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His^{381} of the rat CCK_B receptor is essential for CCK_B versus CCK_A receptor antagonist selectivity

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Abstract

A great interest is devoted to antagonists of the cholecystokinin type B (CCK_B) receptor such as L-365,260, which reduces panic attacks in humans and to antagonists of the cholecystokinin type A (CCK_A) receptor, such as L-364,718 which might be efficient in mental diseases. The A/B specificity of these antagonists was proposed to be mainly dependent on the amino acid sequence of the seventh transmembrane domain (Mantamadiotis and Baldwin (1994) Biochem. Biophys. Res. Commun. 201, 1382). In our study, one of these residues, His^{381} was replaced in the rat CCK_B receptor by leucine (the corresponding residue in the CCK_A receptor), phenylalanine or arginine using site-directed mutagenesis. Changing histidine for leucine or phenylalanine did not modify significantly the affinity of the CCK_B receptor antagonists, L-365,260 and PD-134,308 although both compounds belong to different chemical classes, but strongly improved the affinity of the CCK_A receptor antagonists tested. Interestingly, the A selectivity of these CCK_A receptor antagonists was recovered by substituting His^{381} by arginine. Moreover, these results are discussed on the basis of a three dimentional model of the CCK_B receptor. The mutated receptors possessed unchanged binding properties for agonists, suggesting that determinants confering specificity for agonists and antagonists are different.

Keywords: CCK receptor; Mutation; Histidine

1. Introduction

The peptide cholecystokinin (CCK), originally discovered in the gastrointestinal tract, is also present in high concentrations in the central nervous system (CNS) (Vanderhaeghen et al., 1975), predominantly under the form of a sulfated octapeptide (CCK-8). Binding sites for CCK have been classified as CCK_A and CCK_B receptors. Unlike CCK-8 which has a high affinity for both receptors, CCK-8-related peptides such as CCK tetrapeptide (CCK-4), non-sulfated CCK-8, and gastrin I, show higher affinities for CCK_B than for CCK_A receptors (Inis and Snyder, 1980). The CCK_B receptor is the most abundant type in the CNS whereas the CCK_A receptor predominates in the gastrointestinal system, but also occurs in highly localized areas of the rat CNS (Moran et al., 1986). The complementary DNAs coding for the two CCK receptor types have now been cloned from canine parietal cells (Kopin et al., 1992), rat pancreas (Wank et al., 1992a), rat brain (Wank et al., 1992b), human brain (Pisegna et al., 1992), human stomach (De Weerth et al., 1993), and guinea pig pancreas and gallblader (Wank et al., 1994). These cDNAs encode for proteins containing seven putative transmembrane domains, suggesting that they belong to the G-protein-coupled receptor superfamily. Rat CCK_A receptor cDNA encodes a protein 444 amino acids in length, and rat CCK_B receptor cDNA encodes a protein 452 amino acids in length which shares 48% identity with the CCK_A receptor protein. In contrast, an interspecies homology of 90% is ob-

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served among the CCK_A or CCK_B receptor types (Beinborn et al., 1993; Wank et al., 1994).

There is considerable interest in the development of CCK_B receptor antagonists since these compounds have been shown to inhibit panic attacks triggered by administration of CCK-4 in humans (Bradwejn et al., 1991), to potentiate opioid analgesia (Faris et al., 1983; Maldonado et al., 1993), and to produce antidepressant-like effects in rodents (Derrien et al., 1994). Selective CCK_A receptor antagonists could also have interesting clinical applications, as for instance in the treatment of *anorexia nervosa* (Crawley and Corwin, 1994). Therefore, it is important to elucidate the molecular interactions involved in CCK receptor antagonist binding in order to develop more specific and selective compounds. As for many other G-protein-coupled receptors, the binding domain of CCK receptors is thought to be localized inside the hydrophobic transmembrane domain. Data obtained from mutagenesis experiments have demonstrated that the amino acid in position 349 in the sixth transmembrane domain of the human CCK_B receptor plays a role in the selectivity of benzodiazepine-derived CCK_A L-364,718 and CCK_B L-365,260 antagonists (Beinborn et al., 1993). A recent study using chimaeric CCK receptors suggests also the involvement of the seventh transmembrane domain in L-364,718 binding (Mantamadiotis and Baldwin, 1994). In the present study, His^{381} located within the seventh transmembrane domain, was identified as a possible determinant for CCK_A versus CCK_B receptor selectivity, based on differences between primary sequences of CCK_A and CCK_B receptors, and on a preliminary modeling study of the rat CCK_B receptor. Accordingly, His^{381} in the rat CCK_B receptor, was substituted by site-directed mutagenesis by either leucine, which is located at the corresponding position in the CCK_A receptor, phenylalanine or arginine. Mutant CCK_B receptor cDNAs were then transiently expressed in Cos-7 cells. Affinity and selectivity of several CCK receptor ligands were measured by binding experiments. The results show that the binding of both CCK receptor agonists and CCK_B receptor antagonists were not affected by the various mutations, whereas the replacement of His^{381} by leucine or phenylalanine strongly improved the affinity of two CCK_A receptor antagonists, thus demonstrating the crucial role played by His^{381} in B versus A selectivity of CCK antagonists. The molecular basis for such a role is discussed using a three dimensional model of the CCK_B receptor.

2. Materials and methods

2.1. Reagents

CCK-4 was purchased from Bachem (Buhendorf, Switzerland). CCK-8, pBC 264 (Propionyl-Boc-Tyr(SO_3H)-gNle-mGly-Trp-(NMe)-Nle-Asp-PheNH_2), A-71,623 (Boc-Trp-Lys(Tac)-Asp-MePhe-NH_2), L-365,260 (3*R*(+)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepine-3-yl)-*N'*-(3-methylphenyl)urea), L-364,718 (3*S*(−)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepine-3-yl)-1*H*-indole-2-carboxamide) and PD-134,308 (4-{[2-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo[3.3.1.$1^{3,7}$]dec-2-yloxy)-carbonyl]amino]propyl]amino]-1-phenylethyl]amino}-4-oxo-[*R*-(*R**,*R**)]-butanoate-*N*-methyl-D-glucamine), were synthesized in the laboratory following reported procedures (Charpentier et al., 1988; Lin et al., 1991; Lotti and Chang, 1989; Evans et al., 1986; Hughes et al., 1990). SR-27,897B (1-{[2-[4-(2-chlorophenyl)thiazol-2-yl]amino carbonyl]indolyl}acetic acid) (Gully et al., 1993) was generously provided by Sanofi. Radiolabelled compounds, such as [α-^{33}P]dATP (specific activity, 1000–3000 Ci/mmol) and [^{3}H]pCCK-8 (specific activity, 60–90 Ci/mmol) were purchased from Amersham (Les Ulis, France). Cell culture reagents were from Gibco-BRL (Cergy, France).

2.2. Site-directed mutagenesis

The cDNA of the rat CCK_B receptor was obtained as previously described (Jagerschmidt et al., 1994). For construction of site-directed mutant receptors, a 1.4 Kb *Hind*III-*Eco*RI cDNA fragment containing the entire rat CCK_B receptor coding region was inserted into the expression vector pcDNA3 (Invitrogen, San Diego, CA). Three oligonucleotides of 21 bp were designed to replace the codon for histidine (CAC) located at amino acid position 381 with codons for phenylalanine (5'-TCTTTCATCTTCTTGCTGAGC-3'), leucine (5'-TCTTTCATCCTCTTGCTGAGC-3'), or arginine (5'-TCTTTCATCCGCTTGCTGAGC-3'). Double-strand mutagenesis was carried out using a Transformer^{transmembrane} Site-Directed Mutagenesis kit (Clontech, Palo Alto, CA), following the manufacturer's instructions. Authenticity of each mutation was confirmed by sequencing over the entire protein coding region, using the Sequenase version 2.0 DNA sequencing kit (US Biochemical) and [α-^{33}P]dATP.

2.3. Cell culture and transfections

Cos-7 cells, which do not express CCK_B receptor, were grown in Dulbecco's modified Eagle medium supplemented with 10% foetal calf serum and 50 μg/ml gentamycin, at 37°C. 1 day before transfection, cells were seeded into 24-well plates, at a density of 10^5 cells/well. Cells were transfected with a pcDNA3 vector containing wild-type or mutant CCK_B receptor cDNA, using a calcium phosphate precipitation method (Chen and Okayama, 1987). 48 h after the transfection,

ligand binding analyses were performed directly on the cells.

2.4. Radioligand binding assays

The binding assays were performed in Dulbecco's modified Eagle medium containing 5 mM $MgCl_2$ and 0.2 mg/ml bacitracin. Each assay was performed in a final volume of 0.5 ml. Incubations were carried out for 90 min at 25°C. For equilibrium saturation experiments, the concentration of [^{3}H]pCCK-8 varied from 50 to 8000 pM. For competition experiments, a fixed concentration of 0.5 nM of [^{3}H]pCCK-8 was used, in the presence of various concentrations of the competitor. Non-specific binding was determined in presence of 1 μM CCK-8. Incubations (90 min at 25°C) were stopped by removing the media, followed by two washes with 1 ml of phosphate-buffered saline. 200 μl of trypsin were then added to each well to harvest the cells. After incubation at 37°C, the cells were collected, scintillation mixture was then added and the radioactivity counted. Parameters describing [^{3}H]pCCK-8 sat-

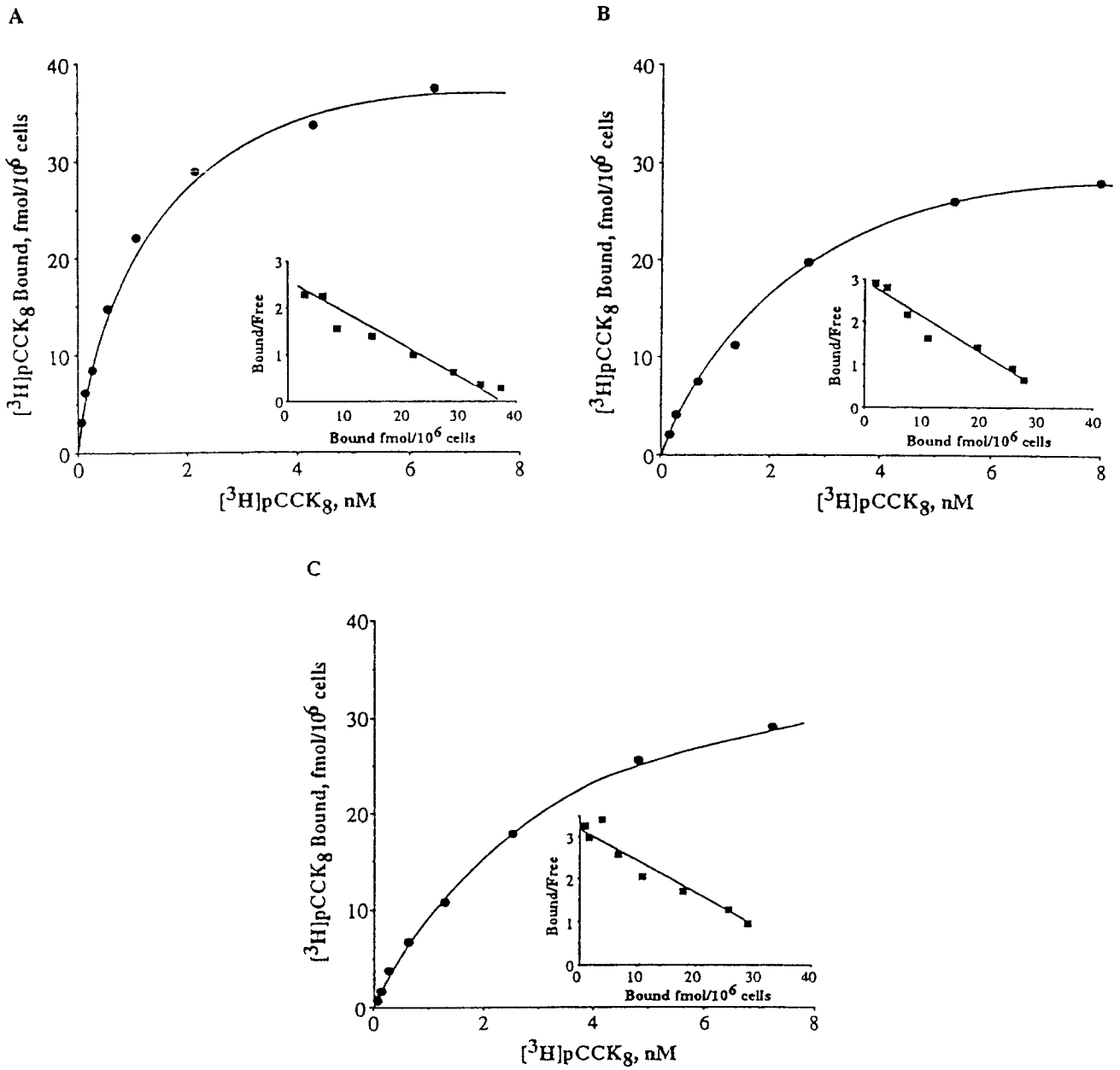

Fig. 1. Saturation analysis of [^{3}H]pCCK-8 binding. Cos-7 cells were transfected with the cDNA encoding wild-type (A), H381L (B), or H381F (C) rat CCK_B receptors. Insert, Scatchard transformation of the data. Assays are described under Materials and methods. Each experiment shown is representative of three similar assays performed in triplicate.

uration binding (ie K_d = dissociation constant; B_{max} = maximal number of binding sites), as well as IC_{50} (concentration of compounds that decreased binding of [^{3}H]pCCK-8 by 50%) were determined using the computer program EBDA (McPherson, 1983).

2.5. Computer modeling

The whole modeling was carried out with Biosym software using the 'Insight', 'Discover' and 'Homology' algorithms. In all these calculations, a distance-dependent dielectric constant of 3.5 r was used to avoid the over evaluation of electrostatic interactions between charged groups. A cutoff of 25 Å was used to truncate the list of interacting atoms to a reasonable size in relation to the available CPU time.

Molecular models of the unliganded CCK_B wild-type and mutant receptors were built using a strategy already used for angiotensin II AT_1 receptor (Joseph et al., 1995) and proved to be a valuable one (Groblewski et al., 1995). These models were constructed using the transmembrane helical positions found in the bacteriorhodopsin crystal structure as a starting point of the optimization procedures. When an energy refined model of the seven transmembrane helices of the CCK_B receptor was obtained, the extra- and intra-cellular loops were then added to the resulting energy-minimized arrangement of the seven-helices domain. This was achieved using the 'homology' loops module which uses a library of loop templates from the PDB database. Because no experimental information about the structure of these loops is yet available, extensive high temperature molecular dynamics at 1000°K followed by annealing to 300°K were next carried on these segments, with the seven transmembrane helices being frozen in their most favorable position as obtained above. The final structure was energy minimized using 10000 steps of a conjugate gradient procedure. During these calculations, the disulphide bridge between Cys^{127} and Cys^{205} of the receptor was imposed as a constraint. Then the whole system was relaxed taking into account possible translation and rotation movements of individual transmembrane helices. The ligands were initially docked inside the receptor transmembrane domains according to the position of the retinal moiety in the bacteriorhodopsin system. This position was further optimized using several rounds of simulated annealing and energy minimization steps. Details of this model building will be presented elsewhere (Maigret et al., manuscript in preparation). This modeling work was performed independently of our experimental work to avoid any bias in the results analysis.

3. Results

3.1. Effects of mutations on the binding of [^{3}H]pCCK-8

As shown in Fig. 1, the binding of [^{3}H]pCCK-8 to transfected cells was specific and saturable. The B_{max} values for wild-type, H381L and H381F mutant receptors were similar (37 ± 10, 32 ± 6 and 32 ± 14 fmol/10^6 cells, respectively) indicating that the mutations have not modified the synthesis, expression and membrane localization of the receptors. The replacement of His^{381} by arginine induced a 10-fold reduction in the B_{max} suggesting some defects in translocation and stable membrane integration.

Scatchard analysis of several binding isotherms showed the occurence of a single class of binding sites, with affinity values for the H381L and H381F mutant receptors ($K_d = 2.51 \pm 0.79$ nM and 2.48 ± 0.64 nM, respectively) essentially unchanged as compared to the wild-type receptor ($K_d = 1.03 \pm 0.30$ nM). In the case

Table 1
Compared pharmacological properties of wild-type (WT), H381L, H381F and H381R mutant CCK_B receptor

Ligand	IC_{50} (nM)			
	WT	H381L	H381F	H381R
Agonists				
pBC 264	0.86 ± 0.17	1.69 ± 0.85	1.35 ± 0.54	1.50 ± 0.12
CCK-4	26.31 ± 2.01	13.51 ± 7.71	54.06 ± 9.12 [a,c]	ND
A-71623	1077 ± 115	867 ± 159	709 ± 362	ND
Antagonists				
PD-134,308	6.52 ± 3.01	3.49 ± 0.32	10.87 ± 2.39 [d]	ND
L-365,260	8.85 ± 0.21	2.97 ± 0.94 [b]	2.60 ± 0.99 [a]	4.52 ± 1.56
L-364,718	101 ± 23	7.61 ± 2.68 [b]	2.20 ± 0.16 [a]	99 ± 37 [d]
SR-27,897B	352 ± 84	19 ± 12 [b]	14 ± 6[b]	60 ± 14 [a,c]

IC_{50} values were calculated from competition binding experiments, using [^{3}H]pCCK-8 as radioligand, as described under Materials and methods. Data are expressed as the means ± S.E.M. of three to four experiments, each performed in triplicate. ND: not determined. [a] $P < 0.05$ and [b] $P < 0.005$ as compare to WT; [c] $P < 0.05$ and [d] $P < 0.01$ as compared to H381L (Student's t-test).

of the H381R mutant, two [^{3}H]pCCK-8 binding sites with $K_d = 2.6$ and 27.8 nM (data not shown) were observed.

3.2. Effects of the mutations on the binding of CCK agonists

Agonist binding to wild-type and mutated receptors was characterized by competition experiments using [^{3}H]pCCK-8 as the radioligand with three different compounds: pBC 264, a pseudopeptide deriving from CCK-8 (Charpentier et al., 1988) and CCK-4, which are both highly selective for CCK_B receptor, and A-71,623 a CCK_A receptor selective agonist which is derived from CCK-4 (Lin et al., 1991). Except for a slightly reduced affinity of CCK-4 for the H381F mutant, the IC_{50} values of these agonists were found to be very similar for both wild-type and mutated receptors (Table 1). Thus, the replacement of His^{381} by leucine, did not reduce the affinity of pBC 264 nor improve that of A-71,623.

3.3. Effects of the mutations on the binding of CCK antagonists

The affinity of the selective CCK_B receptor antagonist L-365,260 was of the same order of magnitude for the wild-type and H381L or H381F receptors (Table 1 and Fig. 2A). Interestingly, the binding of the peptoid CCK_B receptor antagonist PD-134,308 which belongs to a different class of molecules was found unchanged by the mutations.

In contrast, as shown in Table 1 and Fig. 2B, substitution of His^{381} by leucine resulted in a 13-fold increase in the CCK_A receptor selective antagonist L-364,718 affinity (wild-type, $IC_{50} = 101$ nM; H381L, $IC_{50} = 7.61$ nM), and a 19-fold increase in the SR-27,897B affinity (Wild-type, $IC_{50} = 352$ nM; H381L, $IC_{50} = 19$ nM). Substitution of His^{381} by phenylalanine caused 45-fold and 25-fold increases in L-364,718 and SR-27,897B affinity, respectively (Table 1). However, in competition experiments performed with the H381R mutant, the apparent affinity of L-364,718 for the high affinity binding site, was found to be almost identical ($IC_{50} = 99$ nM) as that for the wild-type receptor ($IC_{50} = 101$ nM).

3.4. Molecular modeling

A computer model of the CCK_B receptor was built up using the data on rhodopsin, as recently reported for the AT_1 receptor (Groblewski et al., 1995). Docking of the CCK_A receptor antagonist L-364,718 in the wild-type CCK_B receptor model shows the occurence of a repulsive interaction between the polar His^{381} residue and the phenyl part of the benzodiazepine ring in L-364,718 (Fig. 3A). In contrast, the docking of L-365,260 showed the absence of direct interactions between this CCK_B receptor antagonist and the residue located in position 381.

Replacement of His^{381} in the CCK_B receptor model

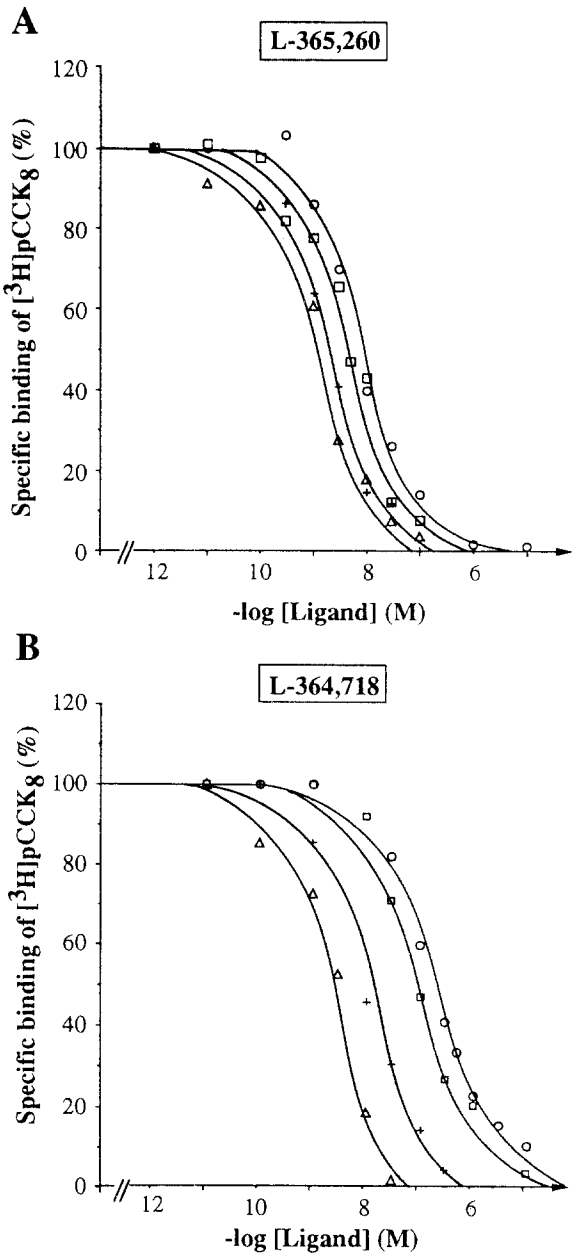

Fig. 2. Competition of CCK antagonists towards the binding of [^{3}H]pCCK-8 to wild-type and mutant rat CCK_B receptor. (A) Displacement curves for L-365,260. (B) Displacement curves for L-364,718. Transformed Cos-7 cells expressing wild-type (○), H381F (△), H381L (+), or H381R (□) mutant receptors were tested as described under Materials and methods. Each point is the average of triplicate determinations. Data which are representative of three to four separate experiments are depicted with S.E.M. values in Table 1.

Fig. 3. Computer-generated model of the CCK_B receptor. Top: longitudinal view of the wild-type CCK_B receptor in which both CCK_A and CCK_B receptor antagonists were docked. Histidine 381 (H381) and valine 354 (V354) are represented in yellow. L-364,718 is represented in red, and L-365,260 is represented in blue. For clarity, only transmembrane domains-III, -V, -VI, and -VII are displayed in green. Bottom: longitudinal stereoview of the wild-type (red), H381L (yellow) and H381F (blue) CCK_B receptors in which the CCK_A receptor antagonist L-364,718 was docked (with the corresponding colors). Only transmembrane domains-III, -V, -VI, and -VII are displayed in green. For each model, valine 354 is shown.

by leucine or phenylalanine (Fig. 3B) led to a slight change in the orientation of the mutant side chains which came into the proximity of other hydrophobic residues of the transmembrane helices, thus removing the repulsive interaction between the residue in position 381 and the CCK_A receptor antagonist. Due to their polarity, the histidine and arginine side chains are unable to reach these local hydrophobic environments which are identical, at least in terms of amino acids, in the rat CCK_A receptor.

4. Discussion

The cloning and sequencing of both CCK_A and CCK_B receptor types from different species have shown a marked amino acid sequence homology. Thus, in the seventh transmembrane domain, there are only five amino acids differing between both receptor types. This local difference has been suggested to play an important role in the preference of the benzodiazepine-derived antagonists L-365,260 for the CCK_B receptor and L-364,718 for the A receptor type (Mantamadiotis and Baldwin, 1994). The aim of this study was therefore to identify the amino acid(s) responsible for this selectivity. Among the five different residues, His^{381} was selected because: (i) based on a rhodopsin-derived model of G-protein-coupled receptor extended here to the CCK_B receptor, His^{381} was found to be embedded within the hydrophobic core formed by the seven helices and located near the position in which a lysine residue was shown to be involved in the retinal Schiff base formation (Bownds, 1967); (ii) site-directed mutagenesis experiments have shown that histidines present in transmembrane domains often play a key role in binding various agonists and/or antagonists to G-protein-coupled receptors (Olah et al., 1992; Fong et al., 1993; Fong et al., 1994; Huang et al., 1994). To determine the function of the His^{381} residue, site-directed mutagenesis was performed on the rat CCK_B receptor, and histidine was replaced by (i) a leucine (H381L), to locally exchange the CCK_B receptor sequence into that of CCK_A receptor; (ii) a phenylalanine (H381F), an amino acid similar to histidine but devoid of polarizable functions; (iii) an arginine (H381R) which is a polar residue with donor-acceptor groups like histidine.

The mutation of the His^{381} residue to leucine or phenylalanine had no apparent effect on the structural integrity of the receptor. Indeed, the affinities of the agonist [^{3}H]pCCK-8 were similar to that found with the wild-type receptor, and the expression level of the mutants were similar to those of the wild-type protein. Moreover, these results indicate that His^{381} does not play a critical role for the binding of the natural peptide ligand CCK-8 and that, at least for the H381F and H381L mutants, the native conformation of the receptor agonist binding site has not been critically affected by these changes.

By contrast, the replacement of His^{381} by arginine led to modification of the CCK-8 binding parameters probably due to the longer side chain and/or the positive charge of arginine. These structural changes of the mutated receptor could allow either two types of interaction of the radiolabelled CCK-8, or a mixture of two receptors, one of them possessing the same spatial organization as the wild-type. Similar findings have already been observed for mutants of various G-protein-coupled receptors (Mantamadiotis and Baldwin, 1994; Shapiro et al., 1993). Moreover, the fact that the mutations performed had no effects on the binding of the three agonists tested, confirms that His^{381} is not involved in the agonist binding site.

Mutations on the His^{381} residue had also no effects on the binding of CCK_B receptor selective antagonists. In contrast, the two antagonists highly selective for CCK_A receptors (L-364,718 and SR-27,897B) had increased affinities for H381L and H381F mutants. These two ligands were chosen because they belong to different chemical classes. This indicates that His^{381} residue could be responsible for the low affinity of these antagonists for the CCK_B receptor. Interestingly, replacement of the seventh transmembrane domain of rat CCK_B receptor was found to produce the same 13-fold increased affinity of L-364,718 for a chimaeric CCK_A/CCK_B receptor (Mantamadiotis and Baldwin, 1994). These results support the assumption that among the five amino acids which differ in the seventh transmembrane domain of CCK_B and CCK_A receptors, the His^{381} residue plays a critical role for CCK_A antagonist affinities in the CCK_B receptor. A similar result was observed in the case of the sixth transmembrane domain of the human CCK_B receptor, which possesses the same sequence as the rat receptor but differs from the canine CCK_B/gastrin receptor by five amino acids. Among all these residues, only one corresponding to Leu^{355} in the canine receptor and Val^{349} in the human receptor was shown to be critical in determining the binding affinity for the two benzodiazepine-derived antagonists (Beinborn et al., 1993).

The size and aromatic properties of His^{381} are not responsible for the lower affinity of the CCK_A receptor antagonists, since these ligands have enhanced affinities for the H381F mutant receptor. In contrast, the results obtained with the H381R mutant receptor suggest that the basic or polar characteristics for His^{381} could be responsible of the weaker affinity of L-364,718 and SR-27,897B for the CCK_B receptor.

In order to investigate the role of the residue in position 381 at the molecular level, a computer model of the CCK_B receptor was built up (Maigret et al., in preparation) using the data on rhodopsin as described

for the AT_1 receptor. The two benzodiazepine-based ligands L-364,718 and L-365,260 were then docked in the CCK_B receptor by taking into account the results of site-directed mutagenesis. Although in the abscence of a high resolution structure, the mutual localization and orientation of the transmembrane helices remain speculative, this approach is now currently used and has been shown useful to explain variations in affinity and specificity of non-peptide antagonists (Fong et al., 1994; Underwood et al., 1994). Moreover, it is important to note that the *C*-terminal part of the seventh transmembrane domain of both CCK receptors present a rather good homology with the corresponding sequence in the bovine opsin (Nathans and Hogness, 1983). In particular, Pro^{392} in CCK_B receptor corresponds to the proline which has been shown critical to maintain the structure of opsins (Strader et al., 1987; and references cited herein).

Structure-activity studies have previously shown that the main difference accounting for the A/B specificity of L-364,718 and L-365,260 is the chirality of the carbon bearing the amido- or ureido-side chains, respectively (Lotti and Chang, 1989; Freidinger, 1989). Docking of both antagonists in the wild-type CCK_B receptor model (Fig. 3A), illustrates these differences in spacial orientation of the two structurally close molecules, since a repulsive interaction between receptor and ligand was observed in the case of L-364,718 (consistent with the reduced affinity of this antagonist for the CCK_B receptor), and not in the case of L-365,260. Replacement of His^{381} by leucine or phenylalanine (Fig. 3B) lead to the removing of the direct interaction between this residue and the ligand, in agreement with the experimental results of binding studies. On the other hand, the slight increase in affinity of the CCK_B receptor antagonist for the H381F receptor, could be due to subtle changes in the structure of the receptor, favourizing ligand binding or, most probably, to thermodynamically improved interactions with an aromatic moiety of the antagonists. It is important to note that the docking of both antagonists into the CCK_B receptor model shows (Fig. 3A) their proximity to Val^{354}, in the sixth transmembrane domain as expected from a previous mutagenesis study (Beinborn et al., 1993). The differences in the position of L-365,260 and L-364,718 versus the valine side chain observed in this study could explain, in terms of van der Waals interactions, the 30-fold preference of the CCK_B receptor antagonist for the human wild-type CCK_B receptor, and the 6-fold increased affinity of L-364,718 following replacement of the valine residue by leucine or isoleucine (Beinborn et al., 1993). The very high transmembrane sequence homology between rat and human CCK_B receptors suggests that the results of site-directed mutagenesis obtained by using the rat receptor could be transposed to the human one.

The fact that the binding affinities of both A and B agonists following His^{381} mutations were unchanged, suggests that this amino acid does not contribute significantly to agonist binding. Differences between chemical determinants for agonist and antagonist binding has already been shown for peptide receptors, including the CCK_B receptor (Beinborn et al., 1993; Mantamadiotis and Baldwin, 1994; Fong et al., 1992; Kong et al., 1993). The affinity of L-364,718 for the H381L mutant was found lower than its affinity for the wild-type CCK_A receptor expressed in Cos-7 cells, ($IC_{50} \sim 1$ nM) (Wank et al., 1994) indicating that some specific chemical interactions other than His^{381} and Val^{355} stabilize the CCK_A receptor antagonist inside the receptor. Identification of the precise site of the chemical interactions for a series of related peptidomimetics or non-peptide ligands could allow the refinement of our three dimensional CCK receptor model. This could facilitate rational design of more potent and selective CCK receptor antagonists which seem to be endowed with promising therapeutic perspectives.

After this study was completed, a similar independent work was published (Kopin et al., 1995) in which the authors predicted the relative positions of residues among the transmembrane helices of the human CCK receptors, which are involved in A/B selectivity. Although eight amino acids seems to be responsible for this selectivity, the histidine residue located in the seventh transmembrane domain appears as the most important. These results are in complete agreement with our present data, except that we did not find any modification in agonists affinities, an apparent discrepancy which could be due to species differences since we used the rat CCK_B receptor instead of the human one. Furthermore, our study employing both CCK_A and CCK_B receptor specific antagonists of different chemical classes extend the critical role played by the histidine residue in A/B antagonist selectivity. Moreover, our proposed three dimensional model of receptor which meets the results of the two site-directed mutagenesis studies could now be used to elucidate the nature of the chemical interactions of A and B antagonists and to possibly improve their affinities. This is now in progress in our laboratory.

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