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Contrasting roles of Leu^{356} in the human CCK$_1$ receptor for antagonist SR 27897 and agonist SR 146131 binding

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Abstract

A new highly specific, potent non-peptide agonist for the cholecystokinin subtype 1 receptor (CCK$_1$), SR 146131 (2-[4-(4-chloro-2,5-dimethoxyphenyl)-5-(2-cyclohexyl-ethyl)-thiazol-2-ylcarbamoyl]-5,7-dimethyl-indol-1-yl-1-acetic acid) was recently described [Bignon, E., Bachy, A., Boigegrain, R., Brodin, R., Cottineau, M., Gully, D., Herbert, J.-M., Keane, P., Labie, C., Molimard, J.-C., Olliero, D., Oury-Donat, F., Petereau, C., Prabonneaud, V., Rockstroh, M.-P., Schaeffer, P., Servant, O.Thurneyssen, O., Soubrié, P., Pascal, M., Maffrand, J.-P., Le Fur, G., 1999. SR 146131: a new, potent, orally active and selective non-peptide cholecystokinin subtype I receptor agonist: I. In vitro studies. J. Pharmacol. Exp. Ther. 289, 742–751]. From binding and activity assays with chimeric constructs of human CCK$_1$ and the cholecystokinin subtype 2 receptor (CCK$_2$) and receptors carrying point mutations, we show that Leu^{356}, situated in transmembrane domain seven in the CCK$_1$ receptor, is a putative contact point for SR 146131. In contrast, Leu^{356} is probably not in contact with the CCK$_1$ receptor specific antagonist SR 27897 (1-[2-(4-(2-chlorophenyl)thiazol-2-yl)aminocarbonyl indoyl]acetic acid), a compound structurally related to SR 146131, since its replacement by alanine, histidine or asparagine gave receptors having wild-type CCK$_1$ receptor SR 27897 binding affinity. Previous mutational analysis of His^{381}, the cognate position in the rat CCK$_2$ receptor, had implicated it as being involved in subtype specificity for SR 27897, results which we confirm with corresponding mutations in the human CCK$_2$ receptor. Moreover, binding and activity assays with the natural CCK receptor agonist, CCK-8S, show that CCK-8S is more susceptible to the mutations in that position in the CCK$_1$ receptor than in the CCK$_2$ receptor. The results suggest different binding modes for SR 27897, SR 146131 and CCK-8S in each CCK receptor subtype.

Keywords: Cholecystokinin receptor; Mutagenesis; Chimeric receptor; Transmembrane domain seven; SR 27897; SR 146131; CCK-8S

1. Introduction

Two subtypes of human cholecystokinin (CCK) receptors have been cloned and characterized (Pisegna et al., 1992; De Weerth et al., 1993). The type 1 receptor, CCK$_1$, (formerly CCK$_A$) is found predominantly in the gastrointestinal system and the type 2, CCK$_2$, (formerly CCK$_B$) is most abundant in the central nervous system (Wank, 1995). Both subtypes belong to the membrane bound heptahelical G protein-coupled receptor (GPCR) superfamily. The tyrosine *O*-sulfated octapeptide CCK-8S (Fig. 1) is the natural agonist for CCK$_1$ receptors, inducing intracellular calcium mobilization through a cascade involving $G_{q/11}$, phospholipase C and inositol 1,4,5-triphosphate production (Wank, 1995; Tarasova et al., 1997). The receptors are implicated in various disease states and clinical disorders affecting both the central and peripheral nervous systems (Wank, 1998), which account for the considerable interest in developing receptor subtype-specific agonists and antagonists.

Recently, two highly specific and potent non-peptide ligands have been developed for the CCK$_1$ receptor, the antagonist SR 27897 (1-[2-(4-(2-chlorophenyl)thiazol-2-yl)aminocarbonyl indoyl]acetic acid) (Gully et al., 1993) and the structurally related agonist SR 146131 (2-[4-(4-chloro-2,5-dimethoxyphenyl)-5-(2-cyclohexyl-ethyl)-thiazol-2-ylcarbamoyl]-5,7-dimethyl-indol-1-yl-1-acetic acid) (Bignon et al., 1999) (Fig. 1). In order to further our

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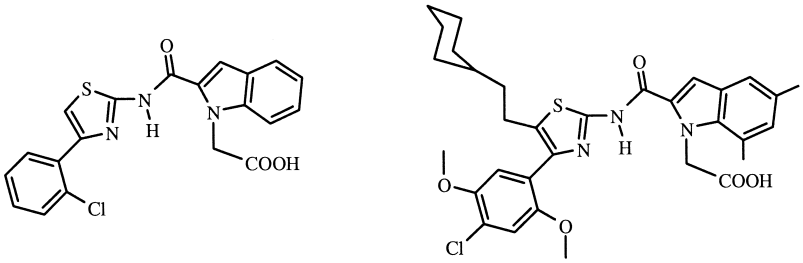
Asp^{1}-$Tyr^{2}(SO_3H)$-Met^{3}-Gly^{4}-Trp^{5}-Met^{6}-Asp^{7}-Phe^{8}-NH_2 CCK-8S



SR 27897 SR 146131

Fig. 1. The structure of CCK-8S and the CCK_1 receptor selective antagonist SR 27897 and CCK_1 receptor agonist SR 146131.

understanding of the factors that govern subtype selectivity for SR 27897 and SR 146131 in the CCK receptors, we have investigated the properties of various CCK_1 and CCK_2 receptor mutants and chimera. Based on previous results from various laboratories we have chosen to explore one position in transmembrane domain seven, that, following a study showing the importance of this domain for non-peptide antagonist binding (Mantamadiotis and Baldwin, 1994), has been shown to affect subtype specificity in the CCK receptors. Previous mutagenesis studies (Kopin et al., 1995; Jagerschmidt et al., 1996) showed that replacement of His^{381} in the rat CCK_2 receptor, equivalent to His^{376} in the human CCK_2 receptor, by the cognate CCK_1 receptor residue, leucine, greatly improved SR 27897 binding affinity, suggesting that this position was critical for receptor selectivity of the antagonist. In those studies, only mutant CCK_2 receptors were constructed and tested for effects on ligand binding. However, the authors did not study the reciprocal mutations in the CCK_1 receptor subtype. We have therefore continued this work and investigated the nature of subtype selectivity of both SR 27897 and SR 146131 in the CCK receptors. The mutagenesis studies we have performed suggest that binding of SR 27897 in the CCK_1 receptor does not involve interaction with Leu^{356}. In contrast, the mutation of Leu^{356} in the CCK_1 receptor had significant effects on the binding and activity of both CCK-8S and SR 146131.

2. Materials and methods

2.1. Drugs and chemicals

CCK-8S was purchased from Neosystem (Strasbourg, France). [^{3}H]CCK-8S (66 Ci/mmol), [^{3}H]SR 27897 (37 Ci/mmol), [^{3}H]SR 146131 (60–90 Ci/mol and [^{3}H]*myo*-inositol (5 μCi/ml) were purchased from Amersham (Les Ulis, France). SR 27987 and SR 146131 were synthesized at Sanofi Recherche (Montpellier, France). Dulbecco's modified essential medium (DMEM), foetal calf serum, phosphate-buffered saline (PBS), and lipofectamine were from Gibco (Paisley, UK). Bovine serum albumin, sodium pyruvate, and soya bean trypsin inhibitor (SBTI) were from Sigma (St. Louis, MO). Monkey kidney COS-7 cells were from the American Tissue Culture Collection (Reference CRL-1651).

2.2. Mutagenesis

A chimeric human CCK_1–CCK_2 receptor was constructed with the N-terminal domain of the CCK_1 receptor from Met^{1} to Leu^{324} in transmembrane domain six joined to Cys^{345} (comparable to Cys^{325} in the CCK_1 receptor from multiple sequence alignment) leading to the C-terminal domain of the CCK_2 receptor. All the point-mutated receptors were constructed using overlap extension polymerization chain reaction (Horton et al., 1989). Constructs were ligated into the p658 expression vector (Miloux and Lupker, 1994) and verified by dye termination sequencing.

2.3. Cell culture, transfections and competition binding

COS-7 monkey kidney cells (1×10^5 cells/well) were transfected directly in 24-well tissue plates using the lipofectamine protocol according to the manufacturer's instructions. Competition binding was performed 24–48 h post-transfection in 1 ml total binding buffer (DMEM supplemented with SBTI 1 mg/l, sodium pyruvate 25ml/l) and varying concentrations of cold ligands. [^{3}H]CCK-8S (1 nM final) or [^{3}H]SR 27897 (1 nM final) was used as radiolabeled ligand. Competition binding with CCK-8S, SR 27897 and SR 146131 was conducted on whole cells at room temperature. After 1 h, the binding media was aspirated and the cells were washed three times with PBS (1 ml) after which 1 ml of sodium hydroxide (3%) was used to lyse the cells. The lysate was analyzed for radiotracer concentration and the data analyzed using the GraphPad Prism software (GraphPad Software, San Diego, CA). [^{3}H]SR 146131 was not displaced by SR 146131, SR 27897 or CCK-8S in competition binding assays under these conditions (manuscript in preparation).

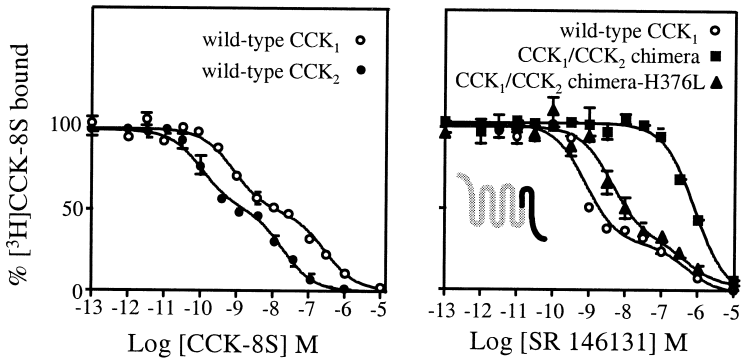


Fig. 2. Left panel: homologous competition binding curves for CCK-8S binding at wild-type CCK_1 and CCK_2 receptors transiently expressed in COS-7 cells showing two affinity states. Right panel: competition binding curves for SR 146131 vs. [^{3}H]CCK-8S at the chimeric CCK_1/CCK_2 receptor construct and the H376L mutated chimeric construct carried out in parallel with the wild-type CCK_1 receptor. The maximum binding is defined as maximal binding in the absence of competing ligand minus the background binding, defined as binding in presence of 10^{-5} M competing ligand. The points are the means ± S.E.M. of at least two separate experiments carried out in triplicate.

2.4. Inositol phosphate assays

COS-7 cells (1.10^5 cells/well) were transfected directly in 24-well tissue plates using the lipofectamine protocol. One day after transfection, the growth media was replaced by DMEM (400 μl) supplemented with [^{3}H]*myo*-inositol (5 μCi/ml). After 24 h, the growth media was removed and the cells washed with PBS (1 ml). Cells were then incubated at 37°C in the presence of DMEM supplemented with 20 mM LiCl (300 μl) for 30 min, after which various concentrations of ligand were added. After a further 60-min incubation in the presence of ligand, the media was aspirated and the cells washed once with PBS (1 ml). Ice-cold methanol/hydrogen chloride 0.1 M (50/50 v/v) was added immediately after washing to lyse the cells. The lysate was added to 1 ml Dowex columns and the inositol phosphates eluted using ammonium formate (0.2 M)/formic acid (0.1 M) and was then analyzed for radio-tracer concentration. The resulting data was analyzed using the GraphPad Prism software (GraphPad Software). Ninety-five percent of the radioactivity quantified was inositol monophosphate (IP1).

3. Results

3.1. Binding characteristics of CCK-8S, SR 146131 and SR 27897 with wild-type and chimeric CCK receptors

To verify the role played by transmembrane domain seven in ligand binding, a chimeric receptor was constructed comprising the amino-terminal region of the CCK_1 receptor fused to the carboxyl-terminal region of the CCK_2 receptor, the point of fusion being in transmembrane domain six between Leu^{324} of the CCK_1 receptor and Cys^{345} of the CCK_2 receptor. The chimera and the wild-type receptors were transiently expressed in COS-7 cells and competition binding assays were carried out on whole cells. The values for homologous competition binding of [^{3}H]CCK-8S with CCK-8S binding for the wild-type CCK_1 and CCK_2 receptors (Fig. 2, left panel) are best represented by curves reflecting two affinity states, with a slightly higher affinity of CCK-8S for the CCK_2 receptor than for the CCK_1 receptor, consistent with the observa-

Table 1
Pharmacological properties of wild-type, chimeric and mutated CCK_1 and CCK_2 receptors

Ligand	IC_{50} (nM)					EC_{50} (nM)	
	CCK-8S		SR 146131		SR 27897	CCK-8S	SR 146131
	High	Low	High	Low			
CCK_1	0.4 ± 0.2	285 ± 96	0.8 ± 0.4	110 ± 18	6 ± 0.2	0.4 ± 0.08	13 ± 0.9
L356A	354 ± 52		310 ± 54		9.6 ± 2.6	4.5 ± 1.2	387 ± 135
L356N	> 1000		600 ± 190		18.6 ± 6.4	19 ± 3.8	247 ± 93
L356H	3.3 ± 0.8	214 ± 94	1111 ± 194		9.8 ± 3	0.18 ± 0.05	289 ± 25
CCK_2	0.1 ± 0.03	14.5 ± 1.5	> 10000		200 ± 30	0.43 ± 0.02	> 10000
H376A	0.3 ± 0.02	89 ± 15	7.5 ± 0.2	236 ± 36	18 ± 5	13 ± 0.7	200 ± 80
H376L	0.3 ± 0.01	34 ± 5	3.5 ± 0.2	358 ± 56	22.6 ± 10	2 ± 0.5	75 ± 20
CCK_1/CCK_2	2.4 ± 1.5	1332 ± 600	493 ± 146		10 ± 2	1.2 ± 0.7	1020 ± 120
H356L	1.9 ± 0.5	326 ± 30	4.2 ± 0.9	511 ± 50	7.8 ± 2.7	0.5 ± 0.05	20 ± 6

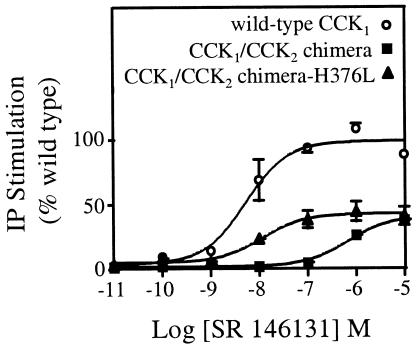


Fig. 3. Dose–response curves of SR 146131-induced inositol phosphate (IP) mobilization at the chimeric CCK_1/CCK_2 receptor construct and the H376L mutated chimeric receptor construct carried out in parallel with the wild-type CCK_1 receptor. Activity is given as a percent of maximum stimulation of the wild-type CCK_1 receptor. Maximum stimulation is defined as stimulation of the wild-type human CCK_1 receptor at 10^{-5} M agonist concentration. The points are the means ± S.E.M. of at least two separate experiments carried out in triplicate. The dpm values obtained were approximately 4500 for wild-type CCK_1 unstimulated, 45,000 for wild-type CCK_1 stimulated with 10^{-5} M CCK-8S and 38,000 for wild-type CCK_1 stimulated with 10^{-5} M SR 146131.

tions of others (Huang et al., 1994; Pandya et al., 1994). The IC_{50} values of CCK-8S for the wild-type CCK_1 and CCK_2 receptors (Table 1) are similar to those previously published (Pandya et al., 1994; Talkad et al., 1994a,b). Competition binding of SR 146131 with [^{3}H]CCK-8S also resulted in curves best representing two affinity sites (Fig. 2, right panel) (Talkad et al., 1994a,b).The affinity of SR 146131 for the CCK_2 receptor was barely detectable at 10 μM. Replacement of the transmembrane domain seven component of the CCK_1 receptor by that of the CCK_2 receptor in the chimeric transmembrane domain six fused construct resulted in a more than 600-fold reduction in SR 146131 binding affinity (Fig. 2, right panel and Table 1), whereas the high affinity of CCK-8S only fell six-fold, perhaps due to different binding modes of CCK-8S in the CCK_1 and CCK_2 receptors, as reported elsewhere (Silvente-Poirot et al., 1998). However, replacement of the transmembrane domain seven histidine, equivalent to His^{376} in the wild-type CCK_2 receptor, by leucine, the cognate CCK_1 receptor residue, Leu^{356}, resulted in a dramatic leftward shift in SR 146131 binding affinity (Fig. 2, right panel), to a value near that of the wild-type CCK_1 receptor (Table 1). At the same time, homologous competition binding assays of SR 27897 with [^{3}H]SR 27897 on the CCK_1 receptor, the chimeric receptor and the His–Leu mutated chimeric receptor gave essentially identical curves

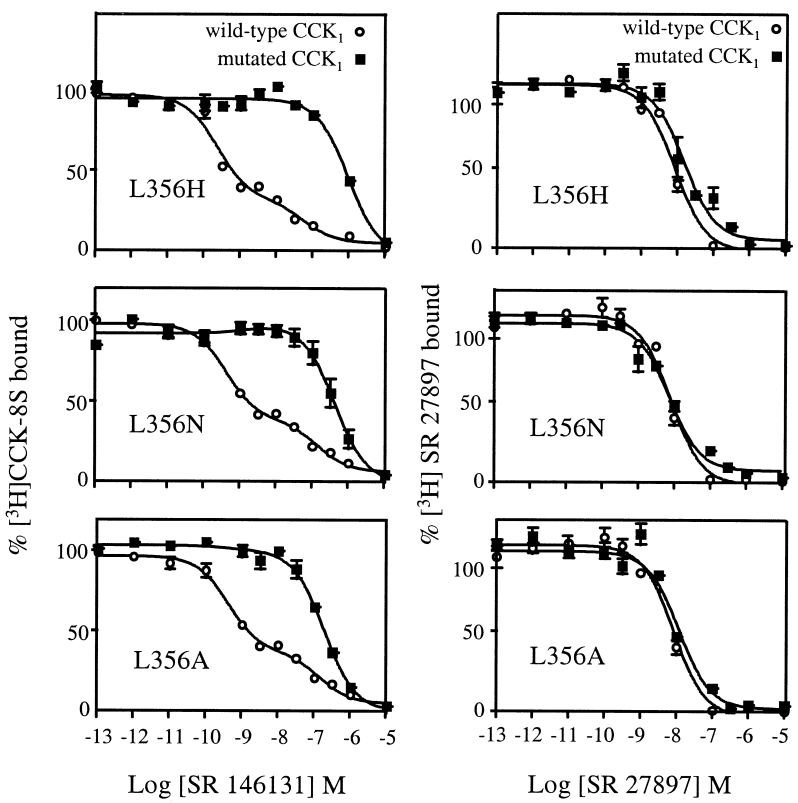


Fig. 4. Left panels: competition binding curves for SR 146131 vs. [^{3}H]CCK-8S at mutant CCK_1 receptors transiently expressed in COS-7 cells. Each experiment was conducted with the wild-type CCK_1 receptor as an internal standard. Right panels: homologous competition binding curves for SR 27897 at mutants CCK_1 receptors transiently expressed in COS-7 cells. Each experiment carried out in parallel with wild-type as an internal standard. The maximum binding is defined as maximal binding in the absence of competing ligand minus the background binding, defined as binding in presence of 10^{-5} M competing ligand. The points are the means ± S.E.M. of at least two separate experiments carried out in triplicate.

showing a single binding site (not shown) and similar IC_{50} values (Table 1). These results indicated dissimilar binding modes for the two, structurally related SR compounds, with Leu^{356} in transmembrane domain seven in the CCK_1 receptor potentially playing a predominant role for binding of the agonist SR 146131, but not of the antagonist SR 27897.

3.2. Inositol phosphate production induced by CCK-8S and SR 146131 in wild-type and chimeric CCK receptors

The potency of SR 146131 to induce inositol phosphate production in COS-7 cells expressing the CCK_1 receptor (Fig. 3) and the CCK_2 receptor (Table 1) was found to be similar to published values (Bignon et al., 1999). Previous values for CCK-8S potency in the two subtypes (Tarasova et al., 1997) were also confirmed. The potency of CCK-8S with the chimeric receptor fell only three-fold from the wild-type value, whereas that of SR 146131 fell 78-fold. The point mutation H376L in the chimera restored wild-type potency of both agonists (Fig. 3 and Table 1). The results obtained from these activity experiments with the wild-type and chimeric receptors were largely in line with the binding results, indicating a putative contact between Leu^{356} and SR 146131, the residue perhaps also playing some role in CCK-8S binding. These results incited us to look more closely at the importance of Leu^{356} in the CCK_1 receptor.

3.3. Binding characteristics of CCK-8S, SR 146131 and SR 27897 with CCK_1 Leu^{356} and CCK_2 His^{376} mutated receptors

It was previously reported that His^{381} in transmembrane domain seven of the rat CCK_2 receptor, corresponding to His^{376} of the human CCK_2 receptor, was not implicated in the high affinity CCK-8S binding site, since its mutation to the cognate residue in the CCK_1 receptor, leucine, or to phenylalanine, gave receptors with wild-type K_d values (Jagerschmidt et al., 1996). However, the mutations largely restored the CCK_1 receptor specific binding affinity of SR 27897. We confirm these observations, by showing that mutations of His^{376} in the human CCK_2 receptor to either leucine or alanine gave receptors with binding affinities for SR 27897 approaching those of the wild-type CCK_1 receptor (Table 1). However, this does not mean that SR 27897 binds in the same site on the two different CCK receptor subtypes. The CCK_2 receptor mutations, H376A and H376L, reduced CCK-8S binding only three-fold, but both had a remarkable effect on SR 146131 binding with more

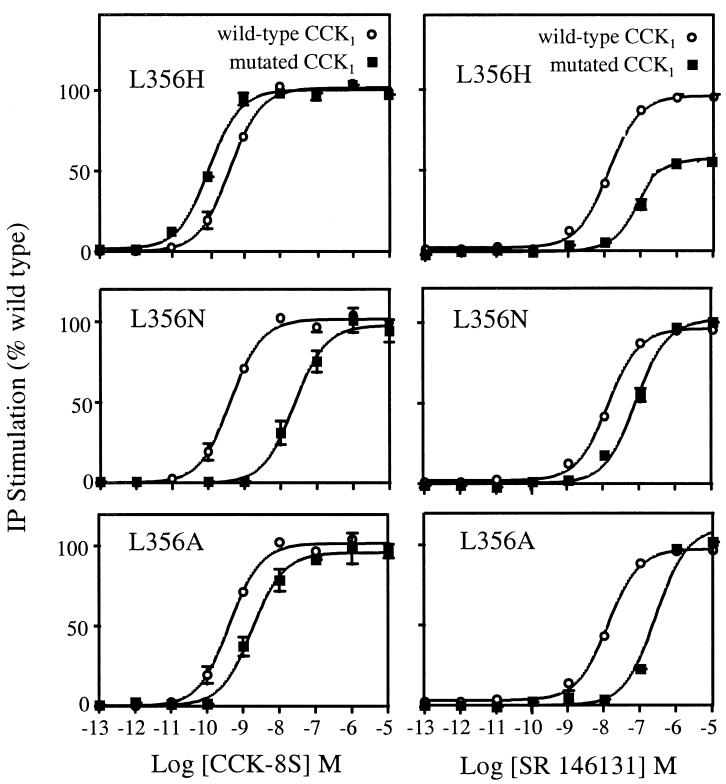


Fig. 5. Dose–response curves of CCK-8S and SR 146131-induced inositol phosphate (IP) mobilization with mutated CCK_1 receptors transiently expressed in COS-7 cells. Each experiment was carried out in parallel with wild-type as an internal standard. Activity is given as a percent of maximum stimulation of wild-type. Maximum stimulation is defined as stimulation of the wild-type human CCK_1 receptor at 10^{-5} M agonist concentration. The points are the means ± S.E.M. of at least two separate experiments carried out in triplicate. The dpm values obtained were approximately 4500 for the unstimulated wild-type CCK_1 receptor, 45,000 for the wild-type CCK_1 receptor stimulated with 10^{-5} M CCK-8S and 38,000 for the wild-type CCK_1 receptor stimulated with 10^{-5} M SR 146131.

than a 1000-fold improvement over the wild-type CCK_2 receptor value.

In contrast to the CCK_2 receptor mutations, the replacement of Leu^{356}, the cognate position of the CCK_1 receptor, by alanine or histidine, gave receptors with essentially wild-type binding affinities for the antagonist. The asparagine replacement of Leu^{356} resulted in a slight loss (three-fold) in affinity for SR 27897 (Fig. 4 and Table 1). These results are compatible with those obtained with the chimeric construct, by confirming that the carboxyl-terminal region of the CCK_1 receptor downstream from the point of fusion of the chimera in the upper part of transmembrane domain six is unimportant for SR 27897 binding to the CCK_1 receptor. Additionally, this would indicate different binding modes for SR 27897 to each receptor subtype.

On the other hand, the effect of the mutations on both CCK-8S and SR 146131 binding was striking. The L356H mutation resulted in an eight-fold reduction in the high affinity binding for CCK-8S compared to the wild-type CCK_1 receptor, whereas L356A and L356N had drastically lowered affinities for CCK-8S. However, unlike CCK-8S, with its eight-fold loss of the high affinity binding for L356H, SR 146131 binding affinity was lowered considerably for this mutation as well as for the L356A and L356N mutations (Fig. 4 and Table 1). The rank order of affinities of SR 146131 was wild-type > L356A > L356N > L356H, strongly suggesting that a hydrophobic interaction with Leu^{356} could be responsible for wild-type receptor affinity and again showing a fundamental difference between the binding mode of SR 146131 and those of CCK-8S and SR 27897.

3.4. Inositol phosphate production induced by CCK-8S and SR 146131 with CCK_1 receptors carrying Leu^{356} mutations

The loss in affinities for the agonists resulting from the point mutations in the CCK_1 receptor were not accompanied by an equivalent loss in potency mediated by the mutated receptors (Fig. 5 and Table 1). This was particularly the case for CCK-8S with the L356A mutation showing an 11-fold loss in potency for an 885-fold loss in high affinity binding; the L356N mutation having a 48-fold loss in potency at higher than micromolar binding affinity; and the L356H mutation showing a gain in potency despite an eight-fold loss in high affinity site binding. SR 146131 had similar potencies with the three mutated receptors. The lack of direct relationship between the high affinity binding values and potency was also observed for CCK-8S with the CCK_2 receptor mutants. The mutations had little effect on binding affinity, but potency was affected, particularly for the H376A mutant. The practically undetectable potency of SR 146131 at the wild-type CCK_2 receptor was partially restored with each of the two point-mutated receptors, the gain-in-activity effect being more striking than the loss-in-activity effect seen with the mutated CCK_1 receptor.

4. Discussion

The subject of the present study, the residue in transmembrane domain seven at position 719 (according to the global numbering scheme for GPCRs of Oliviera et al. (1993), is one that has long been known to play a key role in both ligand binding and subtype specificity in several monoamine and neuropeptide receptors (Suryanarayana et al., 1991; Fong et al., 1992a). Depending on the receptor under consideration, the position has been shown to contact agonists or antagonists or both. In the adrenoceptor family the position is occupied by a phenylalanine in the α subtype and asparagine in the β subtype receptor. Replacement of Phe^{412} in the α_2-adreneroceptor by asparagine increased the affinity for the β-adrenoceptor-specific antagonists pindolol, propranolol and alprenolol (Suryanarayana et al., 1991). It was later demonstrated that the selectivity was due to specific receptor–ligand contacts at position 719 (Kuipers et al., 1997). A similar effect was demonstrated in the 5-HT receptors, based again on β-adrenoceptor specific ligands (Adham et al., 1994). According to that report, replacing the endogenous residue at position 719 in the $5\text{-}HT_{1D\alpha}$, $5\text{-}HT_{1D\beta}$, $5\text{-}HT_{1E}$ and $5\text{-}HT_{1F}$ subtypes for asparagine resulted in mutant receptors that had significantly increased affinity over the wild-type receptor for certain β-adrenoceptor antagonists. In the neuropeptide family, the position 719 has been shown to be critical for natural peptide binding and selectivity for the neurokinin receptors. Mutation of Met^{291} in the tachykinin NK_1 receptor subtype to the cognate tachykinin NK_2 receptor and tachykinin NK_3 receptor residue, phenylalanine, resulted in a change of affinities for the natural peptides substance P, neurokinin A and neurokinin B (Fong et al., 1992b). The tachykinin NK_1 receptor mutant had lowered affinity for substance P, its natural ligand and increased affinity for neurokinin A and B. This result was complemented in the tachykinin NK_2 receptor where replacement of Phe^{293} by alanine resulted in loss of peptide binding affinity and activity (Huang et al., 1995). Replacement of Arg^{306} of the thyrotropin-releasing hormone receptor by alanine, glutamic acid or leucine resulted in 1500-, 3000- and 1200-fold loss of potency, respectively, for the endogenous ligand thyrotropin-releasing hormone receptor; however, substitution by lysine, to conserve the net positive charge, resulted in only a two-fold loss of K_d and a 10-fold loss of potency (Perlman et al., 1995).

Using site-directed mutagenesis we have attempted to define the nature of the subtype specificity associated with the global position 719 in the CCK receptors, Leu^{356} in the CCK_1 receptor and His^{376} in the CCK_2 receptor, for the two non-peptide compounds, SR 27897 and SR 146131. The data clearly show that the position, although impli-

cated in subtype specificity for SR 27897 in the CCK_2 receptor, confirming the results obtained with the rat CCK_2 receptor (Jagerschmidt et al., 1996), surprisingly is not implicated in SR 27987 binding in the CCK_1 receptor. The results of SR 27897 binding with the rat CCK_2 receptor and its mutants were interpreted as showing that the histidine residue was answerable for the low affinity binding of the antagonist to that subtype (Jagerschmidt et al., 1996). However, here we show that placing a histidine residue in this position in the CCK_1 subtype receptor had no effect on SR 27897 binding. The results can be attributed either to structural differences between the CCK_1 receptor and the CCK_2 receptor or to distinct binding sites for SR 27897 in the two receptor subtypes.

In contrast to SR 27897, SR 146131 was found to be susceptible to mutations at position 719 in both the CCK_1 receptor and the CCK_2 receptor. The replacement of Leu^{356} in the CCK_1 receptor by alanine resulted in a loss in affinity and lowered potency, whereas replacement of His^{376} in the CCK_2 receptor by leucine resulted in a receptor having near wild-type properties, with only five-fold less affinity and potency for SR 146131 than the wild-type receptor. The H376L CCK_2 receptor mutant had a better affinity for SR 146131 than H376A, suggesting that a large hydrophobic side chain at this position may play a role in SR 146131 recognition. The affinity of CCK-8S for the L356H CCK_1 receptor mutant was relatively unchanged to wild-type, suggesting the effects on SR 146131 affinity were not due to a misfolded receptor structure. In addition, none of the CCK_1 receptor mutants or chimera exhibited significantly reduced affinity for SR 27897 or B_{max} values (data not shown).

Despite their differences in affinity, all three CCK_1 receptor point mutants showed similar potency with SR 146131, clearly indicating that the potency was not directly related to the affinity. This was also seen with CCK-8S, for which there was no direct relationship between binding affinity for the mutated receptors and resulting potency. As for SR 146131, position 719 was found to be important for CCK-8S binding in the CCK_1 receptor, but not in the CCK_2 receptor, thereby supporting a previously stated hypothesis that the ligand binding site for CCK-8S differs for the two receptor subtypes (Silvente-Poirot et al., 1998).

Although the exact binding sites of CCK-8S or of synthetic ligands on the CCK_1 receptor have not yet been defined, there are growing numbers of reports of possible receptor–ligand contact points, particularly for CCK-8S. Covalent labeling of the CCK_1 receptor with biologically active analogues of CCK-8S have indicated contacts between the carboxyl terminus of the ligand with the amino terminus of the receptor just above transmembrane domain one (Ji et al., 1997) and of the glycine residue of CCK-8S with amino acids in the extracellular loop three-transmembrane domain seven region (Hadac et al., 1998). Site-directed mutagenesis experiments have also indicated CCK-8S contacts in the amino terminus of the CCK_1 receptor (Kennedy et al., 1997) and in extracellular loop one (Silvente-Poirot et al., 1998). The tyrosine of CCK-8S has been shown to contact Met^{195}, but the sulfate group was not directly involved in the contact (Gigoux et al., 1998). In a recent study of the role played by positively charged residues in ligand–receptor binding to the CCK_1 receptor, we found that alanine replacement of Lys^{115}, Arg^{197} and Arg^{336} abolished high-affinity CCK-8S binding and greatly reduced potency of inositol phosphate production (Gouldson et al., submitted for publication). In the same study, we found that mutation of Lys^{115} and Lys^{187} to alanine affected SR 27897 binding without affecting SR 146131-induced inositol phosphate production. Apart from those results and the work described here, the detection of possible contacts between the CCK_1 receptor and non-peptide ligands has not been fruitful. Inspired by a bacteriorhodopsin-based molecular model of the rat CCK_1 receptor into which the benzodiazepine derivative L-364,718 was docked (Van Der Bent et al., 1994), binding and functional studies of three point-mutated receptors, S139A, N349A and S379A were carried out (Smeets et al., 1997). However, the mutations suscitated only minor changes from wild-type behaviour and probably did not reflect important ligand–receptor contacts. In view of the important clinical potential of the CCK_1 receptor agonists and antagonists, efforts to delineate the binding sites of these interesting compounds and to determine their mode of action are well justified.

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