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The agonist SR 146131 and the antagonist SR 27897 occupy different sites on the human CCK_1 receptor

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Abstract

1-[2-(4-(2-Chlorophenyl)thiazol-2-yl) aminocarbonyl indoyl] acetic acid (SR 27897) is an effective CCK_1 receptor antagonist, while the structurally related molecule 2-[4-(4-chloro-2,5-dimethoxyphenyl)-5-(2-cyclohexyl-ethyl)-thiazol-2-ylcarbamoyl]-5,7-dimethyl-indol-1-yl-1-acetic acid (SR 146131) is a highly potent and specific agonist for the same receptor. To discover how the two molecules interact with the human cholecystokinin (CCK) CCK_1 receptor, we have carried out binding and activity studies with 33-point mutated receptors. Only six mutants showed altered [^{3}H]SR 27897 binding properties, Lys^{115}, Lys^{187}, Phe^{198}, Trp^{209}, Leu^{214} and Asn^{333}. In contrast, numerous mutations throughout the receptor either reduced SR 146131 agonist potency, Phe^{97}, Gly^{122}, Phe^{198}, Trp^{209}, Ile^{229}, Asn^{333}, Arg^{336} and Leu^{356} or increased it, Tyr^{48}, Cys^{94}, Asn^{98}, Leu^{217} and Ser^{359}. Only mutations of Phe^{198}, Trp^{209} and Asn^{333} affected both SR 27897 and SR 146131 binding or activity. The collated information was used to construct molecular models of SR 27897 and SR 146131 bound to the human CCK_1 receptor. The clear difference in the binding sites of SR 27897 and SR 146131 offers a molecular explanation for their contrasting pharmacological characteristics.

Keywords: CCK_1 receptor; Binding site; Modelling; SR 27897; SR 146131

1. Introduction

Cholecystokinin (CCK) is a peptide hormone implicated in the regulation of numerous physiological processes throughout the nervous system and in the gastrointestinal tract (Crawley and Corwin, 1994). Various CCK peptides result from the proteolysis of a 115 amino acid preprohormone; the sulfated octapeptide CCK-8S is the shortest fragment having full biological activity with the two CCK receptor subtypes CCK_1 and CCK_2 (Wank et al., 1992a,b), both of which are members of the seven transmembrane domain G-protein-coupled receptor superfamily. The receptors have about 50% amino acid identity and can be readily distinguished by their interaction with nonsulfated CCK-8, for which the CCK_1 receptor has low affinity and with the hormone gastrin, which is specific for the CCK_2 receptor. Recently, two potent and specific non-peptide ligands for the CCK_1 receptor have been described, the antagonist 1-[2-(4-(2-chlorophenyl)thiazol-2-yl) aminocarbonyl indoyl] acetic acid (SR 27897) (Gully et al., 1993) and the agonist 2-[4-(4-chloro-2,5-dimethoxyphenyl)-5-(2-cyclohexyl-ethyl)-thiazol-2-ylcarbamoyl]-5,7-dimethyl-indol-1-yl-1-acetic acid (SR 146131) (Bignon et al., 1999). The two molecules are derivatives of the same chemical class, SR 146131 simply differing from SR 27897 in that it is more highly substituted (Fig. 1b). The availability of two such molecules presents an excellent opportunity for investigating the architecture of the ligand binding sites on a seven transmembrane domain G-protein-coupled receptor.

Despite their structural similarities, previous data obtained from mutated human CCK_1 receptors indicated that the binding site of SR 27897 is quite different from that of SR 146131. It was found that Leu^{356} in transmembrane domain 7 of the CCK_1 receptor could be replaced without affecting the wild-type receptor binding characteristics of SR 27897, while the replacements considerably diminished SR 146131 activity (Gouldson et al., 1999). In addition, the two molecules contact different positively charged

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residues in the extracellular loop regions of the CCK_1 receptor. Arg^{336} was found to be implicated in SR 146131-mediated inositol phosphate accumulation and luciferase production in a reporter gene system, but not SR 27897 binding affinity (Gouldson et al., 2000). In contrast, Lys^{115} and Lys^{187} affected SR 27897 binding, but not SR 146131 activity. Furthermore, Lys^{115}, Arg^{197} and Arg^{336} have been shown to be important for the binding and activity of CCK-8S (Fig. 1b) (Gigoux et al., 1999a,b; Gouldson et al., 2000).

Since the CCK_1 receptor is implicated in various disease states (Wank, 1995, 1998), it was clearly of interest to investigate further the interactions of SR 146131 and SR 27897 with the receptor. To this end, we have conducted alanine scanning mutagenesis of numerous residues throughout the receptor. To discover whether the two agonists SR 146131 and CCK-8S have common points of contact on the CCK_1 receptor, we have also tested the binding and activity of CCK-8S with the mutated receptors. We have used the accumulated mutagenesis data to generate molecular models of SR 27897 and SR 146131 bound to the CCK_1 receptor. According to the model, SR 27897 sits vertically in the receptor, in contact with only the outer part of transmembrane domains 5 and 6 and extracellular loop 2. This orientation contrasts with that of SR 146131, which is found deeper in the receptor, situated perpendicularly to the position of SR 27897. SR 146131, being larger than SR 27897, contacts most of the transmembrane and extracellular loop regions. The clear difference in the binding sites of SR 27897 and SR 146131 would account for their contrasting pharmacological characteristics.

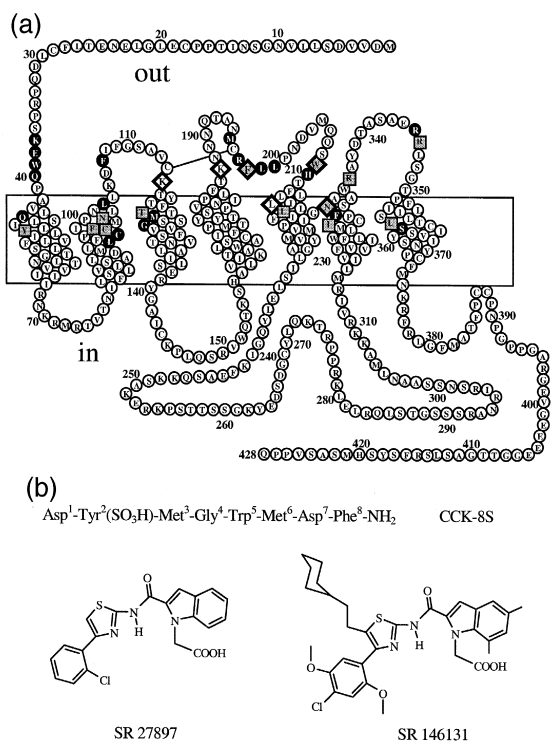

Fig. 1. The human CCK_1 receptor and its ligands. (a) Schematic representation of the human CCK_1 receptor showing the primary sequence with the mutated residues. Residues that have been shown to affect only SR 27897 binding are shown as diamonds with black letters on a white background. Residues that have been shown not to affect the binding of SR 27897 are shown as black circles with white letters. Residues that affect SR 146131 activity are shown as grey squares with black letters. Residues that affect both molecules are shown as diamonds with black letters on a grey background. The putative disulfide bridge is shown as a straight line. The box represents the membrane bilayer. (b) The formulae of CCK-8S and the CCK_1 receptor selective antagonist SR 27897 and agonist SR 146131.

2. Materials and methods

2.1. Drugs and chemicals

SR 27897 and SR 146131 were synthesised at Sanofi-Synthelabo Recherche, Montpellier, France. CCK-8S was purchased from Neosystem (Strasbourg, France). [^{3}H]SR 27897 (37 Ci/mmol), [^{3}H]SR 146131 (60 Ci/mmol). Propionylated [^{3}H]CCK-8S (60–90 Ci/mmol) and [^{3}H]*myo*-inositol (5 μCi/ml) were purchased from Amersham (Les Ulis, France). Dulbecco's modified essential medium, fetal calf serum, phosphate-buffered saline and lipofectamine were from Gibco (Paisley, UK). Bovine serum albumin, sodium pyruvate and soya bean trypsin inhibitor were from Sigma (St. Louis, MO). Monkey kidney epithelial COS-7 cells were from the American Tissue Culture Collection (Reference CRL-1651) and were maintained in Dulbecco's modified essential medium supplemented with sodium pyruvate (5 ml/l), fetal calf serum (5%) and were cycled twice weekly.

2.2. Mutagenesis

The coding region of the human CCK_1 receptor was ligated into the p658-derived expression vector (Miloux and Lupker, 1994) and point mutations were introduced using overlap extension polymerase chain reaction (Horton et al., 1989). All mutant constructs were verified by dye termination sequencing of the full mutant receptor coding region.

2.3. Binding assays

COS-7 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 on whole cells with either [^{3}H]CCK-8S (1 nM final) or [^{3}H]SR 27897 (1 nM final) was performed 48 h post transfection in 1 ml total binding buffer (Dulbecco's modified essential medium supplemented with soya bean trypsin inhibitor 1 mg/l, sodium pyruvate 5 ml/l) and varying concentrations of cold lig-

and. After 1 h, the binding media was aspirated and the cells were washed three times with ice cold phosphate-buffered saline (1 ml), after which 1 ml of sodium hydroxide (3%) was used to lyse the cells and the lysate analysed for radio tracer concentration. For binding data analyses, one- and two-site fits were tested. For all experiments, a two-site fit was considered better at $p < 0.05$. IC_{50} values were calculated by non-linear regression analysis using the PRISM software (GraphPad Software, San Diego, CA) and the significance of the difference compared to wild-type values tested using a one-way ANOVA test. B_{max} values were calculated from [^{3}H]SR 27897 binding experiments. Non-specific binding was determined as the binding in the presence of 10^{-5} M CCK-8S or SR 27897 for binding experiments performed with [^{3}H]CCK-8S and [^{3}H]SR 27897, respectively, and was always less than 25% of the total bound.

2.4. Inositol phosphate assay

COS-7 cells (1×10^5 cells/well) were seeded into 24-well tissue plates and transfected as above. After 24 h of incubation at 37°C/5% CO_2, the growth media was replaced by Dulbecco's modified essential medium (400 μl) supplemented with [^{3}H]*myo*-inositol (5 μCi/ml). After a further 24-h incubation (37°C/5% CO_2), the growth media was aspirated and the cells washed with phosphate-buffered saline (1 ml). Cells were then incubated (37°C/5% CO_2) in the presence of Dulbecco's modified essential medium supplemented with 20 mM LiCl (300 μl) for 30 min, after which various concentrations of agonist were added. After a further 60-min incubation in the presence of agonist, the media was aspirated and the cells washed with 1 ml ice cold phosphate-buffered saline. One milliliter of ice cold methanol/HCl 0.1 M (50/50 v/v) was added immediately after washing to lyse the cells. The lysate was maintained at 4°C until analysed as previously described (Gouldson et al., 1999). The EC_{50} values for each agonist was calculated with PRISM software (GraphPad Software). Ninety five percent of the radioactivity quantified was inositol monophosphate (IP_1). The significance of values obtained from the activity analysis was analysed using a one-way ANOVA test.

2.5. Receptor modelling

The model building and interactive docking of SR 27897 and SR 146131 were performed using WHATIF (Vriend, 1990). The minimisation and molecular dynamics simulations were conducted with the SYBYL modelling package (Tripos, St. Louis, MO 63144) using the TRIPOS force field and Kollman All Atom charges. The initial structures of SR 27897 and SR 146131 were constructed using interactive molecular graphics and energy minimised using MOPAC (keywords: MMOK, AM1), as implemented in SYBYL. The human CCK_1 receptor model was constructed using the method recently described for the rat β_2-adrenoceptor (Gouldson et al., 1997). The initial α-helix bundle was derived from a rat β_2-adrenoceptor model based on the α-carbon template of bovine rhodopsin, but altered to maximise proper helix–helix packing motifs (Gkoutos et al., 1999) and to fit with the recent 5 Å rhodopsin projection structure (Krebs et al., 1998). This resulted in alterations in the rotational and translational positions of transmembrane domain 5, transmembrane domain 6 and transmembrane domain 7. The transmembrane domain 5 region above the well-conserved proline was twisted approximately 20° clockwise (viewed from the extracellular side) and the whole helix translocated approximately 6–8 Å perpendicular to the membrane plane, towards the extracellular side. The alterations were made to allow functionally important positions found in other receptor subtypes, namely Leu^{214} and Leu^{217}, to face into the transmembrane helix bundle cavity (Fig. 1a). Intracellular loop 1 and extracellular loop 3 were added to the initial helix bundle using a protein data bank (PDB) search method as implemented in WHATIF (Vriend, 1990). The remaining, longer loops, extracellular loop 2 and intracellular loop 3 were added using a constrained dynamics method (Gouldson et al., 1997). A disulfide bridge was arbitrarily introduced between Cys^{114} and Cys^{196} (Fig. 1a) based on mutagenesis evidence obtained with the CCK_2 receptor (Silvente-Poirot et al., 1998). After loop addition, the side chain atoms were minimised with 10 steps of steepest descent, and 990 steps of conjugate gradient minimisation. Finally, the entire model was refined with 10 ps of molecular dynamics (298 K, non-bond cut-off 12 Å, distance-dependent dielectric, time step 0.002 ps) followed by energy minimisation to yield an initial human CCK_1 receptor that was used for ligand docking. A copy of the receptor model coordinates has been deposited at GPCRDB.

3. Results

3.1. SR 146131 and CCK-8S do not compete well for the [^{3}H]SR 27897 binding site on the wild-type CCK_1 receptor

The results of binding experiments performed on the wild-type CCK_1 receptor with [^{3}H]SR 27897 competed against SR 27987, SR 146131 and CCK-8S are shown in Fig. 2. The IC_{50} values obtained were: 6.0 ± 0.2 nM for SR 27897, 520 ± 30 nM for SR 146131, and by extrapolation, $> 100{,}000$ nM for CCK-8S. These data show that SR 146131 competes badly and CCK-8S not at all for the binding site of [^{3}H]SR 27897. As an antagonist, SR 27897 is conceivably binding with high affinity to a different site or to a different allosteric state of the receptor for which CCK-8S and SR 146131 have low affinity.

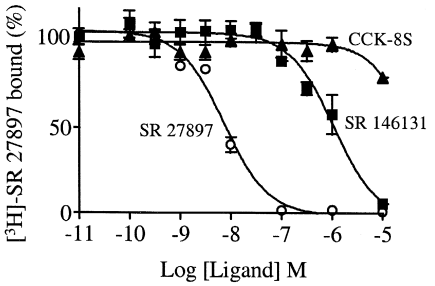

Fig. 2. Competition binding curves for [^{3}H]SR 27897 competed against SR 27897, SR 146131 and CCK-8S. Binding is given as percent total [^{3}H]SR 27897 bound. Non-specific binding was estimated as total [^{3}H]SR 27897 bound in the presence of 10^{-5} M competing ligand. Results are the means ± S.E.M. from two independent experiments performed in triplicate.

3.2. Only six out of 33 mutated residues of the CCK_1 receptor affect [^{3}H]SR 27897 binding affinity

Point mutations to residues in the amino-terminal region and transmembrane domains 1, 2, 3 and 7 of the human CCK_1 receptor resulted in receptors having essentially wild-type expression levels and binding affinity for SR 27897 (Table 1). Mutations that did affect SR 27897 binding affinity were to found in the first and second extracellular loops and in transmembrane domains 5 and 6 (Table 1 and Fig. 1a). Of the two lysine residues occurring in extracellular loop 1, only mutation of Lys^{115} affected SR 27897 binding affinity, reducing it 56-fold. Five residues were mutated in extracellular loop 2; the SR 27897 binding affinity to two of the mutations, K187A and F198A, was reduced 68- and >400-fold, respectively. Of the two neighbouring residues to Phe^{198}, the mutation of Leu^{199} had no effect on SR 27897 binding affinity, whereas the R197A mutant had a 2.8-fold reduced binding affinity. In transmembrane domain 5, two residues were found to be important for SR 27897 binding affinity, Leu^{214}, the mutation of which improved the affinity 6.25-fold and Trp^{209} whose replacement by alanine resulted in a receptor with an unmeasurable affinity. The sixth of the 33 residues mutated that proved to affect SR 27897 binding was Asn^{333} in transmembrane domain 6, the N333A mutant having a 312-fold reduced binding affinity. Apart from the K187A mutation, each of the mutations that affected SR 27897 binding affinity also reduced B_{max} levels two- to threefold (Table 1).

3.3. Several mutants in the amino-terminal region, transmembrane domain 1 and transmembrane domain 2 affect SR 146131 and CCK-8S activity

The K37A, E38A, W39A and Q40A mutations in the amino-terminal region had no effect on either the potency or efficacy of SR 146131-induced inositol phosphate accumulation (Table 2). Apart from a 20-fold loss of high affinity CCK-8S binding observed with W39A, the three mutations had little effect on CCK-8S binding and activity. Q44A, at the top of transmembrane domain 1, improved SR 146131 potency fourfold, but reduced CCK-8S high-affinity binding 10-fold. Interestingly, Arg^{57}, the cognate residue of Gln^{44} in the CCK_2 receptor, exhibited a 20-fold loss of CCK-8S binding affinity (K_d) when mutated to Ala (Silvente-Poirot et al., 1998). Y48A showed a sevenfold loss of potency for SR 146131 and a dramatic loss of CCK-8S binding and activity. The L90A and C91A receptors had essentially wild-type properties with SR 146131 or CCK-8S. The three succeeding mutants in transmembrane domain 2, C94L, F97A and N98A, turned out to be vital for both SR 146131 and CCK-8S activity. However,

Table 1
Results from homologous [^{3}H]SR 27897 competition binding at wild-type and mutant human CCK_1 receptors. Global numbering is according to Oliveira et al. (1993)

Mutation	Position	SR 27897 (IC_{50}, nM)	[^{3}H]SR 27897 (B_{max}, pmol/10^5 cells)
$hCCK_1$	–	5.0 ± 0.2	0.26 ± 0.03
K37A[a]	N-terminal	5.8 ± 0.2	0.25 ± 0.09
E38A	N-terminal	5.6 ± 1.2	0.29 ± 0.09
W39A	N-terminal	5.6 ± 0.3	0.36 ± 0.09
Q40A	N-terminal	5.1 ± 0.1	0.35 ± 0.05
Q44A	115	5.8 ± 1.1	0.30 ± 0.08
Y48A	119	3.5 ± 0.2	0.26 ± 0.02
L90A	227	3.7 ± 0.3	0.22 ± 0.02
C91A	228	7.5 ± 2.5	0.35 ± 0.05
C94L	231	1.5 ± 0.8	0.25 ± 0.01
F97A	234	3.9 ± 0.5	0.30 ± 0.02
N98A	235	10.1 ± 1.1	0.27 ± 0.02
K105A[a]	EL1	12.2 ± 6	0.29 ± 0.05
K115A[a]	EL1	282.3 ± 61[b]	0.15 ± 0.02[c]
T118A	319	2.1 ± 0.3	0.28 ± 0.02
T118S	319	8.9 ± 1.7	0.26 ± 0.06
M121A	322	5.3 ± 1.2	0.35 ± 0.05
G122L	323	5.8 ± 0.3	0.24 ± 0.01
K187A[a]	EL2	338.2 ± 58.3[b]	0.25 ± 0.01
R197A[a]	EL2	14.2 ± 5.1	0.30 ± 0.01
F198A	EL2	> 2000[b]	0.12 ± 0.05[c]
L199A	EL2	3.1 ± 0.6	0.23 ± 0.01
L200A	EL2	2.0 ± 0.1	0.28 ± 0.02
W209A	508	unmeasurable	0.09 ± 0.01[b]
H210A[a]	509	6.6 ± 0.3	0.21 ± 0.05
L214A	513	0.8 ± 0.02[c]	0.29 ± 0.08
L217A	516	6.0 ± 0.2	0.13 ± 0.01[c]
I329A	621	8.0 ± 0.3	0.29 ± 0.02
F330A	622	7.0 ± 0.5	0.13 ± 0.03[c]
N333A	625	1559 ± 300[b]	0.10 ± 0.01[c]
R336A[a]	EL3	9.0 ± 0.5	0.35 ± 0.09
R336D[a]	EL3	12.0 ± 4.1	0.12 ± 0.02[c]
R345A[a]	EL3	5.2 ± 1.2	0.30 ± 0.10
R346A[a]	EL3	5.9 ± 0.9	0.29 ± 0.02
L356A[d]	719	8.6 ± 2.6	0.21 ± 0.05
S359A	722	8.1 ± 2.1	0.29 ± 0.02

[a] Gouldson et al., 2000.
[b] Differs from wild-type CCK_1 receptor with $P < 0.001$.
[c] Differs from wild-type CCK_1 receptor with $P < 0.01$.
[d] Gouldson et al., 1999.

Table 2
Results from homologous CCK-8S binding at wild-type and mutant human CCK_1 receptors and biological activities of CCK-8S and SR 146131. IC_{50} values ± S.E.M from competition binding assays and EC_{50} values ± S.E.M for agonist potency measured using an inositol phosphate production assay were calculated from three to four separate experiments performed in triplicate with wild-type as an internal standard. Agonist efficacies were estimated from the inositol phosphate assays, maximum stimulation being defined as stimulation of wild-type human CCK_1 receptor with 10^{-5} M agonist (either CCK-8S or SR 146131). Global numbering is according to Oliveira et al. (1993)

Mutation	Position	CCK-8S				SR 146131	
		IC_{50high}	IC_{50low}	EC_{50} (IP_1)	Efficacy (%)	EC_{50} (IP_1)	Efficacy (%)
$hCCK_1$	–	0.44 ± 0.2	230 ± 80	0.4 ± 0.08	100	13 ± 0.9	100
K37A[a]	N-term	not done	not done	0.5 ± 0.1	100	10 ± 1.2	100
E38A	N-term	0.4 ± 0.01	100 ± 45	0.3 ± 0.08	100	11 ± 2	100
W39A	N-term	8.3 ± 1.2[b]	264 ± 68	0.6 ± 0.12	100	14 ± 0.8	100
Q40A	N-term	0.32 ± 0.15	430 ± 21	0.45 ± 0.08	100	18 ± 0.5	100
Q44A	115	4.4 ± 0.2	300 ± 35	0.8 ± 0.35	100	3.5 ± 0.7[c]	100
Y48A	119	968 ± 100		180 ± 47[b]	71[c]	88 ± 13[b]	100
L90A	227	0.6 ± 0.1	280 ± 55	0.8 ± 0.2	100	14 ± 1.4	100
C91A	228	0.4 ± 0.15	300 ± 69	1.9 ± 1.0	100	15 ± 1.4	100
C94L	231	5000 ± 600[b]		undetectable		2.5 ± 0.8[c]	22[b]
F97A	234	3994 ± 550[b]		12 ± 2.7[b]	100	> 1000[b]	20[b]
N98A	235	427 ± 86[b]		238 ± 195[b]	34[b]	3.5 ± 0.8[c]	150[c]
K105A[a]	EL1	0.34 ± 0.1	478 ± 65	1 ± 0.16	100	20 ± 0.6	100
K115A[a]	EL1	undetectable		14 ± 5.7[b]	55[c]	12 ± 0.1	35[b]
T118A	319	0.4 ± 0.12	316 ± 35	0.34 ± 0.12	100	3.9 ± 0.89[c]	100
T118S	319	0.6 ± 0.4	325 ± 75	0.26 ± 0.05	100	25 ± 6	100
M121A	322	5.8 ± 0.5[b]		6.6 ± 1.2[b]	100	8.9 ± 2.1	67[c]
G122L	323	1200 ± 132[b]		> 1000[b]	52[c]	> 1000[b]	68[c]
K187A[a]	EL2	0.9 ± 0.05	1476 ± 180[b]	1.0 ± 0.2	100	11 ± 6	100
R197A[a]	EL2	undetectable		356 ± 30[b]	69[c]	20 ± 6	100
F198A	EL2	undetectable		20 ± 1.2[b]	64[c]	65 ± 8[c]	20[b]
L199A	EL2	0.37 ± 0.1	31 ± 8[c]	0.59 ± 0.06	100	12 ± 2.1	100
L200A	EL2	427 ± 49[b]		18 ± 5.1[b]	100	12 ± 1.2	100
W209A	508	undetectable		288 ± 32[b]	100	undetectable	
H210A[a]	509	0.19 ± 0.05	355 ± 85	0.39 ± 0.09	100	17 ± 1.6	100
L214A	513	0.08 ± 0.01[c]	16 ± 2[c]	0.35 ± 0.05	100	10.9 ± 3.7	70[c]
L217A	516	2.2 ± 0.5[c]	480 ± 90	0.25 ± 0.05	60[c]	3.8 ± 1.4[c]	100
I329A	621	undetectable		24 ± 4.8[b]	100	114 ± 10[b]	100
F330A	622	0.03 ± 0.01[c]	13 ± 1[c]	0.6 ± 0.1	60[c]	12 ± 4	60[c]
N333A	625	undetectable		773 ± 222[b]	62[c]	1032 ± 180[b]	60[c]
R336A[a]	EL3	undetectable		570 ± 89[b]	100	138 ± 13[b]	66[c]
R336D[a]	EL3	undetectable		121 ± 50[b]	20[b]	> 2000[b]	50[b]
R345A[a]	EL3	0.25 ± 0.1	91 ± 53[c]	1.0 ± 0.22	100	11.3 ± 3.5	100
R346A[a]	EL3	0.40 ± 0.1	210 ± 45	0.23 ± 0.1	100	5 ± 2[c]	100
L356A[d]	719	354 ± 54[b]		4.5 ± 1.2[b]	100	387 ± 135[b]	100
S359A	722	0.21 ± 0.08	125 ± 22	0.27 ± 0.1	100	4.2 ± 2.1[c]	100

[a] Gouldson et al., 2000.
[b] Differs from wild-type CCK_1 receptor with $P < 0.001$.
[c] Differs from wild-type CCK_1 receptor with $P < 0.01$.
[d] Gouldson et al., 1999.

the type of interactions made by the two agonists in this region are obviously quite different. The C94L mutant resulted in a sevenfold increase in SR 146131 potency, but a 78% reduced efficacy, which contrasted with a dramatic loss of CCK-8S binding affinity and undetectable potency or efficacy. F97A showed a significant loss of SR 146131 potency and CCK-8S affinity, which was accompanied by a small affect on CCK-8S potency. N98A, like Q44A, showed a fourfold increase in potency of SR 146131. The N98A mutant was the only one to show an increased efficacy of SR 146131 (150%), and again this augmentation of SR 146131 activity was matched with diminished CCK-8S binding and activity.

3.4. Several mutants in extracellular loop 1, transmembrane domain 3 and extracellular loop 2 affect SR 146131 and CCK-8S activity

The mutations in transmembrane domain 3 of Thr^{118} and Met^{121} suggest that SR 146131 contacts the receptor in this area (Table 2). T118A displayed a threefold improved potency of SR 146131, but T118S had a twofold loss of potency. Perhaps, a hydrogen bond between this position and SR 146131 prevents the ligand from exerting its full effect. In agreement with published observations concerning the cognate position in the CCK_2 receptor (Kopin et al., 1995), neither of the Thr^{118} mutants affected CCK-8S

binding and activity. M121A, although showing wild-type potency for SR 146131, did show a 33% drop in efficacy compared to wild-type and the effects on CCK-8S binding and activity suggest this ligand might also contact the receptor in this position. G122L resulted in loss of high affinity CCK-8S binding and considerably reduced CCK-8S and SR 146131 potency. The efficacies of both CCK-8S and SR 146131 at G122L were significantly lower than wild type, 52% and 68%, respectively.

Of the mutants in extracellular loop 2, only F198A and W209A affected the binding and activity of the two agonists. F198A exhibited a fivefold loss of potency and 80% reduced efficacy, with the effects on CCK-8S being comparable. W209A had undetectable CCK-8S and SR 146131 potency. The effects of the W209A mutant may be due to low expression or misfolding as suggested by the low B_{max} value. The L199A and L200A mutations had no effect on SR 146131 potency, whereas the L200A mutation showed a loss of CCK-8S binding affinity and potency. It should be noted that the potency of SR 146131 at both K115A and K187A mutant CCK_1 receptors did not diverge significantly from the wild-type value (Table 2), in contrast to the reduced binding affinity observed for SR27897 with both these mutants. A significant difference between SR146131 and CCK-8S is that Lys^{115} is an important contact for CCK-8S, but not for SR146131.

3.5. Several mutants in transmembrane domain 5 and transmembrane domain 6 affect SR 146131 and CCK-8S activity

L214A showed wild-type potency for SR 146131 but reduced efficacy, whereas CCK-8S had increased binding affinity without a change in potency (Table 2). SR 146131 had a fourfold increased potency, but with no change in efficacy with the L217A mutant. In contrast, for this

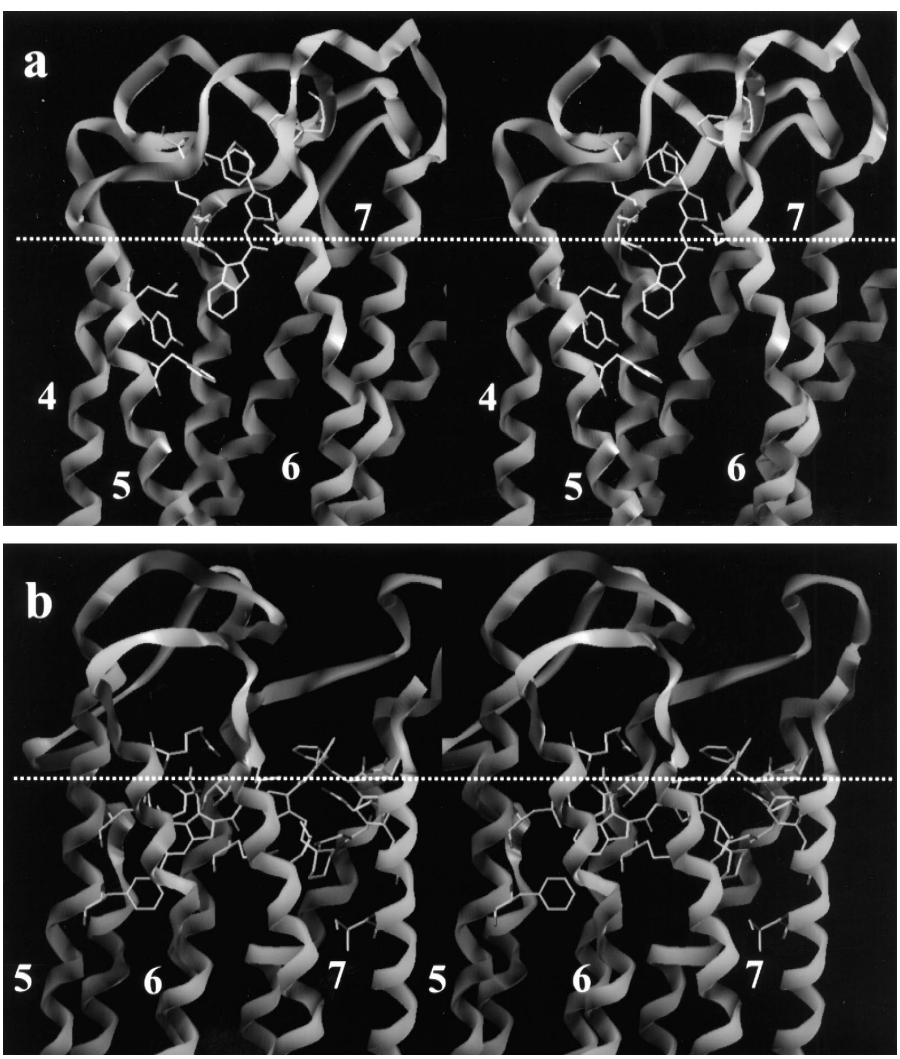

Fig. 3. Stereo views showing the side chain contacts between (a) SR 27897 and (b) SR 146131 with the human CCK_1 receptor model (as listed in Tables 3 and 4). The receptor is shown from slightly different angles to better show the position of the ligands (yellow). The dotted white line shows the approximate outer membrane surface. Transmembrane domains 5, 6 and 7 are labelled.

mutant CCK-8S had 5.5-fold reduced affinity and only 60% efficacy. I329A showed a 10-fold loss of SR 146131 potency without an effect on the efficacy. CCK-8S binding to the I329A mutant was undetectable in the assay used here, although it activated inositol phosphate production with wild-type efficacy and with 60-fold lowed potency. F330A showed wild-type potency for SR 146131 but a 40% loss of efficacy, perhaps due to a lowered B_{max} (Table 1). This was mirrored for CCK-8S potency, but CCK-8S affinity was increased 10-fold, similar to that seen for L214A. Perhaps, Leu^{214} and Phe^{330} are in close proximity and removal of either one allows for better interaction between the receptor and CCK-8S or alternatively these positions are involved in receptor activation. N333A showed much reduced SR 146131 and CCK-8S activity with both potency and efficacy being affected. The effect on efficacy may be due to a lowered B_{max} for this mutant (Table 1).

3.6. Two mutants in extracellular loop 3 and transmembrane domain 7 affect SR 146131 and CCK-8S activity

We previously reported impairment of SR 146131 activity and of CCK-8S binding and activity following mutations of Arg^{336} (Gouldson et al., 2000) and of Leu^{356} (Gouldson et al., 1999) and have incorporated the results in Table 2 for completeness.

3.7. Molecular model of SR 27897 bound to human CCK_1 receptor

Since only six mutated receptors affected SR 27897 binding (Table 1), we were able to exclude many residues as potential contact points for the ligand. SR 27897 was first roughly positioned in the receptor based on the results described here. Our initial CCK_1 receptor model had to be slightly modified in order to respect a putative contact between SR 27897 and Asn^{333} and to rectify a steric clash between SR 27897 and Leu^{214} that was observed in the unmodified model. Consequently, for the docking of SR 27897, these constraints necessitated a translocation of transmembrane domain 6 by approximately 5–6 Å up towards the extracellular side of the receptor. The interaction with Asn^{333} can be through a hydrogen bond acceptor or donor with the SR 27897 through either its peptide amine or carboxyl group. An important question related to the depth of penetration of SR 27897 into the helix bundle. Replacement of Gly^{122} by the more cumbersome Leu had no effect on SR 27987 binding, showing that the antagonist does not contact the receptor in this region and probably does not penetrate down into the receptor below the position of this residue. This reinforces the probability that SR 27897 contacts only the extremities of the transmembrane domains; the results with S359A corroborated this hypothesis.

After the initial positioning of SR 27897 using the information described above, the receptor–ligand complex was energy minimised with 250 ps of constrained molecular dynamics at 298 K, using a non-bonded cut-off of 12 Å, time step of 0.002 ps and a distance-dependent dielectric. The reasons for this are discussed elsewhere (Gouldson et al., 1995). Constraints were used to form the interaction between (i) the carboxylate group of SR 27897 with both Lys^{115} and Lys^{187}, (ii) the peptide amine and carboxyl with Asn^{333} and (iii) the phenyl ring of SR 27897 with Phe^{198}. The extracellular loops and first turn of the α-helices were unconstrained during the molecular dynamics with the rest of the receptor structure fixed. The dynamics protocol was used to alleviate unfavourable steric clashes that arose from the initial positioning of SR 27897 in the receptor model. This energy minimised model was then subjected to 10 cycles of simulated annealing (heating for 10 ps at 500 K, cooling to 200 K over 10 ps using a logarithmic temperature descent curve). After simulated annealing the model was subjected to a final 1000 steps of conjugate gradient minimisation to yield a final model structure.

Fig. 3a shows a stereo view of SR 27897 docked into the receptor and Fig. 4a is a schematic representation of the contact points of the receptor with SR 27897. Table 3

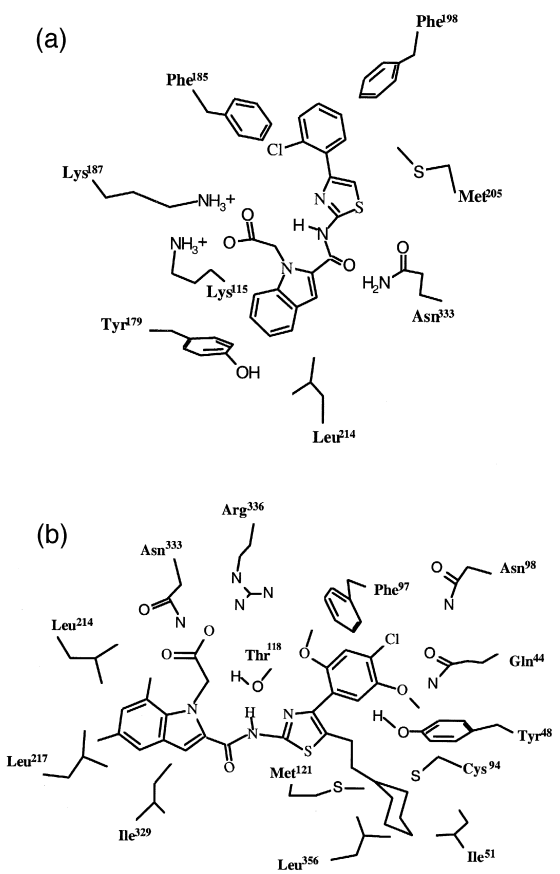

Fig. 4. Schematic representation of the side chain contacts between (a) SR 27897 and (b) SR 146131 and the human CCK_1 receptor as depicted in Fig. 3a and b and as described in Tables 3 and 4.

Table 3
The contacts made between SR 27897 and residues in the human CCK_1 receptor. Contact is defined as side chain or backbone van der Waals interaction i.e. atomic contacts between 3 and 4 Å. Global numbering is according to Oliveira et al. (1993)

Contact in model	Receptor region	Type of contact	Effect of mutation to Ala on SR 27897 binding
Lys^{115}	EL1	charge–charge	reduced affinity, lowered B_{max}
Tyr^{179}	EL2	aromatic	not done
Phe^{185}	EL2	aromatic	not done
Lys^{187}	EL2	charge–charge	reduced affinity
Phe^{198}	EL2	hydrophobic	reduced affinity, lowered B_{max}
Leu^{214}	513	hydrophobic	increased affinity
Asn^{333}	625	H-bond	reduced affinity, lowered B_{max}

lists the human CCK_1 receptor residues in contact with SR 27897 in the final energy minimised model. According to the model, apart from the contacts described above, the phenyl ring of SR 27897 appears to be in close contact with Tyr^{179} and Phe^{185} in transmembrane domain 4 through hydrophobic or aromatic interactions. In addition, Leu^{214} contacts the indole group in SR 27897, probably through hydrophobic interactions. We believe that SR 27897 is situated near the surface of the receptor, because of the lack of effect on binding affinity of mutations situated deeper in the receptor cavity, namely Y48A, C94L, M121A, G122L, I329A, F330A, L356A and S359A.

3.8. Molecular model of SR 146131 bound to the human CCK_1 receptor

SR 146131 was docked into the CCK_1 receptor model with aromatic, charge–charge, hydrophobic and hydrogen bond interactions being taken into consideration. Two putative contacts were used to initially orientate the molecule. We demonstrated previously that R336A was the only charged residue to affect SR 146131 potency (Gouldson et al., 2000). We therefore hypothesised that this residue might form a salt bridge with the carboxylate group of SR 146131. If this were true, the R336D mutation should show a greater loss of potency for SR 146131. This turned out to be the case (Table 2), as reported previously (Gouldson et al., 2000). The remaining charged residues in the extracellular domains of the receptor, Lys^{37}, Lys^{105}, Arg^{197}, Arg^{345} and Arg^{346} can be excluded from forming part of either the SR 146131 or SR 27897 binding sites (Table 2). The second contact was Leu^{356} in transmembrane domain 7, following the discovery that SR 146131 activity, but not SR 27897 binding, seemed to be dependent on a hydrophobic residue at this position (Gouldson et al., 1999). An obvious candidate for the contact with Leu^{356} is the cyclohexyl group of SR 146131 (Fig. 1b), which in the model fits into a hydrophobic pocket formed by Ile^{51}, Met^{121}, Ile^{355} and Leu^{356}. This interaction would be improved in the C94L mutant, thereby providing a possible explanation for the increased potency obtained with this mutant.

One possible reason for the increase in potency of SR 146131 at Q44A and N98A is that unfavourable side chain

Table 4
The contacts made between SR 146131 and residues in the human CCK_1 receptor. Contact is defined as side chain or backbone van der Waals interaction i.e. atomic contacts between 3 and 4 Å. Global numbering is according to Oliveira et al. (1993)

Contact in model	Receptor region	Type of contact	Mutant	Effect of mutation on SR 146131 activity
Gln^{44}	115	H-bond	Ala	increased potency
Tyr^{48}	119	H-bond	Ala	reduced potency
Ile^{51}	122	hydrophobic	not done	unknown
Cys^{94}	231	hydrophobic	Leu	increased potency
Phe^{97}	234	aromatic	Ala	reduced potency and efficacy
Asn^{98}	235	H-bond	Ala	increased potency and efficacy
Thr^{118}	319	H-bond	Ala	increased potency
			Ser	reduced potency
Met^{121}	322	hydrophobic	Ala	reduced efficacy
Gly^{122}	323	hydrophobic	Leu	reduced potency and efficacy
Phe^{198}	EL2	aromatic	Ala	reduced potency and efficacy
Leu^{217}	516	hydrophobic	Ala	increased potency
Phe^{218}	517	aromatic	not done	unknown
Ile^{329}	621	hydrophobic	Ala	reduced potency
Asn^{333}	625	H-bond	Ala	reduced potency
Arg^{336}	EL3	charge–charge	Ala	reduced potency and efficacy
			Asp	reduced potency and efficacy
Ile^{352}	715	hydrophobic	not done	unknown
Leu^{356}	719	hydrophobic	Ala	reduced potency

interactions are removed, thereby facilitating a better interaction of SR 146131 with other residues. Alternatively, these residues are implicated in maintaining the inactive state of the receptor, and their replacement favours receptor activation. However, since lowered potency and affinity were observed for CCK-8S at these mutants, it seems unlikely that the latter argument is correct. According to the model, the candidates for interaction with these residues on SR 146131 are the methoxy groups, consistent with the insertion of the cyclohexyl group of SR 146131 into the hydrophobic pocket formed by Ile^{51}, Cys^{94}, Met^{121} and Leu^{356}.

After the initial positioning of SR 146131 using the information described above, the receptor–ligand complex was energy minimised with 250 ps of constrained molecular dynamics at 298 K, using a non-bonded cut-off of 12 Å and a distance-dependent dielectric, as for SR 27897. Constraints were used to form interactions between (i) the carboxylate group of SR 146131 with Arg^{336}, (ii) the cyclohexyl group of SR 146131 with Ile^{51}, Cys^{94}, Met^{121} and Leu^{356}, and (iii) the methoxy groups with Gln^{44} and Asn^{98}. The same dynamics protocol described above for the SR 27897–receptor complex was used to energy minimise the SR 146131–receptor complex. Fig. 3b shows a stereo view of SR 146131 docked into the CCK_1 receptor and Fig. 4b is a cartoon of the contact points of SR 146131 with the receptor. Table 4 lists the human CCK_1 receptor residues in contact with SR 146131 in the final energy minimised model and the effect of their mutation on SR 146131 binding if known.

4. Discussion

The objective of the present investigation was to discover the structural features of the binding sites of two closely related molecules, SR 146131 and SR 27897, on the human CCK_1 receptor that account for their opposing pharmacological properties. The basic structure of the two molecules is the same, but SR 146131 has an additional cyclohexylethyl group attached to the thiazol ring, two methyl groups on the indole ring and two methoxy groups on the phenyl ring (Fig. 1b), making this the larger of the two molecules. The calculated volume of SR 146131 is 498 $Å^3$ compared to only 278 $Å^3$ for SR 27897. Additionally, a certain degree of flexibility could result in the two molecules having quite different overall spatial characteristics. Clearly, a combination of size, shape and electrostatic properties must account for the difference in biological properties. To understand how these factors influence the interactions of SR 146131 and SR 27897 with the CCK_1 receptor, it was necessary to try and map their respective binding sites through analysis of a large number of point-mutated receptors. The data obtained were used to construct molecular models of the interaction between the ligands and the CCK_1 receptor. The docking models for the two synthetic molecules presented in Figs. 3 and 4 are a result of these experiments.

As can be clearly seen, SR 27987 and SR 146131 are positioned quite differently on the receptor. SR 27987 is orientated vertically and seems only to have contacts in the extracellular extremities of transmembrane domains 5 and 6, in extracellular loop 1 and particularly in extracellular loop 2, as listed in Table 3. In contrast to SR 27897, in line with its larger size, SR 146131 contacts numerous residues throughout the transmembrane domains, as listed in Table 4 and is at an angle of roughly 90° to the SR 27897 binding position. According to the final model, both molecules are essentially planar, but the substituted phenyl group of SR 146131 is twisted 12° out of the plane of the rest of the molecule, probably because of interactions of the methoxy groups with residue side chains on the receptor and internal steric forces.

Despite their resemblance, the two molecules have few common contact points with the CCK_1 receptor. There does appear to be a slight overlap between the binding sites of SR 27897 and SR 146131, concentrated in the extracellular extremities of transmembrane domains 3, 5, 6 and extracellular loop 2. This small overlap of binding sites may account for the poor competition of SR 146131 with [^{3}H]SR 27897. It is noteworthy that of 33-point mutations, only six affected SR 27897 binding. Two of these involve Lys^{115} and Lys^{187}, situated in two adjoining extracellular loops, which in our model form charge–charge interactions with the carboxylate group on the indole ring. In contrast, the corresponding carboxylate group of SR 146131 interacts with Arg^{336} and not with Lys^{115} and Lys^{187}, this being one of the most striking differences between the contacts made by the two ligands.

It would seem that to induce or stabilise the active state of the receptor numerous interactions with the agonist are necessary, but their identification is not straightforward. A comparison of the binding site of the agonist SR 146131 with that of the endogenous agonist CCK-8S may help towards their comprehension. The residues identified as putative SR 146131 or CCK-8S contacts in transmembrane domain 1 (Gln^{44}, Tyr^{48}), transmembrane domain 2 (Cys^{94}, Phe^{97}, Asn^{98}) and transmembrane domain 7 (Leu^{356}) do not seem to be in contact with SR 27897. Therefore, these residues may be the key to the differences in pharmacological behaviour. The methoxy groups and cyclohexyl group of SR 146131 favour interactions with these six residues, thereby dictating the difference in orientation of SR 146131 compared to SR 27897. We found that residues implicated in SR 146131 potency were also implicated in CCK-8S binding and activity, suggesting the two agonists have a large degree of overlap in their binding sites on the CCK_1 receptor and/or in their mechanism of action. However, although certain residues were found to affect both agonists, the effects were not always unidirectional. For example, the N98A mutated receptor exhibited a loss in CCK-8S binding and activity, but a significantly in-

creased SR 146131 potency and efficacy. Similar effects were seen for Q44A, C94L, L214A and L217A. It would seem that although the two agonists occupy a similar site in the receptor, the nature of their interactions with the receptor differ considerably and vary in importance for each ligand, as would be expected considering that we are comparing a peptide with a non-peptide.

Other ligands of the same structural class having contrasting pharmacological properties have been described, notably for the bradykinin B2 receptor (Asano et al., 1998) and for the angiotensin II receptor (Perlman et al., 1997). However, the precise ligand–receptor interactions responsible for their divergent properties have not yet been elucidated. In addition, non-peptide agonists for other peptide receptors have been developed, including the neurotensin subtype 2 receptor (Vita et al., 1998), somatostatin SST2 (Yang et al., 1998) and SST4 (Liu et al., 1999) receptor subtypes and a large number are known for the various opioid receptor subtypes. The existence of such molecules provides an unique opportunity for studying receptor function and to develop rational explanations at the molecular level for agonist and antagonist activity.

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