L365,260, 2.8 ± 1.1 nM; YM022, 0.392 ± 0.006 nM; GV150,013X, 11.3 ± 1.2 nM; PD135,158, 1.3 ± 0.2 nM; CR2945, 8.0 ± 2.9 nM; RPR101,048, 2.8 ± 1.1 nM; and CCK, 0.66 ± 0.09 nM (data not shown). For all competitions, the Hill number was near that of the unit (data not shown).

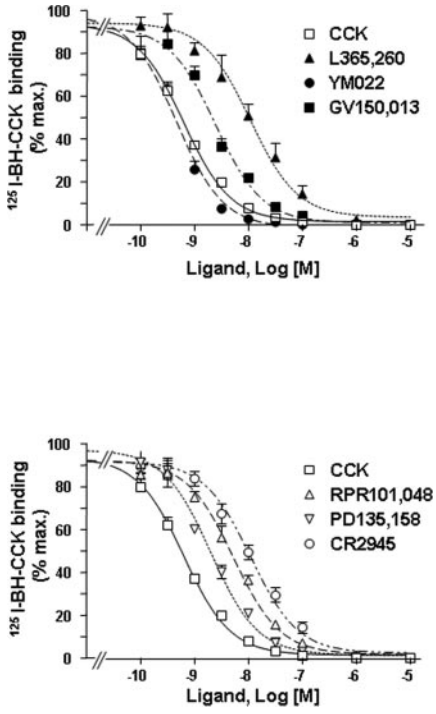

Fig. 2. Competition of ^{125}I-BH-(Thr,Nle)-CCK9 binding to the human wild-type CCK2R by synthetic ligands. Binding is expressed as the percentage of specifically bound ^{125}I-BH-CCK-9. Results are expressed as mean ± S.E. of three to five separate experiments performed in duplicate.

Effects of Nonpeptide Ligands on CCK-Induced Stimulation of Inositol Phosphates by the Wild-Type and E151A-CCK2R. The ability of the different synthetic ligands to decrease CCK-stimulated Ins-P accumulation was assessed on COS-7 cells expressing the wild-type or the constitutively active human CCK2R (Fig. 3, a–d). Given the number of molecules to be tested, a single concentration of CCK was used for stimulation, namely 0.5 nM, which corresponds to concentrations eliciting half-maximal responses (EC_{50}). Three compounds, YM022, RPR101,048 and GV150,013X, inhibited CCK-stimulated production of Ins-P by the wild-type CCK2R to a level that was either close or below the basal values. In contrast, L365,260 and CR2945 only partially inhibited the CCK effect, whereas PD135,158 did not cause any change in Ins-P accumulation. The inhibitory effect of YM022, RPR101,048, and GV150,013X was confirmed upon CCK stimulation of E151A-CCK2R (Fig. 3, c and d). In contrast, with this mutant, CR2945, L365,260, and PD135,158 slightly increased Ins-P production obtained with 0.5 nM CCK.

Effects of Nonpeptide Ligands on Basal Activities of the Wild-Type and E151A-CCK2R on Inositol Phosphate Production. Results showed that three of the tested ligands, L365,260, CR2945, and PD135,158, at a concentration of up to 100-fold their K_i values, did not fully reverse

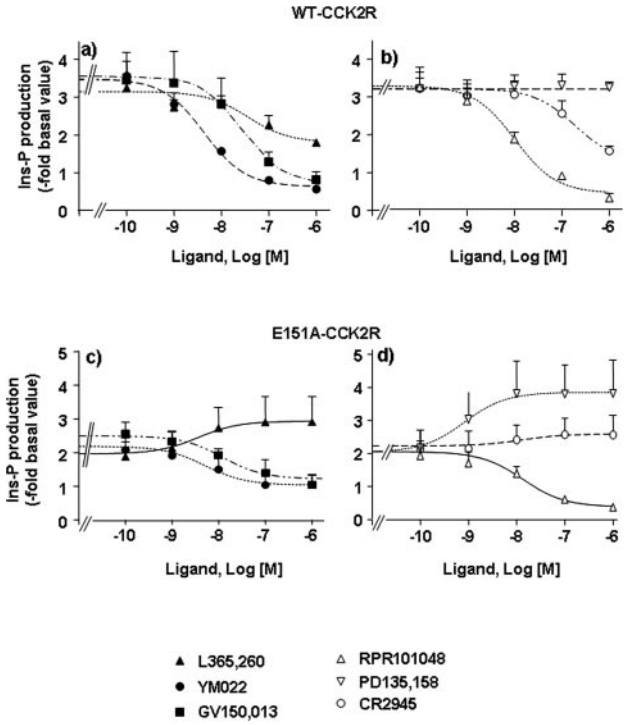

Fig. 3. Effects of synthetic ligands on CCK-induced stimulation of Ins-P in COS-7 cells expressing the wild-type human CCK2R (a and b) or the constitutively active E151A-CCK2R (c and d). Cells were stimulated by 0.5 nM CCK. Values for Ins-P accumulation are expressed as the fold number of the basal value with transfected cells and are mean ± S.E. of three to six separate experiments performed in duplicate. The value 0 corresponds to the inositol phosphate level in nontransfected COS-7 cells, and the value 1 corresponds to basal inositol phosphate level in COS-7 cells expressing the wild-type or mutated CCK2R.

CCK stimulation; this suggested that these compounds were endowed with intrinsic agonist activity. We therefore tested all molecules at increasing concentrations on both nontransfected cells and cells expressing the wild-type or the constitutively active CCK2R. Results from these experiments (Fig. 4, a–d) show different profiles of dose-response curves. Although none of the tested ligands produced any effect on nontransfected cells, PD135,158, L365,260, and CR2945 dose-dependently stimulated the accumulation of Ins-P in COS-7 cells expressing the WT-CCK2R to a maximum, which represented 61, 34, and 28% of that achieved with 1 μM CCK, respectively. YM022 maintained the level of Ins-P to the basal value of the cells expressing the CCK2R. In contrast, RPR101,048 and GV150,013X decreased the production of Ins-P to a level that tended toward that of nontransfected cells. On cells expressing constitutively active E151A-CCK2R, which showed a basal level of Ins-P content reaching 2.5-fold that of the cells expressing the WT-CCK2R, PD135,158, L365,260, and CR2945 displayed stimulatory effects (Fig. 4, c and d). YM022 did not change the basal activity, and RPR101,048 and GV150,013X inhibited it by 61 to 64%. Thus, according to the current experimental data, PD135,158, L365,260, and CR2945 are partial agonists with the following order of potency: PD135,158 > L365,260 > CR2945; YM022 is a neutral antagonist, and RPR101,048 and GV150,013X are inverse agonists.

Dependence of Basal and Maximal Inositol Phosphate Stimulations on CCK2R Expression Levels in COS-7 Cells. To determine whether the activity of nonpeptide ligands of the CCK2R were dependent on the expression level of the WT- and E151-CCK2R by COS-7 cells, we investigated the effects of these ligands on cells transfected with various amounts of plasmid encoding the receptor. Results are illustrated in Fig. 5, top and bottom graphs, for some of the molecules tested. They show that for transfected amounts of plasmids (from 50 to 1000 ng) yielding expression levels of WT-CCK2R or E151A-CCK2R from 1.5 to 8.0 pmol/10^6 cells, basal activity of both WT-CCK2R and E151A-CCK2R was dependent on receptor expression and was significantly more elevated than in nontransfected cells ($p < 0.001$). Moreover, pharmacological behavior of each synthetic ligand was maintained at the three levels of receptor expression.

Effect of CCK2R Mutation on Affinity of Nonpeptide Ligands. The effect of mutation of four reference amino acids (Blaker et al., 1998; Gales et al., 2003a; Langer et al., 2005) of the CCK binding site on the affinity of synthetic ligands was evaluated for docking refinement. Mutation factors, which account for shifts of affinity, varied for the different nonpeptide ligands and were generally lower than for CCK-4 (Table 1). Exchange of Asn353 (TM6) for an alanine decreased the affinity of all compounds except for CR2945. Mutation of Tyr189 (TM4), which decreased affinity of CCK by 227-fold, also dramatically decreased affinity of RPR101,048. On the other hand, mutation of Arg336 (TM6) and His207 caused minor modifications of the affinity of nonpeptide ligands.

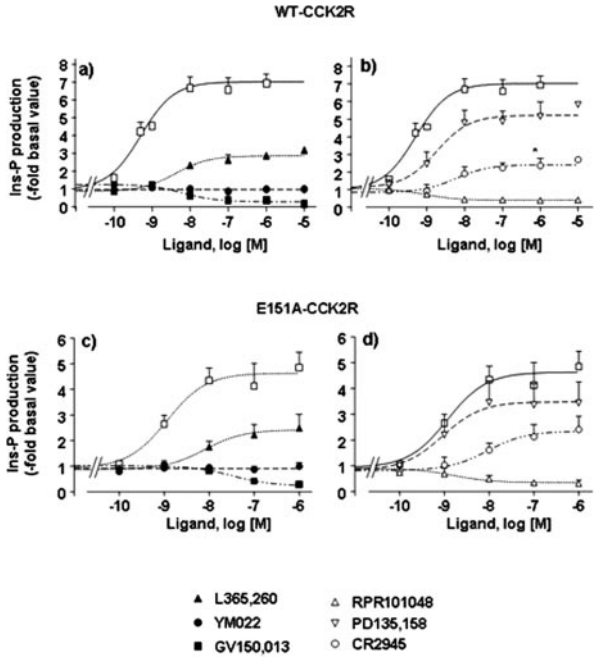

Fig. 4. Effects of synthetic ligands on Ins-P accumulation in COS-7 cells expressing the wild-type human CCK2R (a and b) or the constitutively active E151A-CCK2R (c and d). Values for Ins-P accumulation are expressed as the -fold number of the basal value, with COS-7 cells expressing the wild-type or the mutated CCK2R, and are mean ± S.E. of three to six separate experiments performed in duplicate. The value 0 correspond to Ins-P level in nontransfected COS-7 cells, and the value 1 corresponds to basal inositol phosphate level in COS-7 cells expressing the wild-type or the mutated CCK2R.

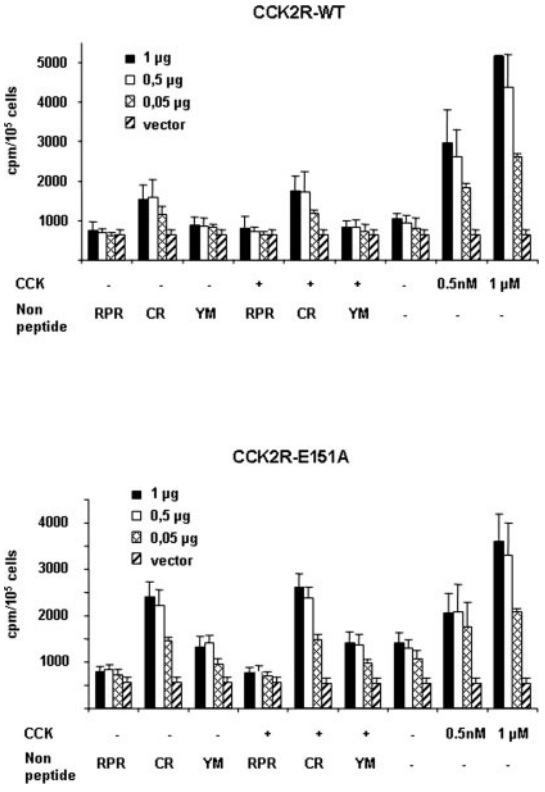

Fig. 5. Effects of expression levels of WT-CCK2R or E151A-CCK2R on the effects of nonpeptide ligands on Ins-P accumulation. COS-7 cells which were transfected by various amounts of plasmid containing CCK2R (0.05, 0.5, and 1.0 μg/plate) or empty vector (1.0 μg/plate) were incubated in the presence of 1 μM nonpeptide ligand alone or with 0.5 nM CCK plus 1 μM nonpeptide ligand. For comparison, inositol phosphates obtained with 0.5 nM or 1 μM CCK are also shown. The basal level of inositol phosphates was significantly more elevated in cells transfected with 1.0 μg/plate of plasmid encoding WT-CCK2R or E151A-CCK2R compared with cells transfected with an empty vector ($p < 0.001$).

Docking of Nonpeptide Ligands into the Binding Pocket of the Modeled CCK2R. For flexible docking of synthetic ligands, the CCK2R.CCK-4 complex was built using a CCK2R.CCK9 complex previously modeled and validated on the basis of site-directed mutagenesis data (Gales et al., 2003a; Langer et al., 2005). By doing so, a number of distinct positions with similar scoring functions were generated (data not shown). To choose between the different possibilities, pharmacophore analysis was performed using CCK2R-bound CCK-4 structure as a reference. As shown in Fig. 6, synthetic ligands of the CCK2R mimicked some of the determinants of CCK-4 structure known to be important for binding and/or biological activity. These are indol moiety of tryptophan, methionine/leucine, negative charge of aspartic acid, and aromatic ring of phenylalanine. Results from pharmacophore orientation fitted at least one solution from the flexible docking approach, except for CR2945 and YM022 (data not shown). With these last two ligands, the pharmacophore-based docking result was selected. Because the above site-directed mutagenesis results supported that L365,260, PD135,158, YM022, GV150,013X, and RPR101,048 but not CR2945 are probably in interaction with Asn353, although no contact could be seen after the first docking round, refinement was carried out by constraining interaction between Asn353 side chain and the closest atoms capable of hydrogen bonds in L365,260, PD135,158, YM022, GV150,013X, and RPR101,048. For all complexes, further molecular dynamic simulation yielded refined complexes in which the ligand kept a position close to its initial one.

Figure 7 presents the refined complexes and their superimposition with CCK2R.CCK-4 complex. The common binding pocket for all ligands is composed of residues Arg356, Asn353, His207, Tyr189, His376, Ser131, Thr193, and Gln216, which are in the distance interval 1.5 to 10 Å from the closet ligand atom. However, the precise binding site of each ligand and the possibilities of interactions were only partially overlapping. In addition to the many hydrophobic contacts that contributed the most to binding, ionic and hydrogen bonds could be identified. PD135,158 was ionic and hydrogen-bond with Arg356 (TM6) and His207 (EL2) through their carboxyl group. In addition, phenylalanine amide bond of PD135,158 was hydrogen bound to the carbonyl group of Asn353 side chain (TM6) and to the hydroxyl of Tyr189 (TM4). CR2945 formed hydrogen bonds with the Arg356 carbonyl group, Tyr189 hydroxyl, and Ser131 and Gly135 NH backbone. In the case of L365,260, there was only one close contact between the carbonyl group of the benzodiazepine core and Asn353 (TM6), whereas YM022 interacted with both Asn353 (TM6) and Arg356 side chains through carbonyl group and the NH group of the amide bond. In the complex with GV150013X, two carbonyl groups of the ligand interacted with Arg356, Asn353, and Ser131 side chains, whereas RPR101,048 had a carboxyl group in strong interaction with Asn353, Arg356, and Tyr189.

Molecular Dynamic Simulation Study of the Effects of Ligand Binding to CCK2R. Previous studies, including ours, provided evidence that the proximity of the phenylalanine side chain of CCK with the aromatic network of CCK2R binding site composed of amino acids Tyr189 (TM4), Tyr192 (TM4), Phe227 (TM5), Phe342 (TM6), Trp346 (TM6), and Tyr350 (TM6) was a key feature for CCK2R activation (Blaker et al., 1998; Jagerschmidt et al., 1998; Pommier et al., 2003; Langer et al., 2005). Analysis of the different complexes after docking and dynamic simulation procedure (Fig. 7) shows that the aromatic moiety of each ligand which, in the pharmacophore analysis, mimicked the phenylalanine of CCK-4, reached different positions with respect to the aromatic network of the CCK2R (Fig. 7). Indeed, in complexes with PD135,158, L365,260, or CR2945, the phenyl or naphtyl rings partially overlapped the phenylalanine of CCK, whereas in YM022, the phenyl ring laid in an upper site close to the Tyr192 side chain. It is noteworthy that L365260 and YM022 have the same 1,4-benzodiazepine scaffold but reached different sites in the CCK2R binding pocket. This is obviously caused by the presence of the bulky substituent on the 1N-benzodiazepine core in YM022, which is in close contact with Val349 (TM6) and Leu375 (TM7) (data not illustrated). Indeed, the position of YM022 is maintained above that of L365260 (Fig. 7), and this changes the orientation of the phenyl ring expected to mimic the phenylalanine of CCK-4. On the other hand, in complexes with RPR101,048 and GV150,013X, the ligands displayed deeper positions in the binding pocket; hence, their phenyl moieties were located deeper relative to the phenylalanine of CCK-4. As a consequence, orientation of CCK2R aromatics found after molecular dynamic simulations was different in the various complexes. To account for these differences, we measured the dihedral angles (C-Cα-Cβ-C3, Cα-Cβ-Ca-C3a for Trp346) of residues Phe227, Phe342, Trp346, and Tyr350. Data showed on Fig. 7 and summarized in Table 2 indicate that binding of partial agonists L365,260, PD135,158, and CR2945 as well as that of the neutral antagonist YM022 to the "active state" of CCK2R taken from the CCK2R.CCK-4 complex did not sig-

TABLE 1

Effect of CCK2R mutations on binding affinity of synthetic ligands

COS-7 cells expressing the WT-CCK2R or mutated CCK2R were incubated with ^{125}I-BH-(Thr, Nle)-CCK9 alone or in the presence of increasing concentrations of synthetic ligands. Binding was expressed as a percentage of specific binding in absence of competitor. Results are the mean ± S.E.M. of three to four individual experiments.

	WT	Y189A		H207A		N353A		R356A	
	K_i	K_i	F_{mut}	K_i	F_{mut}	K_i	F_{mut}	K_i	F_{mut}
	nM	*nM*		*nM*		*nM*		*nM*	
CCK	0.52 ± 0.05	98.6 ± 3.9	188	12.9 ± 2.9	25	21.9 ± 3.8	42	16.6 ± 3.6	32
L365,260	12.4 ± 6.0	7.6 ± 2.6	0.6	9.0 ± 2.4	0.7	65.6 ± 14.4	5.3	6.5 ± 4.4	0.5
YM022	0.38 ± 0.05	3.0 ± 1.5	7.9	0.73 ± 24	1.9	3.1 ± 0.4	8.1	3.6 ± 2.0	9.6
GV150,013X	2.1 ± 0.4	14.9 ± 13.0	7.0	3.7 ± 1.5	1.8	8.8 ± 2.5	4.1	8.0 ± 4.9	3.8
RPR101,048	4.2 ± 0.4	167 ± 82	39	1.6 ± 0.5	0.4	3460 ± 1390	816	33.7 ± 7.6	8.0
PD135,158	1.6 ± 0.2	7.3 ± 1.9	4.7	1.6 ± 0.1	1.0	23.5 ± 5.4	15	3.0 ± 0.2	1.9
CR2945	14.3 ± 4.2	68.4 ± 11.9	6.9	20.0 ± 5.8	2.0	2.6 ± 0.9	0.3	14.3 ± 4.2	1.4

nificantly affect the orientation of Phe227, Phe342, Trp346, and Tyr350 side chains. In contrast, the binding of inverse agonists GV150,013X and RPR101,048 modified the aromatic network, especially the orientation of Trp346 and Phe342 side chains. The docking of L365,260, PD135,158, and CR2945 to this last CCK2R structure (taken from the CCK2R.RPR101,048 complex), which can be considered as a "nonactive state", followed by dynamic simulation restored the aromatic network closer to that observed in the CCK2R.CCK-4 complex. On the other hand, insertion of

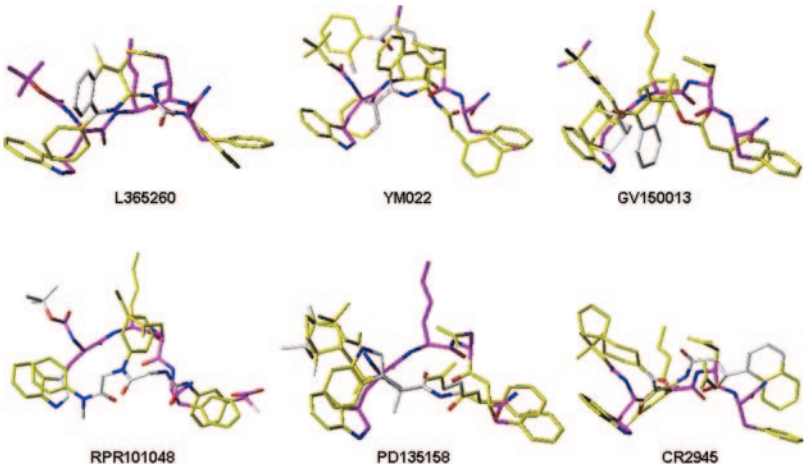

Fig. 6. Pharmacophore overlaps of nonpeptide ligands with CCK-4. The common elements between ligands are presented in yellow, and carbon atoms CCK-4 are in pink.

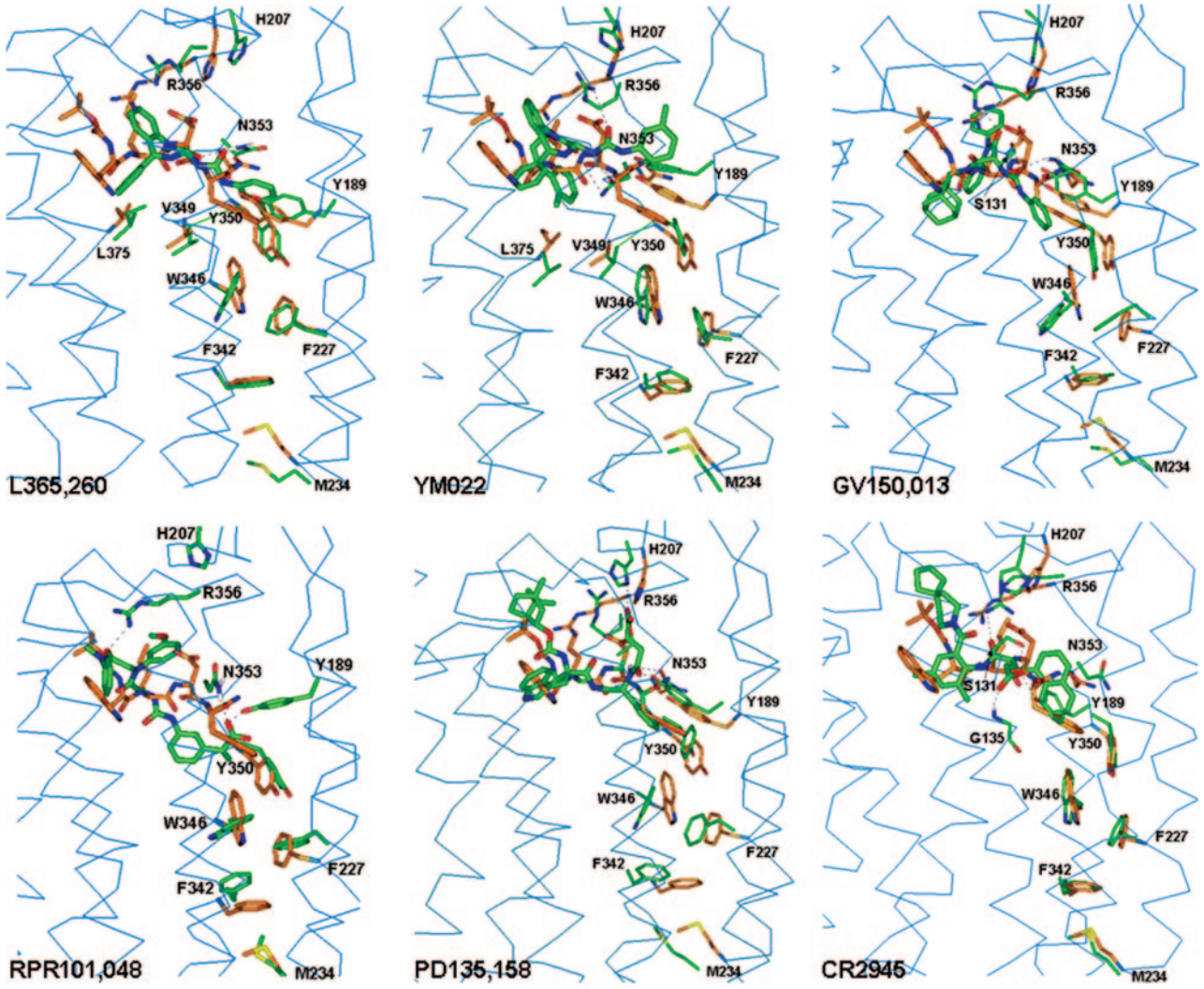

Fig. 7. Final models of CCK2 receptor complexes with nonpeptide ligands superimposed with CCK-4.CCK2R. CCK-4.CCK2R is shown in orange. For simplicity, only the carbon trace for CCK-4.CCK2R is shown.

YM022 did not affect the aromatic network (Table 2 and Fig. 8).

Discussion

The aim of the current study was to precisely define pharmacological features of a series of referenced so-called nonpeptide antagonists of the CCK2R by analyzing their activity either on basal and CCK-stimulated Ins-P production by COS-7 cells expressing the human wild-type or a constitutively active CCK2R. Furthermore, we aimed to relate the pharmacological activity of the tested ligands with their precise site of binding on the CCK2R using state-of-the-art methods of molecular modeling.

Apart from these objectives, this study is the first to clearly demonstrate that the human wild-type CCK2R presents a significant constitutive (ligand-independent) activity. Indeed, transient transfection of COS-7 cells with a plasmid containing cDNA encoding the CCK2R led to an increase of Ins-P production relative to cells expressing the plasmid lacking a CCK2R sequence. Hence, the human CCK2R joins the group of GPCR, the native form of which exhibits a constitutive activity. In fact, spontaneous activity of native GPCRs has long been known (Cerione et al., 1984; Costa and Herz, 1989). Since the earliest examples, a number of native GPCRs with constitutive activity have been identified (Seifert and Wenzel-Seifert, 2002). However, spontaneous activity of the wild-type CCK2R remained less robust than that of E151A-CCK2R mutant, which exhibited a 2.5-fold higher activity than the wild-type CCK2R and near 20% of the maximal response achieved upon CCK stimulation. The first described constitutive activation of a G protein-coupled receptor generated by mutation was achieved with the α1-adrenergic receptor (Cotecchia et al., 1990). Concerning the human CCK2R, several point mutations within the third intracellular loop and transmembrane helices, which caused constitutive activity, have been described previously (Beinborn et al., 1998, 2004). In the current study, the use of E151A-CCK2R mutant to test intrinsic activity of synthetic ligands allowed easy and accurate detection of inverse agonists, namely antagonists capable of blocking ligand-independent activity of CCK2R and to distinguish them from partial agonists and neutral antagonists. In this respect, our results unambiguously demonstrated that RPR101,048 and GV150,013X are inverse agonists, whereas YM022 is a neutral antagonist. On the other hand, tests with both wild-type and E151A-CCK2R indicated that PD135,158, L365,260, and CR2945 are partial agonists.

Concerning binding properties of the tested ligands, competition binding experiments using labeled CCK only allowed us to compare apparent affinity of the nonpeptide ligands for the active states of the wild-type and E151A-CCK2R. They showed that each nonpeptide ligand bound to both receptors with very close affinities. This result supports the view that conformation of the binding site of the active state of the wild-type CCK2R resembles that of E151A-CCK2R. Because of unavailability of labeled antagonist of the CCK2R, we did not measure the affinity of the nonpeptide ligands for the inactive state of the CCK2R, and therefore we could not provide any data that would establish a relationship between the activity of these compounds and their affinity for each receptor state.

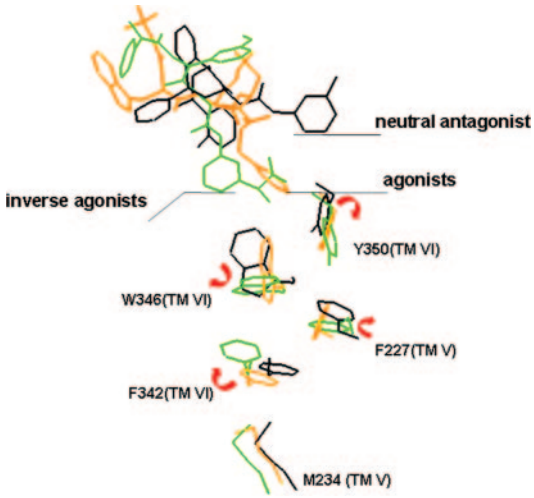

Fig. 8. Schematic representation of the effect of agonists (CCK-4), neutral antagonist (YM022), or inverse agonists (RPR101,048) on the position of aromatic residue side chains. The figure shows how binding of the ligands differently influenced side-chain position of aromatic residues playing the role of "sensor" during molecular dynamic simulations of liganded CCK2R.

TABLE 2

Dihedral angles (C-Cα-Cβ–C1, C-Cα-Cβ-C3 and Cα-Cβ-Ca-C3a) of hydrophobic residues Phe227, Phe342, Trp346, and Tyr350 in the complexes with full agonist (CCK4), partial agonists (PD135,158, CR2945, and L365,260), inverse agonists (GV150,013 and RPR101,048) and a neutral antagonist (YM022)

The first part of the table shows dihedral angles achieved after the binding of nonpeptides to the "active state" of the receptor (CCK4.CCK2R) and the second to the "nonactive state" of the receptor taken from RPR101,048.CCK2R complex.

	CCK2R Residues				
	Phe227	Phe342	Trp346		Tyr350
Dihedral angles	C-Cα-Cβ–C1 ± 6	C-Cα-Cβ-C1 ± 6	C-Cα-Cβ-C3 ± 6	Cα-Cβ-Ca-C3a±6	C-Cα-Cβ-C1 ± 6
RPR101,048	39	89	129	225	160
GV150,013X	53	72	148	238	162
YM022	301	38	125	279	155
L365,260	312	60	144	272	151
PD135,158	305	59	149	295	155
CR2945	327	56	136	283	168
CCK4	317	57	127	303	155
Ligand-nonactive-receptor complexes					
CR2945	146	58	152	263	167
PD135158	123	63	125	288	171
L365260	121	67	142	277	162
YM022	321	33	156	211	160

Since their initial synthesis, there have been a number of reports regarding pharmacological features of CCK2R nonpeptides ligands. YM022 has been used in several cell or animal models to block CCK2R-related biological effects. This compound was generally found to be very efficient to reverse CCK- and gastrin-elicited biological activities (Herranz, 2003). Furthermore, in contrast to our findings, YM022 has been found recently to exhibit an inverse-agonist activity on constitutive and Src-dependent internalization of a splice variant of the CCK2R (Chao et al., 2005). On the other hand, this compound was classified as a neutral antagonist on the basis of absence of inhibition of the basal Ins-P production induced by two distinct engineered CCK2R mutants (Beinborn et al., 1998, 2004). Among the two inverse agonists, RPR101,048 and GV150,013X, only RPR101,048 was found previously by us to reverse constitutive activity of E151A-CCK2R in NIH 3T3 cells. RPR101048 was able not only to inhibit ligand-independent production of Ins-P but also to reverse the transforming activity of the mutated E151A-CCK2R on the cells (Gales et al., 2003b). Among the three other ligands that must be classified as partial agonists, L365,260 has been long considered as a reference antagonist of the CCK2R because, when used at adequate doses or concentrations, it reversed CCK- or gastrin-induced biological activities (Herranz, 2003). Here, we confirmed that despite its intrinsic activity, L365,260 also efficiently reversed Ins-P production elicited by 0.5 nM CCK. Our results showing that L365,260 possesses an intrinsic agonist activity are in accordance with data from several studies (Beinborn et al., 1998; Blaker et al., 1998; Kopin et al., 2003). New generations of CCK2R ligands that we could not yet test were synthesized on the basis of L365,260 structure (Herranz, 2003). Several of them were reported as true antagonists and even, for one of them (L740093R), as an inverse agonist on a constitutively active human CCK2R mutant (Beinborn et al., 1998). Concerning the peptoid PD135,158, despite initial studies demonstrating its anxiolytic activity, this compound was further showed to stimulate second-messenger formation in cells transiently expressing the human, mouse, or dog CCK2R (Hughes et al., 1990; Kopin et al., 1997; Kopin et al., 2000). Moreover, PD135,158 was found to stimulate rat pancreatic acinar cell secretion through the CCK1R (Hocker et al., 1993). In the family of peptoid CCK2R ligands, several molecules were released subsequently to PD135,158 (Herranz, 2003). It would be interesting to evaluate them using our sensitive tests with the human target (Schmassmann et al., 1994; Blaker et al., 2000). Finally, this is the first and only study showing weak intrinsic agonist activity of CR2945.

L365,260, YM022, and GV150013X belong to the same family of nonpeptide ligands, having a common 1,4-benzodiazepine core but with different substituents on the 1*N*-benzodiazepine core (Fig. 1). These ligands, which exhibit a wide spectrum of activity, namely partial agonism, neutral antagonism, and inverse agonism, respectively, join the group of nonpeptide ligands of GPCRs in which very subtle modifications in the structure have been shown to induce dramatic changes in the intrinsic activity (Soudijn et al., 2005). Our current findings complete previous observations with this series of benzodiazepine ligands, in particular those showing that more pronounced structural modifications such as a change of C3-stereochemistry can give rise to compounds having opposite activities. This phenomenon was illustrated for enantiomers of L740093, which are partial (*S*-enantiomer) or inverse agonists (*R*-enantiomer), respectively, and was further generalized to a larger series of benzodiazepine-related compounds (Kopin et al., 2003).

An important part of the current study addressed the challenged issue of the molecular basis for partial agonism, neutral antagonism, and inverse agonism at CCK2R. For this purpose, we carried out in silico docking of nonpeptide ligands into the modeled CCK2R binding site using a procedure combining data from flexible docking, pharmacophore analysis on the basis of CCK2R-bound CCK conformation, and site-directed mutagenesis information. Then, we submitted each obtained complex to a procedure of molecular dynamic simulations to account for the pharmacological features of the ligand. The rationale for this study was based on the facts that 1) relative positioning of the ligands in the binding pocket is determined by the structure of these ligands and their ability to interact with chemical partners within the CCK2R binding pocket; 2) the differences in ligand positioning achieved by docking and dynamic simulations can be translated in silico into changes in the side-chain orientation of aromatic residues; and 3) changes in side-chain orientation of aromatic residues represent a "local sensor" of the overall conformational changes that occur after binding of agonist, neutral antagonist, or inverse agonist to the native CCK2R.

As we documented previously with the CCK1R, automated docking of small ligands, such as those tested here, is a hazardous task because of their "chemical symmetry", which produced scoring functions similar to those of the binding energy (Martin-Martinez et al., 2005). Indeed, for most of the nonpeptide ligands tested, several plausible positions were generated by in silico docking in the modeled CCK2R binding site. To overcome the difficulty, we introduced data from pharmacophore analysis using a method that is distinguishable from others by the fact that distances between key chemical elements important for activity and efficacy of the ligands were generated based on the 3D structure of the C-terminal end of CCK (CCK-4), taken from the validated CCK2R.CCK complex (Langer et al., 2005). This procedure of ligand alignment with CCK-4 structure completed data from automated docking, allowing us to determine with more confidence the orientation of the different nonpeptide ligands within the CCK2R binding site.

An attempt to build a common pharmacophore for several CCK2R ligands has been carried out previously using the active analog approach on the basis of eight peptidoid derivatives (Ursini et al., 2000). The superimposition of GV150,013X to these peptidoid structures indicated that the phenyl ring of benzodiazepine moiety and the ureidic substituent were superimposed to phenylalanine and tryptophan of CCK, respectively, whereas in our work, the opposite situation was obtained (Fig. 6). Another pharmacophore study was applied recently to a set of 33 antagonists of CCK receptors comprising 6 classes of structurally distinct compounds. In this later work, the conformational search was carried out for all compounds, leading to conformational models that were used for the generation of pharmacophore models. The authors selected the best one, which has two hydrogen bond donors, one aliphatic, and one aromatic moiety (Chopra and Mishra, 2005), which differs from our finding.

Finally, it is noteworthy that earlier studies of gastrin fragments in the absence of the structural receptor information showed that CCK-4 could exist in a 3_{10} helix with two aromatic rings of tryptophan and phenylalanine interacting in a π-stacking arrangement at a distance of 5 to 7 Å (Kalindjian et al., 1994), whereas in our study, the conformation of CCK-4 in its binding site is more extended because the distance between tryptophan and phenylalanine is approximately 10 to 12 Å. These different results highlight difficulties in drawing a reliable pharmacophore of nonpeptide ligands of the CCK2R independently of information regarding the structure of bound ligands.

Another lesson learned from our study of nonpeptide docking in the CCK2R binding site is related to the difficulty encountered to define acceptor/donor elements in nonpeptide ligands and surrounding residues of the CCK2R binding site, such as Asn353. In fact, results from the site-directed mutagenesis study and molecular modeling indicated that in the CCK2R.CCK complex, Asn353 (TM6) interacted as an acceptor with the NH group of the CCK C-terminal amide (Langer et al., 2005). Site-directed mutagenesis results from the current study supported that L365,260, PD135,158, YM022, GV150,013X, and RPR101,048 but not CR2945, were likely to interact with Asn353. However, first results from automated docking of these nonpeptide ligands followed by dynamic simulation did not show any direct contact with Asn353. A likely explanation for this result lies in the fact that in the docking procedure, the receptor structure was rigid, and during the process of dynamic simulation in the vacuum, Asn353 competed with neighboring charged residues such as Arg356 and His205. In addition, pharmacophore analysis with these molecules could not provide any satisfactory match with the donor part of CCK corresponding to the NH amide. This situation led us to assume that Asn353 side chain can play a role of either donor or acceptor, depending on the bound ligand, what allowed us to perform further refinement of the complexes by constraining the interaction between Asn353 side chain and the closest atoms in nonpeptide ligands capable of hydrogen bond, so that the position of the different compounds fits site-directed mutagenesis results. Therefore, conducted refinements show that Asn353 played a donor role in complexes with L365,260, YM022, GV150,013X, and RPR101,048 and an acceptor role in complexes with PD135,158. In accordance with these results, previous study about the identification of the binding site of pyridopyrimidine antagonists on the human CCK1R using a different strategy involved Asn333 (corresponding residues to Asn353 of CCK2R), and this residue was found to play a donor role (Martin-Martinez et al., 2005).

Pharmacophore studies with nonpeptide ligands also revealed that all compounds have in their structure a part that will mimic phenylalanine side chain of CCK once bound to the CCK2R. However, refined docking of these ligands followed by dynamic simulation generated significant variations in the location of phenylalanine mimetic relative to the aromatic network of the CCK2R. This was a result of both 3D structure of the ligands and possibilities of interactions within the binding pocket. As a consequence, orientation of aromatic side chains varied according to the bound ligand. One can propose that, as in other GPCRs, local motions of amino acid side chains revealed by dynamic simulation on CCK2R complexes can account for conformational rearrangements that occur during the switch of the receptor from the active form to the inactive form and vice versa. Indeed, whereas the neutral antagonist YM022 did not significantly influence the aromatic network in the active CCK2R taken from the CCK2R.CCK-4 complex or the inactive CCK2R taken from the CCK2R.RPR101,048 complex, partial agonists L360,260, PD135,158, and CR2945 were able to position the aromatic cluster in the inactive CCK2R in a way similar to the peptide agonist CCK-4. Inverse agonists RPR101048 and GV150013X similarly influenced the aromatic network in the active CCK2R.

These new insights represent a good basis for further studies aimed at the delineation of the subtle switch mechanisms that involves aromatic network in the CCK2R. Our findings are in line with previous studies, which showed the importance of aromatic residues Tyr189 (TM4), Tyr192 (TM4), Phe227 (TM5), Phe342 (TM6), Trp346 (TM6), and Tyr350 (TM6) in the activation process of the CCK2R (Blaker et al., 1998; Jagerschmidt et al., 1998; Pommier et al., 2003; Langer et al., 2005). It is noteworthy that in other receptors, a hydrophobic cluster between transmembrane helices V and VI constrains the receptor in an inactive conformation via interhelical interactions and is involved in activation by agonists (Fanelli and De Benedetti, 2005). For instance, in the TRH receptor, disruption of hydrophobic constraints by TRH binding or by mutations leads to changes in the relative positions of TM5 and TM6 and to the formation of an active form of TRH receptor (Colson et al., 1998). Moreover, in the serotonin receptor 5-hydroxytryptamine-2A, orientation of the indoleamine moiety of the ligands in the binding pocket was shown to determine their relative efficacy (Ebersole et al., 2003). A hydrophobic motif in TM6 of this receptor that is conserved in some other GPCRs was proposed to play the role of sensor of such positioning of the agonist (Visiers et al., 2002). In the CCK1R, we demonstrated previously that agonist efficacy of CCK analogs is dependent on the position of the phenylalanine side chain of CCK relative to a cluster of phenylalanines of TMs 5 and 6 (Archer-Lahlou et al., 2005).

In conclusion, in the context of longstanding interest in drugs that target and block the CCK2R, this pharmacological study confirms that several referenced so-called antagonists are in fact partial agonists, and because of this undesired activity, we suggest that the use of newly generated molecules should be preferred to efficiently block CCK2R-related physiological effects. This study also allowed the identification of two inverse agonists and one neutral antagonist of the human CCK2R. Furthermore, in silico experiments using a validated model of the CCK2R and state-of-the-art modeling methods provide the first plausible explanations for the different features of the tested nonpeptide ligands of the CCK2R.

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