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Essential role of extracellular charged residues of the human CCK_1 receptor for interactions with SR 146131, SR 27897 and CCK-8S

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Abstract

We hypothesized that charge–charge interactions may be important for the binding of the human cholecystokinin type 1 (CCK_1) receptor-specific non-peptide full 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), the competitive antagonist SR 27897, (1-[2-(4-(2-chlorophenyl)thiazol-2-yl) aminocarbonyl indoyl] acetic acid) and the natural octapeptide CCK-8S to the CCK_1 receptor. Alanine replacement studies of positively charged residues in the extracellular domains of the receptor showed that only the R336A mutation affected SR 146131 potency of mutated receptors transiently expressed in monkey kidney epithelial COS-7 cells. Two residues, Lys^{115} and Lys^{187}, were implicated in SR 27897 binding. Only the replacement of Lys^{115}, Arg^{197} and Arg^{336} significantly affected CCK-8S binding or activity. These results clearly indicated the importance of certain charged residues, but not others, in SR 146131, SR 27897 and CCK-8S binding. Furthermore, although these molecules probably occupy different binding sites on the CCK_1 receptor, we show that a small non-peptide agonist, SR 146131, can stimulate the dual signaling pathways mediated by the CCK_1 receptor.

Keywords: Cholecystokinin CCK_1 receptor; Mutagenesis; Charged residue; SR 27897; SR 146131

1. Introduction

Cholecystokinin (CCK) is a peptide functioning as a hormone and/or neurotransmitter in the gastrointestinal tract and the peripheral and central nervous systems (reviewed in Wank, 1998). Although multiple forms of CCK exist through proteolysis of a 115 amino acid precursor, the shortest form of CCK with full biological activity is the tyrosine O-sulfated octapeptide CCK-8S (Fig. 1b). The effects of CCK-8S on target cells are mediated through integral membrane receptors belonging to the heptahelical G protein-coupled receptor superfamily, characterized by seven transmembrane domains joined by extracellular and intracellular loops. Two subtypes of the receptor, having about 50% structural identity, denoted as the CCK_1 receptor and the CCK_2 receptor (formerly CCK_A and CCK_B) (Wank, 1995), have been cloned (Wank et al., 1992a,b) and characterized. The two receptors are clearly distinguishable in that (i) the CCK_1 receptor has an approximately 500-fold higher affinity for CCK-8S than for the nonsulfated peptide, (ii) the CCK-8 related peptide gastrin has a 1000-fold higher affinity for the CCK_2 receptor than for the CCK_1 receptor, and (iii) numerous subtype-specific synthetic ligands have been developed, among which are the competitive non-peptide antagonist SR 27897 (Fig. 1b; Gully et al., 1993) and the structurally related full agonist SR 146131 (Fig. 1b; Bignon et al., 1999).

The existence of two structurally dissimilar agonists, CCK-8S and SR 146131, provides an excellent opportunity for investigating the mechanism(s) by which the receptor attains the activated state that triggers the intracellular signaling cascade. One common feature of the two agonists is the presence of negatively charged groups, three on CCK-8S, two aspartic acid residues and the tyrosine sulfate and one on the carboxylate group of SR 146131. The antagonist SR 27897 also carries a carboxylate group. Consequently, as a first step, we have investigated the importance of positively charged residues in the extracellular domains of the human CCK_1 receptor in the binding of

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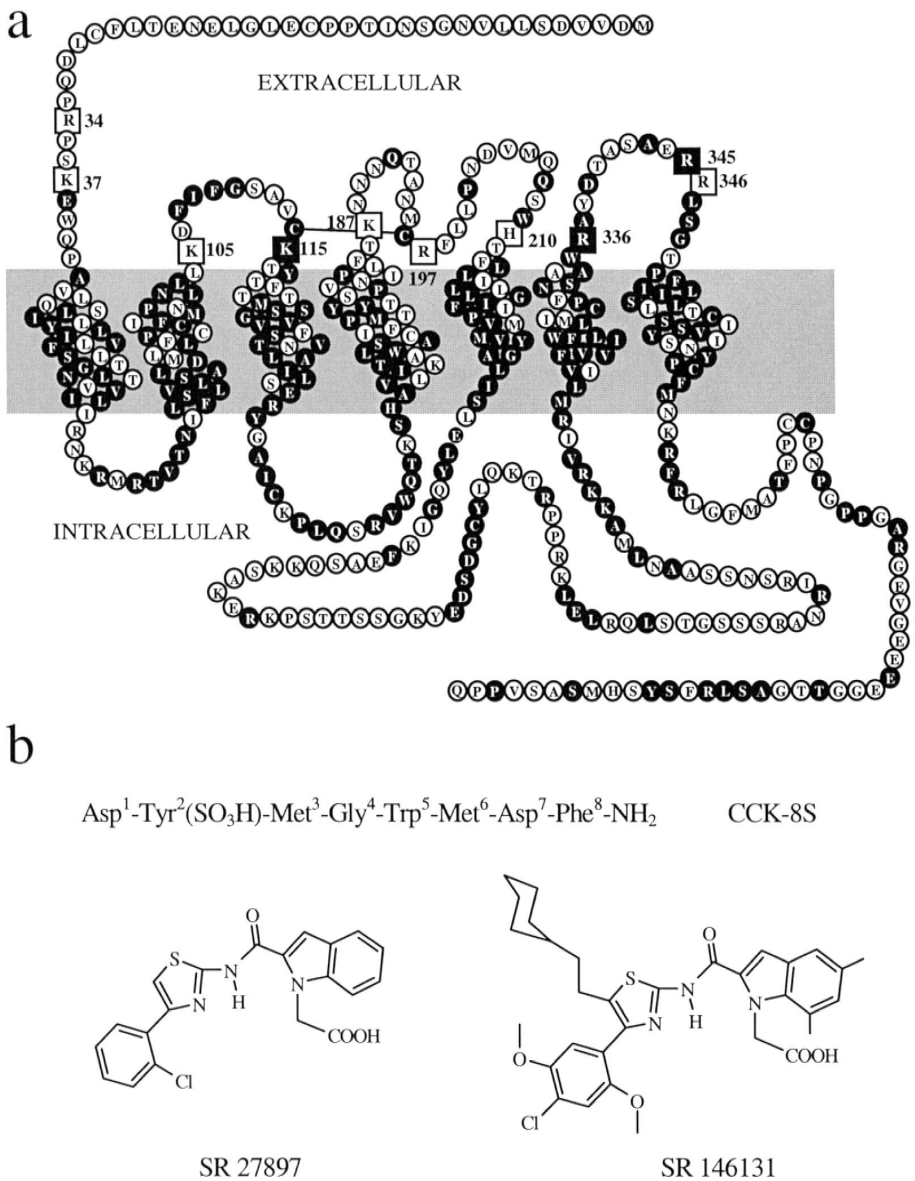

Fig. 1. (a) Schematic representation of the human CCK_1 receptor showing the primary sequence with the nine mutated residues in numbered squares. Residues common to both human CCK_1 and CCK_2 subtype receptors are in *white* on a *black* background. The putative disulfide bridge is shown as a straight line. The *gray* box represents the membrane bilayer. (b) The formulae of CCK-8S and the CCK_1 receptor selective antagonist SR 27897 and the CCK_1 receptor selective agonist SR 146131.

CCK-8S, SR 146131 and SR 27897, together with the biological activities activated by the two agonists.

Ten positively charged residues, including the potentially positively charged His^{210}, occur in the extracellular domains of the human CCK_1 receptor (Fig. 1a). Recently, one counterion has been experimentally identified, Arg^{336}, postulated to interact with the penultimate aspartic acid of CCK-8S and the carboxylate group of SR 27897 (Gigoux et al., 1999). The same researchers have also reported an interaction between the sulfated tyrosine of CCK-8S and Met^{195} and Arg^{197} of the CCK_1 receptor (Gigoux et al., 1998). In another study using molecular modeling, it was suggested that Lys^{105} forms a salt bridge with the CCK-8S tyrosine sulfate (Ji et al., 1997). However, this hypothesis was not tested by mutation of Lys^{105}. His^{210} was of potential interest because the cognate residue in the CCK_2 receptor has been implicated in the subtype specificity of gastrin (Kopin et al., 1995). Previous studies with truncated CCK_1 receptors have shown that Arg^{34} and Lys^{37} in the amino-terminus of the CCK_1 receptor are not involved in CCK-8S binding (Kennedy et al., 1995).

We have individually mutated to alanine all the extracellular positively charged residues in the human CCK_1 receptor, except Arg^{34}, transiently expressed the mutated receptors in monkey kidney epithelial COS-7 cells, and carried out binding and inositol phosphate accumulation

assays on intact cells. In addition, since it is known that the CCK_1 receptor mediates cAMP accumulation as well as inositol phosphate formation, we have assayed the effect of the receptor-mediated induction of luciferase activity by co-expressing the mutated receptors with a cAMP response element–luciferase reporter gene system. The main findings were that only R336A affected SR 146131 inositol phosphate stimulation potency, with K105A and R345A affecting its luciferase stimulation potency. Only K115A and K187A significantly affected SR 27897 binding affinity, and only three of the mutations, K115A, R197A and R336A, markedly reduced CCK-8S binding and activity. The results strongly suggest that the three ligands contact different sites on the CCK_1 receptor.

2. Materials and methods

2.1. Drugs and chemicals

The CCK_1 receptor-specific antagonist SR 27897 (1-[2-(4-(2-chlorophenyl)thiazol-2-yl) aminocarbonyl indoyl] acetic acid) and 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) were synthesized at Sanofi Recherche, Montpellier, France. Propionylated [^{3}H]CCK-8S (66 Ci/mmol) and [^{3}H]SR 27897 (37 Ci/mmol) were purchased from Amersham (Les Ulis, France). CCK-8S was purchased from Neosystem (Strasbourg, France). Dulbecco's modified essential medium, fetal calf serum, phosphate-buffered saline and Lipofectamine were from Gibco/BRL Life Technologies (Paisley, UK). Bovine serum albumin, sodium pyruvate and soya bean trypsin inhibitor were from Sigma (St Louis, MO). *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide · 2HCl (H-89), 2-[2-amino-3-methoxyphenyl]-4*H*-1-benzopyran-4-one (PD 98059) and bisindolylmaleimide (GF 109203X) were from Biomol Research Laboratories (Plymouth Meeting, PA) and were used according to the manufacturers' recommendations. Luciferase activities were determined using the Luciferase Assay System (Promega). Monkey kidney COS-7 cells were from the American Tissue Culture Collection (Reference CRL-1651).

2.2. Mutagenesis

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

2.3. Transfection of COS-7 cells and binding protocol

COS-7 cells (1×10^5 cells/well) were seeded into 24-well tissue plates and transfected with lipofectamine according to the manufacturer's instructions. Competition binding was performed 48 h post transfection on whole cells at room temperature in 1 ml total binding buffer (Dulbecco's modified essential medium supplemented with soya bean trypsin inhibitor 1 mg/l, sodium pyruvate 25 ml/l and various concentrations of cold ligand. Either [^{3}H]CCK-8S (1 nM final) or [^{3}H]SR 27897 (1 nM final) was used as radiolabeled ligand. After 1 h, the binding media was aspirated and the cells were washed three times with phosphate buffered saline solution (1 ml) after which 1 ml of sodium hydroxide (3%) was used to lyse the cells. The lysate was analyzed for radio tracer concentration.

2.4. Inositol phosphate assays

COS-7 cells (1×10^5 cells/well) were seeded into 24-well tissue plates and transfected with lipofectamine according to the manufacturer's instructions. After 24 h, 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, the growth media was aspirated and the cells washed with phosphate buffered saline solution (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 ligand were added. After a further 60-min incubation in the presence of ligand, the media was aspirated and the cells washed with PBS (1 ml). Ice cold methanol/0.1 M HCl (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). Ninety five percent of the radioactivity quantified was inositol monophosphate (IP).

2.5. Firefly luciferase assay (index for cAMP formation)

COS-7 cells (3×10^4 cells/well) were seeded into 96-well plates (opaque), and co-transfected with expression vectors for the wild-type CCK_1 or mutated receptors (10 μg/plate) and cAMP response element (CRE)-luciferase (5 μg/plate; Bouaboula et al., 1997). After 24 h, the cells were incubated with varying concentrations of CCK-8S or SR 146131. To determine the effects of second messenger inhibitors, these were added 30 min before CCK-8S treatment. Four hours after the addition of agonist, the cells were washed with 1 ml PBS before adding luciferin (25 μl, 1% Triton 1000). After 3 min, light emission was accumulated for 30 s in a Hamamatsu MTP Reader. Mean values from sextuplicate samples were ex-

Table 1
Results from homologous competition binding for the agonist [^{3}H]CCK-8S and for the non-peptide antagonist [^{3}H]SR 27897 with the wild-type and mutant human CCK_1 receptors. All values are given in nM ± S.E.M. and are from three to four separate experiments performed in triplicate. B_{max}, were estimated from binding experiments performed with both agonist [^{3}H]CCK-8S and antagonist [^{3}H]SR 27897. Binding data were analyzed using the Graphpad Prism software. (–) undetectable; (nd) not determined

Ligand	[^{3}H]CCK-8S			[^{3}H]SR 27897	
Mutation	IC_{50high}, nM	IC_{50low}, nM	B_{max}, pmol/10^5 cells	IC_{50}, nM	B_{max}, pmol/10^5 cells
$hCCK_1$	0.44 ± 0.2	230 ± 80	0.032 ± 0.012	6.0 ± 0.2	0.26 ± 0.02
K37A	nd	nd	nd	6.8 ± 0.2	0.25 ± 0.09
K105A	0.34 ± 0.1	478 ± 65	0.037 ± 0.011	13 ± 6	0.29 ± 0.05
K115A	–	–	–	283 ± 60[a]	0.15 ± 0.02[a]
K187A	0.9 ± 0.05	1476 ± 180[a]	0.029 ± 0.005	339 ± 60[a]	0.25 ± 0.01
R197A	–	–	–	15 ± 5	0.30 ± 0.01
H210A	0.19 ± 0.05[b]	355 ± 85	0.039 ± 0.014	7.6 ± 0.3	0.21 ± 0.05
R336A	–	–	–	10 ± 0.5	0.35 ± 0.09
R345A	0.25 ± 0.1	91 ± 53[b]	0.029 ± 0.01	6.2 ± 1.2	0.30 ± 0.10
R346A	0.40 ± 0.1	110 ± 45	0.038 ± 0.004	6.9 ± 0.9	0.29 ± 0.02

[a] Statistically different from wild-type with $P < 0.001$.
[b] Statistically different (one-way ANOVA test) from wild-type with $P < 0.01$.

pressed as a percentage of mean maximal wild-type CCK_1 receptor stimulation obtained with 10^{-5} M CCK-8S or 10^{-5} M SR 146131.

2.6. Data analysis

Curves were fitted to the data with the Prism nonlinear least squares curve-fitting program (GraphPad Software, San Diego, CA). For binding data analyses, one- and two-site fits were tested. For all experiments, a two-site fit was considered better at $P < 0.05$. The values given in Tables 1 and 2 and in Figs. 1–5 are the means ± S.E.M. from three to four experiments. The significance of the values obtained was analyzed using a one-way ANOVA test.

3. Results

3.1. Binding characteristics of the wild-type CCK_1 receptor with CCK-8S and inositol phosphate accumulation and luciferase stimulation by CCK-8S and SR 146131

The competition binding curves obtained for [^{3}H]CCK-8S and CCK-8S with the wild-type CCK_1 and the mutant receptors are shown in Fig. 2a–f. Two binding affinities

Table 2
Results from CCK-8S and SR 146131 induced inositol phosphate mobilization and luciferase stimulation with the wild-type and mutant human CCK_1 receptors. All values are given in nM ± S.E.M. and are from three to four separate experiments performed in triplicate (sextuplicate for the luciferase assay).(–) Undetectable

Ligand	CCK-8S		SR 146131	
Mutation	inositol phosphate EC_{50} nM (efficacy wt.%)	luciferase EC_{50} nM (efficacy wt.%)	luciferase EC_{50} nM (efficacy wt.%)	luciferase EC_{50} nM (efficacy wt.%)
$hCCK_1$ wt	0.4 ± 0.08 (100)	25 ± 5 (100)	13 ± 0.9 (100)	90 ± 18 (100)
K37A	0.5 ± 0.10 (100)	76 ± 16 (200)	10 ± 1.2 (100)	89 ± 9 (100)
K105A	1.0 ± 0.26 (100)	130 ± 8[a] (100)	20 ± 6 (86)	413 ± 38[a] (100)
K115A	14 ± 5.7[a] (55)[a]	38 ± 6 (23)[b]	12 ± 0.1 (35)[a]	67 ± 13 (32)[b]
K187A	1.0 ± 0.20 (100)	36 ± 10 (100)	11 ± 2 (100)	151 ± 20 (100)
R197A	356 ± 30[b] (69)[a]	> 5000[b] ([c])[b]	20 ± 6 (100)	66 ± 12 (100)
H210A	0.39 ± 0.09 (100)	64 ± 5 (100)	17 ± 6 (100)	145 ± 21 (100)
R336A	576 ± 89[b] (66)[b]	> 10,000[b] ([c])[b]	138 ± 13[b] (100)	–
R345A	1.0 ± 0.22 (100)	20 ± 2 (100)	11.3 ± 3.5 (100)	262 ± 50[a] (100)
R346A	0.23 ± 0.01 (100)	15 ± 5 (100)	5.0 ± 2.0 (100)	54 ± 4 (100)

[a] Statistically different from wild-type (one way ANOVA test) with $P < 0.01$.
[b] Statistically different from wild-type with $P < 0.001$.
[c] Plateau not attained at 10^{-5} M CCK-8S.

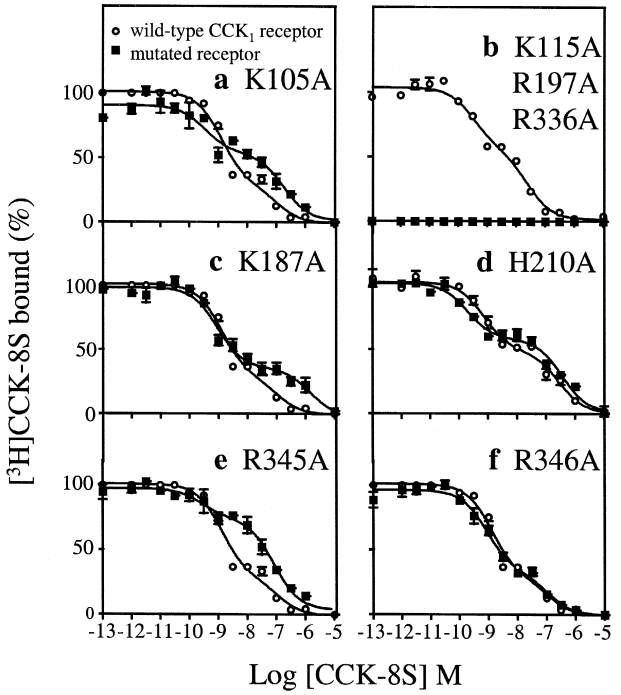

Fig. 2. Competition binding curves of [^{3}H]CCK-8S with CCK-8S with mutated CCK_1 receptors transiently expressed in COS-7 cells. Binding is given as a percentage of maximal binding of [^{3}H]CCK-8S. Maximal binding is defined as total [^{3}H]CCK-8S binding in the absence of competing ligand minus non-specific binding, defined as binding in presence of 10^{-5} M competing ligand. Each experiment was conducted in parallel with the wild-type CCK_1 receptor as an internal standard. The results are the means ± S.E.M. of three to four separate experiments carried out in triplicate. (b) Results of competition binding for three individual CCK_1 receptor mutants, K115A, R197A and R336A.

for CCK-8S with the wild-type human CCK_1 receptor, termed high and low, were found. The two binding affinities (Table 1) were consistent with published values (Huang et al., 1994; Pandya et al., 1994; Talkad et al., 1994a,b). The dose–response curves for CCK-8S-induced inositol phosphate formation are shown in Fig. 3a–h. The EC_{50} of CCK-8S-induced inositol phosphate production at wild-type human CCK_1 (Table 2) agreed with that published elsewhere (Tarasova et al., 1997). The Hill slope for the wild-type receptor was less than unity, reflecting the known complex binding mechanism. The dose–response curves for CCK-8S-induced luciferase activity are shown in Fig. 4a–h. The EC_{50} for the CCK-8S-stimulated luciferase response (25 ± 5 nM, Table 2) with the wild-type CCK_1 receptor was very close to the published EC_{50} value of 20 ± 4 nM for CCK-8S-stimulated cAMP formation (Wu et al., 1997). To verify if the luciferase response was a direct consequence of downstream signaling dependent on cAMP accumulation, we tested the effects on CCK-8S-induced luciferase activity of H-89, GF 109203X and PD 98059, inhibitors of protein kinase A, protein kinase C and mitogen-activated protein kinase kinase 1, respectively. Only H-89 at 10^{-5} M significantly reduced the CCK-8S-induced luciferase level, a result fully compatible with a cAMP/protein kinase A/gene transcription pathway (Fig. 5).

SR 146131 induced inositol phosphate accumulation in the COS-7 cells expressing the wild-type CCK_1 receptor with 30-fold less potency than CCK-8S (Table 2) and with 70% of its efficacy (data not shown). The EC_{50} values obtained (Table 2) can be compared to those of 2.2 ± 0.4 nM for CCK-8S and 18 ± 4 nM for SR 146131 obtained in a 3T3-CCK_1 cell line, SR 146131 having about 85% of the efficacy of CCK-8S (Bignon et al., 1999). The efficacy of luciferase stimulation by SR 146131 in the COS-7 cells was also 70% that of CCK-8S, but its potency was only 4-fold less than that of CCK-8S (Table 2).

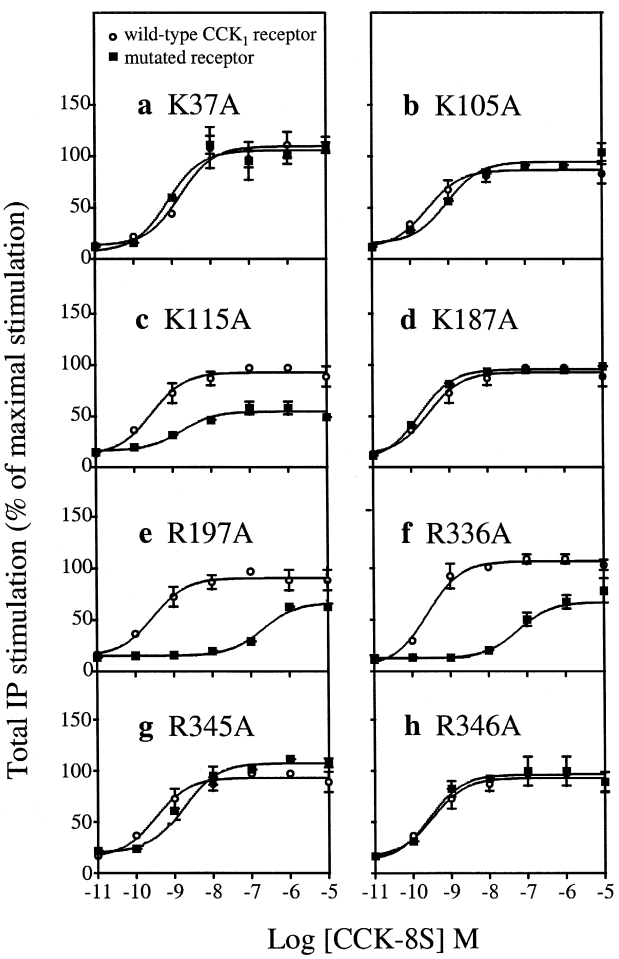

Fig. 3. Dose–response curves of CCK-8S-induced inositol phosphate (IP) mobilization with mutated CCK_1 receptors transiently expressed in COS-7 cells. Activity is given as a percentage of maximum stimulation of the wild-type CCK_1 receptor with 10^{-5} M CCK-8S. Basal activity is defined as level of radioactivity in the absence of agonist. Each experiment was conducted with the wild-type CCK_1 receptor as an internal standard. The results are the means ± S.E.M. of three to four 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.

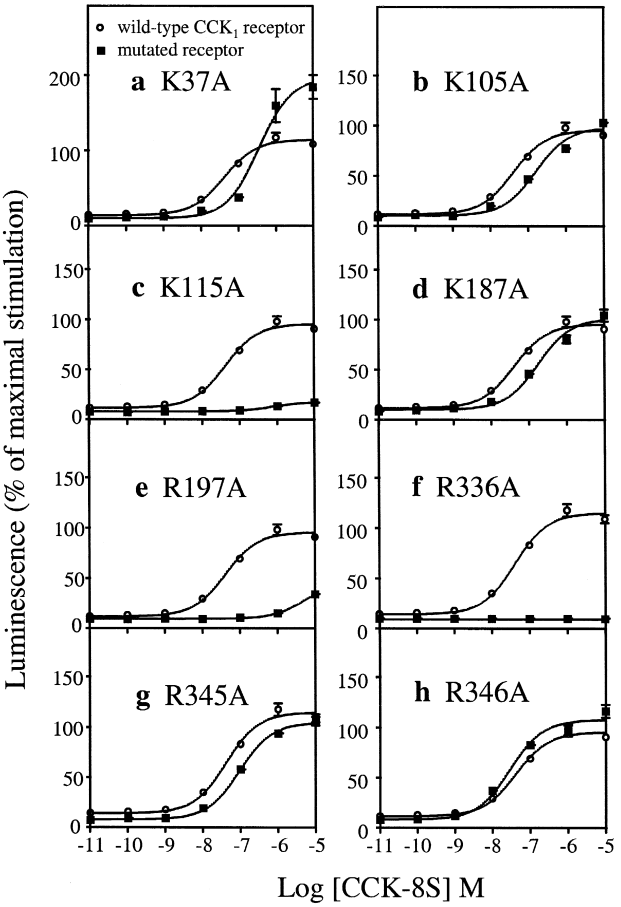

Fig. 4. Dose–response curves of CCK-8S-induced luciferase activity in COS-7 cells transiently co-expressing mutated CCK_1 receptors and a CRE–luciferase response element reporter gene construct. Activity is expressed as a percentage of maximum stimulation of the wild-type CCK_1 receptor with 10^{-5} M CCK-8S. Basal activity is defined as level of luminescence in the absence of agonist. Each experiment was conducted with the wild-type CCK_1 receptor as an internal standard. The results are the means ± S.E.M. of three to four separate experiments carried out in sextuplicate.

3.2. Binding and activity assays with the K37A (N-terminal) and K105A and K115A (extracellular loop 1) CCK_1 receptor mutants

The results of all binding assays for the mutated receptors are presented in Table 1 and activity measurements in Table 2. It was previously reported that a truncated CCK_1 receptor lacking the first 37 amino-terminal amino acids showed wild-type CCK_1 receptor properties with CCK (Kennedy et al., 1995). In accordance with this report, in a control experiment we found that with CCK-8S the K37A mutated receptor had the same EC_{50} value and efficacy for inositol phosphate formation as the wild-type CCK_1 receptor (Fig. 3a). SR 146131 gave wild-type CCK_1 receptor values for both inositol phosphate formation and luciferase stimulation. In contrast, the potency for luciferase stimulation by CCK-8S was reduced 3-fold, while the efficacy was increased 2-fold (Fig. 4a), suggesting that Lys^{37} somehow influences CCK_1 receptor-mediated cAMP accumulation with CCK-8S, but not with SR 146131. The K37A CCK_1 receptor was expressed at wild-type CCK_1 receptor levels and bound SR 27897 with wild-type CCK_1 receptor affinity.

The K105A mutation in extracellular loop 1 resulted in near wild-type CCK_1 receptor binding affinity for CCK-8S for both binding states (Fig. 2a) and no significant effect on either potency or efficacy of inositol phosphate formation (Fig. 3b). With both CCK-8S (Fig. 4b) and SR 146131, the potency of luciferase activation was reduced 4- to 5-fold, although efficacy remained at wild-type CCK_1 receptor levels. In contrast, K115A situated at the extracellular loop 1-transmembrane domain 3 interface exhibited a drastic loss of affinity for CCK-8S (Fig. 2b), accompanied by a 35-fold drop in potency and a 2-fold loss of efficacy for inositol phosphate stimulation (Fig. 3c). The potency for luciferase stimulation was at the wild-type level, but the efficacy was reduced 4-fold (Fig. 4c). This mutant also showed a drop in affinity for SR 27897 compared to wild-type and was the only mutant receptor that showed a significantly lower B_{max} compared to cells expressing the wild-type receptor. This lower expression possibly explains the loss in efficacy of CCK-8S at the K115A CCK_1 receptor. The results suggested either that Lys^{115} was a common binding residue for CCK-8S and SR 27897 or that the mutation resulted in a misfolded receptor structure. However, SR 146131 stimulated both inositol phosphate formation and luciferase activity with wild-type CCK_1

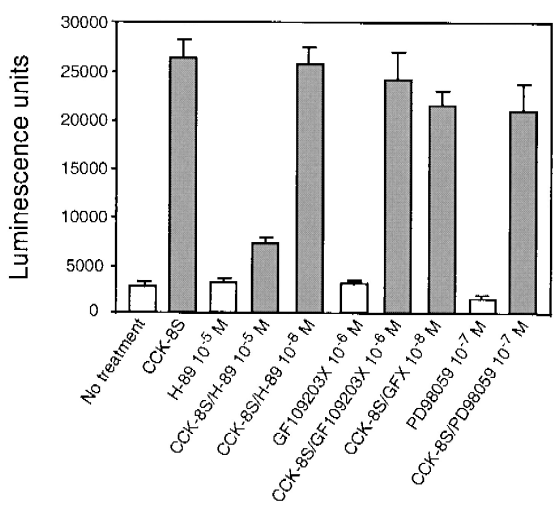

Fig. 5. Inhibition of luciferase activity induced by CCK-8S in COS-7 cells transiently coexpressing CRE–luciferase and the human CCK_1 receptor. The histogram shows the effect of second messenger inhibitors (pre-treatment for 30 min) on induced luciferase activity in arbitrary light units induced in the absence, *white*, and the presence, *gray*, of 10^{-5} M CCK-8S. The results are the means ± S.E.M. of three to four separate experiments carried out in sextuplicate.

receptor potency. The efficacy of SR 146131 was 35% that of wild-type for both functional assays, suggesting that the receptor was correctly folded, but was not present at wild-type CCK_1 receptor levels at the cell surface.

3.3. Binding and activity measurements with the K187A, K197A and H210A (extracellular loop 2) CCK_1 receptor mutants

The K187A mutation in extracellular loop 2 resulted in a 2-fold decrease in the IC_{50} of the high affinity binding site and a 6.4-fold decrease in IC_{50} of the low affinity CCK-8S binding state (Fig. 2c), accompanied by a 2.5-fold reduction in CCK-8S potency for inositol phosphate production (Fig. 3d). Additionally, the affinity of SR 27897 was reduced 56-fold. However, the B_{max} obtained with [^{3}H]SR 27897 was not significantly different from that of the wild-type receptor. Here again, with SR 146131, the EC_{50} and efficacies for inositol phosphate production and luciferase stimulation indicated that the mutated receptor functioned as well as the wild-type CCK_1 receptor. Lys^{187} may be another contact point for SR 27987, but probably not for SR 146131 or CCK-8S.

The R197A mutant showed a loss of affinity for CCK-8S (Fig. 2b) and a large decrease in potency for inositol phosphate formation, although the efficacy was not significantly lowered (Fig. 3e). Likewise, the effect of the mutation on luciferase stimulation was drastic and neither potency nor efficacy could be quantified because the dose–response curve did not attain a plateau (Fig. 4e). In contrast, the binding of SR 27897 was not altered significantly. Similarly, the potency and efficacy of SR 146131 were similar to that of the wild-type CCK_1 receptor. Clearly, Arg^{197} in the CCK_1 receptor is an important point of contact for CCK-8S, but not for SR 146131 or SR 27897.

The H210A mutant showed wild-type CCK_1 receptor binding characteristics (Fig. 2d), inositol phosphate and luciferase stimulation potency for CCK-8S and SR 146131 and wild-type CCK_1 receptor properties for SR 27987 binding.

3.4. Binding and activity measurements with the R336A, R345A and R346A (extracellular loop 3) CCK_1 receptor mutants

Replacement of Arg^{336} in extracellular loop 3 by alanine resulted in a loss of affinity for CCK-8S (Fig. 2b), with an accompanying 1500-fold drop in potency of inositol phosphate formation and decreased efficacy (Fig. 3f). Luciferase production was undetectable (Fig. 4f). The effect of the mutation on the SR 146131-induced potency of inositol phosphate formation was considerable, but less drastic, with only a 10-fold reduction, the efficacy remaining at the wild-type CCK_1 receptor level. In contrast, the R336A mutant bound SR 27897 with almost wild-type receptor affinity. The B_{max} value was similar to that of the wild-type receptor, suggesting the loss of potency and reduced efficacy of CCK-8S, and to a lesser extent that of SR 146131, was due to a direct effect on agonist binding, not low receptor levels at the cell surface. The binding result for the R336A mutated receptor with SR 27897 contrasted with that of Gigoux et al. (1999), who reported that the R336M and R336D mutations resulted in 8-fold and 92-fold losses, respectively, in [^{3}H]SR 27897 binding. We constructed the R336D mutant and found that it bound [^{3}H]SR 27897 with near wild-type affinity although the B_{max} was slightly reduced (IC_{50} 13 ± 4 nM, B_{max} 0.18 ± 0.02 pmol/10^5 cells).

The final two mutants, R345A and R346A, both in extracellular loop 3, exhibited essentially wild-type CCK_1 receptor behavior in the binding (Fig. 2e,f) and activity assays (Figs. 3g,h and Figs. 4g,h) suggesting little or no involvement in ligand binding or activity.

4. Discussion

Six of the positively charged residues in the extracellular region of the CCK_1 receptor, Lys^{37}, Lys^{105}, Lys^{187}, His^{210}, Arg^{345} and Arg^{346}, clearly do not play a major role in the interaction of either SR 146131 or CCK-8S with the receptor. Lys^{105} was suggested from modeling studies to be implicated in CCK-8S binding (Ji et al., 1997), but the results presented here contradict this theory. Three residues, Lys^{115}, Arg^{197} and Arg^{336}, are important for the binding and the biological activity of CCK-8S. Although there was no detectable binding of [^{3}H]CCK-8S to K115A, R197A and R336A, the persistence of some CCK-8S-induced inositol phosphate accumulation showed that the peptide was still able to activate the mutated receptors to some extent, particularly the K115A mutant. This implies that some activating binding sites are still present, perhaps at lower levels than in the wild-type, that are undetectable with competition binding. In the K115A mutant, SR 27897 binding affinity was also reduced along with the B_{max} value. However, the receptor showed wild-type potency for SR 146131 in both functional assays, although efficacy was reduced to 35% of the wild-type level, in line with the reduced B_{max} value. Therefore, apart from its participation in CCK-8S binding, Lys^{115} in the wild-type receptor also appears to play some structural or functional role that remains to be elucidated. The three mutations had a much greater effect on the cAMP–luciferase pathway than on inositol phosphate accumulation. Neither Lys^{115} nor Arg^{197} influences SR 146131 potency for either pathway to a significant extent, pointing to a fundamental difference in the ways CCK-8S and the small molecule bind to, and possibly activate, the CCK_1 receptor. The cAMP–

luciferase pathway was particularly sensitive to certain point mutations in the CCK_1 receptor. The 2-fold increase in the efficiency of the luciferase response in the CCK-8S-induced K37A mutated receptor, and the loss in SR 146131-induced potencies with the K105A and R345A mutations, merit further investigation.

Both agonists were adversely affected by the R336A mutation, with the SR 146131-induced potency of inositol phosphate accumulation being reduced 10-fold and the luciferase response being undetectable. Since this was the only replacement of a charged residue in the present study that affected SR 146131-induced activity, Arg^{336} is a good candidate for an interaction with the carboxylate group on SR 146131. Molecular modeling (manuscript in preparation) suggests that SR 146131 can contact both Arg^{336} and Leu^{356}, also a putative contact (Gouldson et al., 1999). The large effects on the observed CCK-8S binding, potency and efficacy suggest a key role for Arg^{336} in receptor–CCK-8S interactions or in maintaining the receptor in an active state. If we exclude Asp^1, being implicated as an interaction point between CCK-8S and the receptor (Bodanszky et al., 1980; Pearson and Miller, 1987; Silvente-Poirot et al., 1998), the alternative types of contact for Arg^{336} in CCK-8S are charge–π interactions with the aromatic ring of Tyr^2 or Trp^5 of CCK-8S, hydrogen bonds with the backbone or charge–charge interactions with the sulfate group or with the carboxylate group of Asp^7. Data strongly supporting the latter possibility were recently presented (Gigoux et al., 1999). However, unlike Gigoux et al. (1999), our data did not indicate an interaction between Arg^{336} and SR 27897, since the R336A and R336D mutations resulted in receptors with essentially wild-type CCK_1 receptor characteristics. In fact, of the 10 charged residues mutated here, only K115A and K187A affected SR 27897 binding.

The present work suggests that CCK-8S makes contact with charged residues situated in each of the three extracellular domains. Without further investigation, we cannot establish the nature of the contacts or decide which of the two conflicting models for the orientation of CCK-8S in the CCK_1 receptor is correct (Ji et al., 1997; Kennedy et al., 1997). Following site-directed mutagenesis experiments, a model was proposed for the docking of CCK-8 in which Asp^1 of CCK-8S was in direct contact with Gln^{40} in the N-terminus of CCK_1 and the C-terminus was situated in the middle of the transmembrane region of the receptor (Kennedy et al., 1997). Recent two-dimensional mutation experiments have reinforced this model (Gigoux et al., 1999). From results using crosslinking techniques a different orientation of CCK-8S has been proposed (Ji et al., 1997; Hadac et al., 1998). Using a CCK analogue carrying a photolabile group at the C-terminus, Ji et al. (1997) specifically labeled Trp^{39} in the amino terminus of rat CCK_1. Using another probe, Hadac et al. (1998) labeled residues in transmembrane domain 7. The molecular model resulting from these two studies positioned the C-terminus of CCK-8S in close contact with the N-terminus of CCK_1 and Gly^4 of CCK-8S in extracellular loop 3.

It is interesting that Arg^{197} is adjacent to Cys^{196}, a cysteine residue that may be involved in disulfide bridge formation with Cys^{114}, adjacent to Lys^{115}. The existence of a disulfide bridge has not been demonstrated in CCK_1, but some evidence exists for its presence in CCK_2, since mutation of the cognate CCK_2 cysteines resulted in non-functional receptors (Silvente-Poirot et al., 1998). Molecular modeling (manuscript submitted) shows that as a result of a covalent S–S bond, Lys^{115} and Arg^{197} may form a small cluster of positive charge that could be of importance for CCK-8S binding. In addition, these residues are in close proximity to Met^{195} in extracellular loop 2 of CCK_1, a residue that has been shown to be crucial for CCK-9S binding and activity (Gigoux et al., 1998). The substitution by alanine of Met^{195} was attributed to a loss of a quadropole–quadropole interaction between the sulfur atom and the π electron cloud of the aromatic ring of Tyr^3 of CCK-9S (equivalent to Tyr^2 of CCK-8S) (Gigoux et al., 1998). Although we find here that Arg^{197} clearly interacts with CCK-8S, it cannot be concluded that it is involved in a charge–charge interaction with the ligand.

Charged residues situated in the extracellular domains have been identified experimentally or proposed from modeling as important for ligand binding in other 7 transmembrane domain G-protein-coupled peptide receptors. For instance, studies of the corticotrophin releasing factor subtype 1 receptor (Sydow et al., 1999) and opiate receptor-like 1 receptor (Topham et al., 1998) have indicated the importance of charged residues in peptide ligand binding. In addition, acidic residues in the extracellular loops of the human neuropeptide Y1 subtype receptor have been shown through site directed mutagenesis to be important for the binding and activity of the natural peptide agonist neuropeptide Y (Walker et al., 1994). Four negatively charged residues, Asp^{104} in extracellular loop 1, Asp^{194} and Asp^{200} in extracellular loop 2 and Asp^{287} in extracellular loop 3 were shown to be critical for neuropeptide Y binding. Consistent with our results for the CCK_1 receptor, the majority of the charged residues, located in the extracellular domain of the Y1 receptor, were found not to be involved in ligand recognition. Another study on the Y1 receptor implicated two positively charged residues, Arg^{33} and Arg^{35}, in neuropeptide Y binding (Becksickinger and Jung, 1995). Mutagenesis of the neuromedin B receptor revealed the implication of two charged residues, Arg^{127} and His^{294}, in the high affinity binding of the natural agonist neuromedin (Sainz et al., 1998). Arg^{127} is situated in transmembrane domain 3 and His^{294} is positioned at the transmembrane domain 6–extracellular loop 3 interface. An alignment of the neuromedin B and neuropeptide Y1 receptor sequences with that of the CCK_1 receptor shows that His^{294} of the neuromedin B receptor is in the same position as Arg^{336} in CCK_1. It is shown here to be critical for CCK-8S binding, and Asp^{287} of the Y1 receptor is only

one residue away. This suggests that some of the neuropeptide receptors may share common contact points for their endogenous peptide agonists, perhaps pointing to a common mode of receptor activation. Here, we show for the first time that a small non-peptide molecule, SR 146131, is capable of eliciting the dual intracellular responses mediated by the CCK_1 receptor. For the purpose of elucidating the subtle mechanisms of peptide receptor activation, non-peptide agonists such as SR 146131 that share some, but not all, of the binding sites and stimulatory processes of the natural agonists may prove themselves to be useful tools.

References

Becksickinger, A.G., Jung, G., 1995. Structure–activity relationships of neuropeptide Y analogues with respect to Y-1 and Y-2 receptors. Biopolymers 37, 123–142.

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. SR146131: 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.

Bodanszky, M., Tolle, J.C., Gardner, J.D., Walker, M.D., Mutt, V., 1980. Cholecystokinin (pancreozymin). Synthesis and properties of the N alpha-acetyl-derivative of cholecystokinin 27–33. Int. J. Pept. Protein Res. 16, 402–411.

Bouaboula, M., Perrachon, S., Milligan, L., Canat, X., Rinaldi-Carmona, M., Portier, M., Barth, F., Calandra, B., Pecceu, F., Lupker, J., Maffrand, J.P., Le Fur, G., Casellas, P., 1997. A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1 — evidence for a new model of receptor/ligand interactions. J. Biol. Chem. 272, 22330–22339.

Gigoux, V., Escrieut, C., Fehrentz, J.A., Poirot, S., Maigret, B., Moroder, L., Gully, D., Martinez, J., Vaysse, N., Fourmy, D., 1999. Arginine 336 and asparagine 333 of the human cholecystokinin-A receptor binding site interact with the penultimate aspartic acid and the C-terminal amide of cholecystokinin. J. Biol. Chem. 274, 20457–20464.

Gigoux, V., Escrieut, C., Silvente-Poirot, S., Maigret, B., Gouilleux, L., Fehrentz, J.A., Gully, D., Moroder, L., Vaysse, N., Fourmy, D., 1998. Met-195 of the cholecystokinin-A receptor interacts with the sulfated tyrosine of cholecystokinin and is crucial for receptor transition to high affinity state. J. Biol. Chem. 273, 14380–14386.

Gouldson, P.R., Legoux, P., Carillon, C., Delpech, B., Le Fur, G., Ferrara, P., Shire, D., 1999. Contrasting roles of Leu356 in the human CCK1 receptor for antagonist SR 27897 and agonist SR 146131 binding. Eur. J. Pharmacol. 383, 341–348.

Gully, D., Fréhel, D., Marcy, C., Spinazzé, A., Lespy, L., Neliat, G., Maffrand, J.P., Le Fur, G., 1993. Peripheral biological activity of SR-27,897: a new potent non-peptide antagonist of CCK-A receptors. Eur. J. Pharmacol. 232, 13–19.

Hadac, E.M., Pinon, D.I., Ji, Z.S., Holicky, E.L., Henne, R.M., Lybrand, T.P., Miller, L.J., 1998. Direct identification of a second distinct site of contact between cholecystokinin and its receptor. J. Biol. Chem. 273, 12988–12993.

Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K., Pease, L.R., 1989. Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77, 61–68.

Huang, S., Fortune, K.P., Wank, S.A., Kopin, A.S., Gardner, J.D., 1994. Multiple affinity states of different cholecystokinin receptors. J. Biol. Chem. 269, 26121–26126.

Ji, Z.S., Hadac, E.M., Henne, R.M., Patel, S.A., Lybrand, T.P., Miller, L.J., 1997. Direct identification of a distinct site of interaction between the carboxyl-terminal residue of cholecystokinin and the type A cholecystokinin receptor using photoaffinity labeling. J. Biol. Chem. 272, 24393–24401.

Kennedy, K., Escrieut, C., Dufresne, M., Clerc, P., Vaysse, N., Fourmy, D., 1995. Identification of a region of the N-terminal of the human CCKA receptor essential for the high affinity interaction with agonist CCK. Biochem. Biophys. Res. Commun. 213, 845–852.

Kennedy, K., Gigoux, V., Escrieut, C., Maigret, B., Martinez, J., Moroder, L., Frehel, D., Gully, D., Vaysse, N., Fourmy, D., 1997. Identification of two amine acids of the human cholecystokinin-A receptor that interact with the N-terminal moiety of cholecystokinin. J. Biol. Chem. 272, 2920–2926.

Kopin, A.S., McBride, E.W., Quinn, S.M., Kolakowski, L.F., Beinborn, M., 1995. The role of the cholecystokinin-B gastrin receptor transmembrane domains in determining affinity for subtype-selective ligands. J. Biol. Chem. 270, 5019–5023.

Miloux, B., Lupker, J.H., 1994. Rapid isolation of highly productive recombinant Chinese hamster ovary cell lines. Gene 149, 341–344.

Pandya, P.K., Huang, S.C., Talkad, V.D., Wank, S.A., Gardner, J.D., 1994. Biochemical regulation of the three different states of the cholecystokinin (CCK) receptor in pancreatic acini. Biochim. Biophys. Acta 1224, 117–126.

Pearson, R.K., Miller, L.J., 1987. Affinity labeling of a novel cholecystokinin-binding protein in rat pancreatic plasmalemma using new short probes for the receptor. J. Biol. Chem. 262, 869–876.

Sainz, E., Akeson, M., Mantey, S.A., Jensen, R.T., Battey, J.F., 1998. Four amino acid residues are critical for high affinity binding of neuromedin B to the neuromedin B receptor. J. Biol. Chem. 273, 15927–15932.

Silvente-Poirot, S., Escrieut, C., Wank, S.A., 1998. Role of the extracellular domains of the cholecystokinin receptor in agonist binding. Mol. Pharmacol. 54, 364–371.

Sydow, S., Flaccus, A., Fischer, A., Spiess, J., 1999. The role of the fourth extracellular domain of the rat corticotrophin-releasing factor receptor type 1 in ligand binding. Eur. J. Biochem. 259, 55–62.

Talkad, V.D., Fortune, K.P., Pollo, D.A., Shah, G.N., Wank, S.A., Gardner, J.D., 1994a. Direct demonstration of three different states of the pancreatic cholecystokinin receptor. Proc. Natl. Acad. Sci. U.S.A. 91, 1868–1872.

Talkad, V.D., Patto, R.J., Metz, D.C., Turner, R.J., Fortune, K.P., Bhat, S.T., Gardner, J.D., 1994b. Characterization of the three different states of the cholecystokinin (CCK) receptor in pancreatic acini. Biochim. Biophys. Acta 1224, 103–116.

Tarasova, N.I., Stauber, R.H., Choi, J.K., Hudson, E.A., Czerwinski, G., Miller, J.L., Pavlakis, G.N., Michejda, C.J., Wank, S.A., 1997. Visualization of G protein-coupled receptor trafficking with the aid of the green fluorescent protein — endocytosis and recycling of cholecystokinin receptor type A. J. Biol. Chem. 272, 14817–14824.

Topham, C.M., Mouledous, L., Poda, G., Maigret, B., Meunier, J.C., 1998. Molecular modelling of the ORL1 receptor and its complex with nociceptin. Protein Eng. 11, 1163–1179.

Walker, P., Munoz, M., Martinez, R., Peitsch, M.C., 1994. Acidic residues in extracellular loops of the human Y1 neuropeptide Y receptor are essential for ligand binding. J. Biol. Chem. 269, 2863–2869.

Wank, S.A., 1995. Cholecystokinin receptors. Am. J. Physiol. 32, G628–G646.

Wank, S.A., 1998. G protein-coupled receptors in gastrointestinal physiology i — CCK receptors — an exemplary family. Am. J. Physiol. 37, G607–G613.

Wank, S.A., Harkins, R.T., Jensen, R.T., Shapira, H., De Weerth, A., Slattery, T., 1992a. Purification, molecular cloning, and functional

expression of the cholecystokinin receptor from rat pancreas. Proc. Natl. Acad. Sci. U.S.A. 89, 3125–3129.

Wank, S.A., Pisegna, J.R., De Weerth, A., 1992b. Brain and gastrointestinal cholecystokinin receptor family: structure and functional expression. Proc. Natl. Acad. Sci. U.S.A. 89, 8691–8695.

Wu, V., Yang, M., McRoberts, J.A., Ren, J., Seensalu, R., Zeng, N.X., Dagrag, M., Birnbaumer, M., Walsh, J.H., 1997. First intracellular loop of the human cholecystokinin-A receptor is essential for cyclic AMP signaling in transfected HEK-293 cells. J. Biol. Chem. 272, 9037–9042.