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Binding Sites and Transduction Process of the Cholecystokinin$_B$ Receptor: Involvement of Highly Conserved Aromatic Residues of the Transmembrane Domains Evidenced by Site-Directed Mutagenesis

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ABSTRACT

The functional significance of the extracellular amino-terminal region and of three highly conserved aromatic residues present in the fifth (TM-V) and sixth (TM-VI) transmembrane domains of the rat cholecystokinin (CCK)$_B$ receptor, transfected in Cos-7 cells, was investigated by site-directed mutagenesis. The amino-terminal region of the CCK$_B$ receptor seemed to be weakly involved in CCK binding in that the affinities of CCK$_8$ and selective agonists and antagonists were not modified by truncation of this region. Substitution of Phe347 in TM-VI with alanine produced a mutant receptor that displays the same affinity and selectivity as the wild-type receptor for agonists, but a slightly increased affinity for the selective CCK$_B$ antagonist L-365,260. However, the addition of saturating CCK$_8$ concentrations to cells expressing this mutant did not result in the production of inositol phosphates, demonstrating the critical role of Phe347 in CCK$_B$ receptor to G protein coupling. Substitution of Phe227 with alanine was without effect on the affinities of CCK$_B$ ligands and on phosphoinositide turnover but modified the affinity of the CCK$_A$ antagonist L-364,718. Residue Trp351 located within the CCK$_B$ receptor TM-VI is involved in the binding of CCK$_8$ and CCK$_4$ and of the CCK$_4$-based antagonist PD-134,308, as illustrated by the decreased affinities of these ligands in W351A mutant. The lower affinity for CCK$_8$ observed with this mutated CCK$_B$ receptor accounts for the higher EC$_{50}$ value for phosphotidylinositol hydrolysis. This study suggests that at least part of the binding site for the agonist is located inside the transmembrane domain of the CCK$_B$ receptor, partially overlapping that of antagonists, and gives new insights into the regions involved in the transduction process.

The effects of CCK$_8$, the carboxyl-terminal octapeptide fragment of cholecystokinin, are mediated by two distinct receptors: the CCK$_A$ receptors, mainly found in the gastrointestinal system, and the CCK$_B$ receptors, predominant in the central nervous system (see review in Wank, 1995). Both receptors, cloned from various species, belong to the GPCR superfamily, characterized by seven membrane-spanning segments and a extracellular NH_2-terminal domain. On binding of CCK$_8$, the CCK$_B$ receptor activates PLC, triggering an increase in IP production (Jagerschmidt *et al.*, 1995; Wank, 1995). The brain CCK$_B$ receptor is involved in several important adaptational processes. Thus, selective CCK$_B$ antagonists were shown capable to block the panic attacks induced by administration of CCK$_4$ (Bradwejn *et al.*, 1992) and to be endowed with antidepressant-like properties in rodents (Derrien *et al.*, 1994). Furthermore, due to the negative feedback control achieved by CCK$_8$ via CCK$_B$ receptor activation on the opioid system, the CCK$_B$ antagonists were shown to produce a strong potentiation of the antinociceptive effects induced by opioids (Maldonado *et al.*, 1993). On the other hand, potent and selective CCK$_B$ receptor agonists such as BC 264 (Charpentier *et al.*, 1988) were shown to improve vigilance and memory processes (see review in Daugé and Roques, 1995). All these data account for the great interest devoted to the design of selective CCK$_B$ ligands.

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ABBREVIATIONS: CCK, cholecystokinin; CCK$_8$, cholecystokinin octapeptide; CCK$_4$, cholecystokinin tetrapeptide; pBC 264, propionyl-Boc-Tyr(SO_3H)-gNle-mGly-Trp-(NMe)-Nle-Asp-PheNH_2; L-365,260, 3*R*-(+)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepine-3yl)-*N*′-(3-methylphenyl)urea; L-364,718, 3*S*-(−)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepine-3yl)-1*H*-indole-2-carboxamide; PD-134,308, 4-[2-([3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-{[(tricyclo[3.3.1.1^{3,7}]dec-2yloxy)carbonyl]aminopropyl}amino]-1-phenyl ethyl)amino]-4-oxo-]R-(R*,R*)]-butanoate *N*-methyl–glucamine; GPCR, G protein-coupled receptor; PI, phosphoinositide; PLC, phospholipase C; TM, transmembrane domain; WT, wild-type; IP, inositol phosphate.

Although numerous results support the localization of binding sites for small amine ligands (e.g., histamine, 5-hydroxytryptamine, dopamine) within the TM domains of GPCRs (Schwartz and Rosenkilde, 1996), several peptide ligands were reported to interact with both TM and extracellular domains of these receptors. Thus, for instance, it has been shown by point mutagenesis and chimeric studies that opioid receptors interact with their ligands at multiple sites, both extracellular and intramembranous (Befort *et al*., 1996; Pepin *et al*., 1997). Likewise, numerous studies have provided evidence for an important role of residues in the amino-terminal domain, the extracellular loops, and the transmembrane helices for the ability of several receptors to bind their ligands, such as neurokinin receptor (Fong *et al*., 1992), V_{1a} vasopressin receptor (Chini *et al*., 1995), oxytocin receptor (Chini *et al*., 1996), somatostatin receptor (Kaupman *et al*., 1995), and vasoactive intestinal peptide receptor (Du *et al*., 1997).

Few studies have been devoted to the CCK_B receptor. Using site-directed mutagenesis, eight residues located in the TM helices of the human CCK_B receptor (Kopin *et al*., 1995) and His381 located in the TM-VII of the rat CCK_B receptor (Jagerschmidt *et al*., 1996) have been suggested to be involved in CCK_B versus CCK_A antagonist selectivity. In addition, a segment of five amino acids in the second extracellular loop of the CCK_B receptor was shown to be essential for the high affinity of the natural peptide agonist gastrin, suggesting that determinants of the binding site of the CCK_B receptor are situated, at least partially, within the extracellular domains (Silvente-Poirot and Wank, 1996). This result has been confirmed in the case of the CCK_A receptor, in which two amino acids in the amino-terminal region have been identified as critical components of the agonist binding site (Kennedy *et al*., 1997). Moreover, we recently reported that the Asp100 residue, located within the second TM segment of the rat CCK_B receptor and highly conserved among the GPCR superfamily, was involved in agonist-induced IP production (Jagerschmidt *et al*., 1995). All these findings were in good agreement with docking experiments performed with a three-dimensional model of the CCK_B receptor (Jagerschmidt *et al*., 1995, 1996).

On the other hand, several aromatic residues were shown to be highly conserved within the transmembrane domains of GPCRs, in particular, in helices IV, V, and VI (Probst *et al*., 1992; Underwood *et al*., 1994; Befort *et al*., 1996) (Table 1). These conserved aromatic residues were proposed to play an important role in the spatial organization of the binding site (Underwood *et al*., 1994). Moreover, these amino acids could be involved in the signal transduction mechanism occurring after agonist-induced receptor activation as described in the case of the neurokinin type 1 receptor (Huang *et al*., 1994), the 5-hydroxytryptamine$_2$ receptor (Choudhary *et al*., 1993), and the angiotensin II type AT_1 receptor (Marie *et al*., 1994).

To gain further insights into the mechanism of ligand recognition and transduction process for the CCK_B receptor, we investigated the role of the amino-terminal region and of three conserved aromatic residues located on TM-V (Phe227) and TM-VI (Phe347 and Trp351) helices of the rat CCK_B receptor (Table 1 and Fig 1). Using site-directed mutagenesis, the aromatic residues were replaced by alanine, and a amino-terminally truncated (Δ1,53) CCK_B receptor was constructed. The WT, the truncated, and the F227A, F347A, and W351A mutated rat CCK_B receptors were transiently expressed in Cos-7 cells, and radioligand binding experiments and IP assays were performed. This enabled us to demonstrate that Trp351 interacts with the CCK agonists and antagonists, whereas Phe227 and Phe347 are more important for CCK antagonists binding. Moreover, the amino-terminal region of the CCK_B receptor seems not to be involved in CCK ligands recognition. On the other hand, at least one residue (Phe347) localized in the sixth TM domain was identified as essential for the signal transduction process.

Materials and Methods

Reagents. CCK_4 was purchased from Bachem (Buhendrof, Switzerland). CCK_8, pBC 264, L-365,260, L-364,718, and PD-134,308 (Fig. 2) were synthesized in the laboratory according to reported procedures (Evans *et al.,* 1986; Charpentier *et al.,* 1988; Lotti and Chang, 1989; Hughes *et al.,* 1990). Radiolabeled compounds, [α-^{33}P]dATP (specific activity, 1000–3000 Ci/mmol), [^{3}H]pCCK_8 (specific activity, 60–90 Ci/mmol), and *myo*-[2-^{3}H]inositol (specific activity, 60–90 Ci/mmol) were purchased from Amersham (Les Ulis, France). [^{3}H]pBC 264 (specific activity, 20–30 Ci/mmol) was from NEN (Les Ulis, France). Cell culture reagents were from GIBCO-BRL (Cergy, France), and the AG1-X8 Dowex resin was from BioRad (Ivry/Seine, France).

Site-directed mutagenesis. The cDNA of the rat CCK_B receptor was obtained as described previously (Jagerschmidt *et al*., 1995). For construction of the amino-terminally truncated (1–53) CCK_B receptor, the *Pvu*II site at residue 53 was used for restriction endonuclease cleavage. Three oligonucleotides were designed to replace the codon for phenylalanine (TTC) located at amino acid positions 227 and 347 with a codon for alanine (5′-CTGCTTTTGGCCTTCATCCCG-3′ and

TABLE 1

Sequence comparison of TM-V and TM-VI helices within various GPCRs

The predicted TM helices of the rat CCK_B receptor are compared with the corresponding sequences of other representative members of the GPCRs. Numbers above the sequences refer to amino acid positions within the rat CCK_B receptor (NK-1, neurokinin-1 receptor; BK, bradykinin receptor; AT-1, angiotensin receptor type 1; 5HT2, 5-hydroxytryptamine receptor type 2; M1, muscarinic receptor type 1; β2-AR, β_2 adrenergic receptor). The single-letter amino acid code is used. The highly conserved aromatic residues are indicated in *bold*.

	TM-V		TM-VI	
	227	238	347	351
CCK_B (rat)	SVLLLLLL**F**FI**P**GVVIAVA**Y**GLIS		MLLVIVLL**F**FLC**W**L**P**VYSVNT	
CCK_A (human)	HTFLLLIL**F**LI**P**GIVMMVA**Y**GLIS		MLIVIVVL**F**FLC**W**M**P**IFSANA	
NK-1 (human)	HICVTVLI**Y**FL**P**LLVIGYA**Y**TVVG		MMIVVVCT**F**AIC**W**L**P**FHIFFL	
BK (human)	NMLLNVVG**F**LL**P**LSVITFC**T**MQIM		LVLVVLLL**F**IIC**W**L**P**FQISTF	
AT1 (human)	GLTKNILG**F**LF**P**FLIILTS**Y**TLIW		IIMAIVLF**F**FFS**W**I**P**HQIFTF	
5-HT_2 (human)	VLIGSFVS**F**FI**P**LTIMVIT**Y**FLTI		VLGIVFFL**F**VVM**W**C**P**FFITNI	
M1 (human)	TFGTAMAA**F**YL**P**VTVMCTL**Y**WRIY		TLSAILLA**F**ILT**W**T**P**YNIMVL	
β_2-AR (human)	AIASSIVS**F**YV**P**LVIMVFV**Y**SRVF		TLGIIMGT**F**TLC**W**L**P**FFIVNI	

5′-GTTTTGCTTGCCTTCCTGTGT-3′, respectively) and to replace the codon for tryptophan (TGG) located at amino acid position 351 with a codon for alanine (5′-TTCCTGTGTGCGCTGCCAGTG-3′). Double-strand mutagenesis was carried out as described previously (Jagerschmidt *et al.*, 1995). Authenticity of the mutations was confirmed by sequencing the constructions over the entire protein-coding region with the Sequenase Version 2.0 DNA sequencing kit (United States Biochemical, Cleveland, OH) and [α-^{33}P]dATP.

Radioligand binding assays. Cos-7 cells, which do not express CCK_B receptor, were grown onto 24-well plates and transfected, using the calcium phosphate precipitation method, with 0.5 μg of plasmid DNA/10^5 cells as described previously (Jagerschmidt *et al.*, 1996). Then, 8 μg of plasmid containing either the WT or truncated receptor cDNA also were transfected into Cos-7 (10^5 cells) using the calcium phosphate precipitation method as described by Graham and van der Erb (1973). At 48 hr after the transfections, the binding assays were performed directly on cells in Dulbecco's modified Eagle's medium containing 5 mM $MgCl_2$ and 0.2 mg/ml bacitracin. Each assay (90 min, 25°) was performed in a final volume of 0.5 ml. For saturation binding experiments, the concentration of the [^{3}H]pCCK_8 or [^{3}H]pBC 264 varied from 50 to 6000 pM. For competition experiments, a fixed concentration of 500 pM of the radioligand [^{3}H]pCCK_8 or [^{3}H]pBC 264 were used in the presence of various concentrations of the competitor. Nonspecific binding was determined by using 1 μM CCK_8. Incubations were stopped by removing the media. The cells were harvested and the radioactivity was counted as described previously (Jagerschmidt *et al.*, 1995). Parameters describing [^{3}H]pCCK_8 saturation binding (i.e., K_d and B_{max}) were determined using the computer program EBDA. K_i values were determined by using the Cheng-Prussof equation: $K_i = IC_{50}/[1 + (\text{radioligand concentration}/K_d \text{ value of the radioligand})]$.

IP assays. Cos-7 cells transiently expressing WT and mutated CCK_B receptors were assayed for CCK_8-stimulated PI hydrolysis, as described previously (Jagerschmidt *et al.*, 1995). Briefly, transfected cells were grown in the presence of 1 μCi/ml *myo*-[2-^{3}H]inositol for 16 hr at 37°. Cells then were treated with 10 mM LiCl for 30 min at 37° and various concentrations of CCK_8 were added to the cells. After 45 min at 37°, the incubation medium was removed, and the cells were washed twice with 1 ml of PBS. The reaction was stopped by adding 400 μl of ice-cold 75% methanol and 300 μl of 0.12% Triton. The cells were scraped, and the suspension was subjected to chloroform extraction. Then, 0.5 ml of the aqueous phase was added to 4.5 ml of H_2O. The solution was loaded onto a 0.5-ml column containing AG1-X8 Dowex anion exchange resin. The column was washed with 1 ml of distilled water followed by 5 ml of 5 mM sodium borate/60 mM sodium formate. Total [^{3}H]IP then were eluted into scintillation vials with 5 ml of 1 M ammonium formate/0.1 M formic acid. After the addition of scintillation mixture, radioactivity was counted.

Statistical analysis. The results presented are the mean ± standard error. Data were analyzed using one-way analysis of variance and paired Student's *t* tests. Differences were considered statistically significant at $p < 0.05$.

Results

Identification of aromatic residues of the rat CCK_B receptor important for the binding of CCK ligands. The WT and mutant CCK_B receptors were expressed transiently in Cos-7 cells. To determine whether the mutated receptors were expressed correctly by the cells and displayed CCK affinities comparable to that of the WT receptor, saturation studies were first performed. Except for cells expressing the W351A mutated receptor, the binding of [^{3}H]pCCK_8 to transfected cells was specific and saturable. In the case of the W351A mutant receptor, the binding experiments performed with [^{3}H]pCCK_8 were difficult to interpret due to high nonspecific binding. Moreover, the absence of saturation at a concentration of 6 nM of this radioligand suggested a decrease

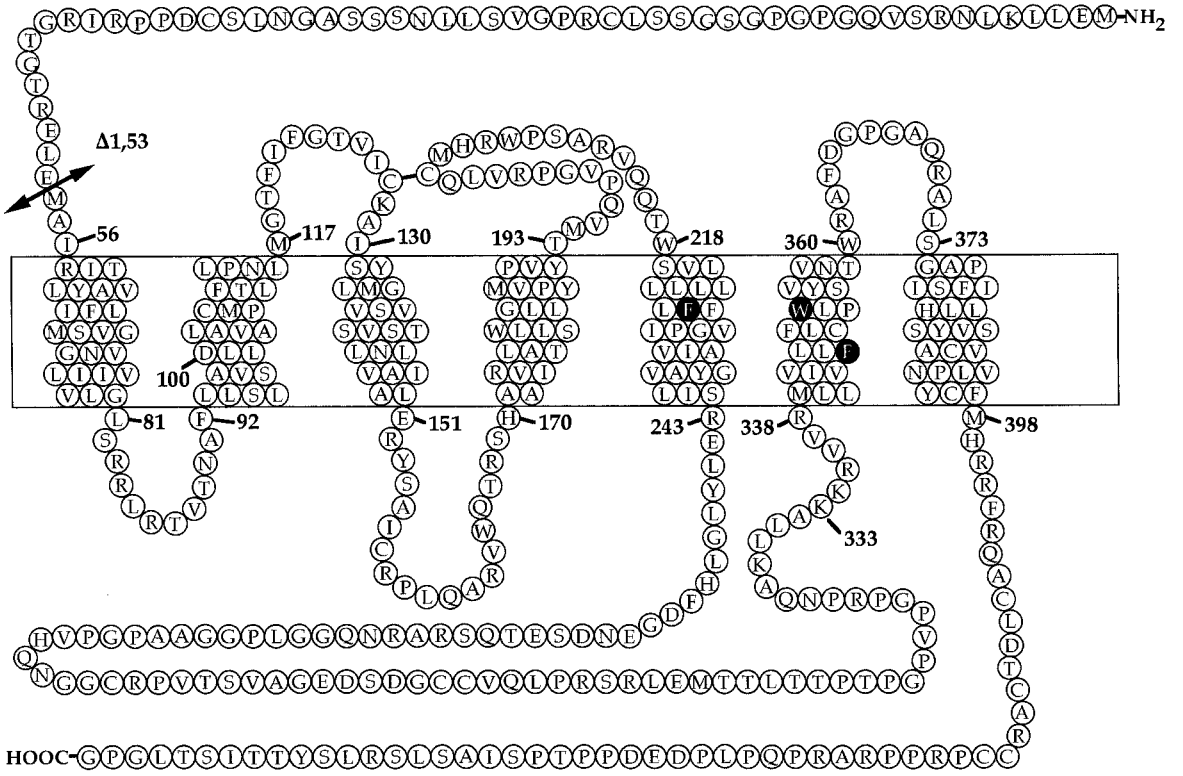

Fig. 1. Schematic representation of the rat CCKB receptor showing the postulated transmembrane topology. The phenylalanine (F227, F347) and tryptophan (W351) residues are highlighted; these residues were individually converted to alanine, as described in Experimental Procedures. *Boxed,* seven TM domains.

in its affinity (data not shown). To overcome this problem, we used the CCK_8-derived peptidomimetic [^{3}H]pBC264, a selective CCK_B receptor agonist endowed with both a higher affinity and a lower nonspecific binding than [^{3}H]pCCK_8 (Charpentier *et al.*, 1988; Durieux *et al.*, 1992). With this radioligand, specific and saturable binding was observed for both WT and W351A receptors. [^{3}H]pBC264 was then chosen for further characterization of the W351A mutant receptor. On the other hand, in the experimental conditions used (0.5 μg of DNA transfected), the B_{max} value for the amino-terminal truncated CCK_B receptor (Δ1,53) was strongly decreased. Thus, to facilitate the determination of the binding characteristics of this mutant, the amount of DNA used in the transfection was increased (8 μg).

Table 2 shows the K_d and B_{max} values obtained for the WT and mutant CCK_B receptors. [^{3}H]pCCK_8 and [^{3}H]pBC264 binding to the WT receptor yielded K_d values of 1.03 ± 0.30 and 0.78 ± 0.16 nM, respectively, and B_{max} values of 37 ± 10 and 35 ± 8 fmol/10^6 cells, respectively. No binding of both radiolabeled probes was detected for nontransfected cells (data not shown), demonstrating the absence of CCK receptor on native cells. The mutation of Phe227 to alanine did not modify the expression of CCK_B receptors in Cos-7 cells. The mutation of Phe347 to alanine gave only 33% expression, whereas the W351A mutation strongly decreased the expression of receptor, yielding 12% of the WT level. The affinity constants (K_d values) of the F227A, F347A, and W351A mutants showed no significant change as compared with the WT value. The truncation of the amino-terminal domain in Δ1,53 yielded also a large reduction in amounts of expressed receptors, with only 10% of that of the WT receptor. Again for this mutant, changes occurred in the level of binding sites rather than in the affinities for CCK agonists (Table 2).

The effects of mutations on the pharmacological properties of the mutant receptors were studied by competition binding experiments using [^{3}H]pCCK_8 or [^{3}H]pBC264 as radioligands. In these conditions, both K_d (1.03 nM or 0.78) and K_i (0.58 or 1.40 nM) values of pBC264 or CCK_8, respectively, were found to be almost identical, as expected (Tables 2-4). Moreover, the four mutations, F227A, W351A, F347A, and Δ1,53, have no effect on the binding of the highly selective CCK_B agonist pBC264 (Tables 3 and 4). However, the exchange of Trp351 for an alanine caused a 6-fold decrease in the affinity of the natural ligand CCK_8 as determined in the competition experiments using [^{3}H]pBC264 as radioligand (Table 4). Interestingly, the carboxyl-terminal tetrapeptide CCK_4 showed also a reduced K_i value (10-fold) for this mutant receptor compared with the WT receptor (Table 4, Fig. 3).

The K_i values of several antagonists that belong to different chemical classes were determined. In this study, the CCK_4-derived peptoid PD-134,308 and the benzodiazepine-derived compound L-365,260, which are selective CCK_B receptor antagonists, and the CCK_A receptor-selective antagonist L-364,718, a benzodiazepine-derived compound, have been used. As shown in Table 3, the exchange of Phe227 for an alanine caused a 3-fold decrease in affinity of L-364,718 (K_i = 68 and 207 nM for WT and F227A, respectively) without affecting the affinity of the CCK_B antagonists L-365,260 and PD-134,308 (Table 3). The mutant F347A demonstrated only increased binding for L-365,260 (Table 3). The exchange of Trp351 for an alanine produced a 3- and 10-fold decrease in the affinity of the benzodiazepine-derived CCK_A antagonist L-364,718 and of the CCK_4-derived peptoid PD-134,308 (Table 4, Fig. 3), respectively, without affecting the affinity of the benzodiazepine CCK_B antagonist L-365,260 (Table 4). The deletion of the amino-terminal region of the CCK_B receptor had no effect on the binding of the CCK_B and CCK_A receptor antagonists (Table 3).

Functionality of the mutant receptors. Rat CCK_B receptors expressed in Cos-7 cells display agonist-mediated dose-dependent increases in PI hydrolysis (Jagerschmidt *et al.*, 1995; Wank, 1995). To determine the functionality of each of the mutant receptors, the CCK_8-induced accumulation of IP in transfected Cos-7 cells was measured.

As illustrated in Fig. 4, CCK_8 induced a dose-dependent stimulation of the production of IP to a 7-fold maximal increase in Cos-7 cells transfected with the WT, whereas no change in PI hydrolysis was observed in control cells transfected with pcDNA3 alone (Fig. 5). Moreover, as pre-

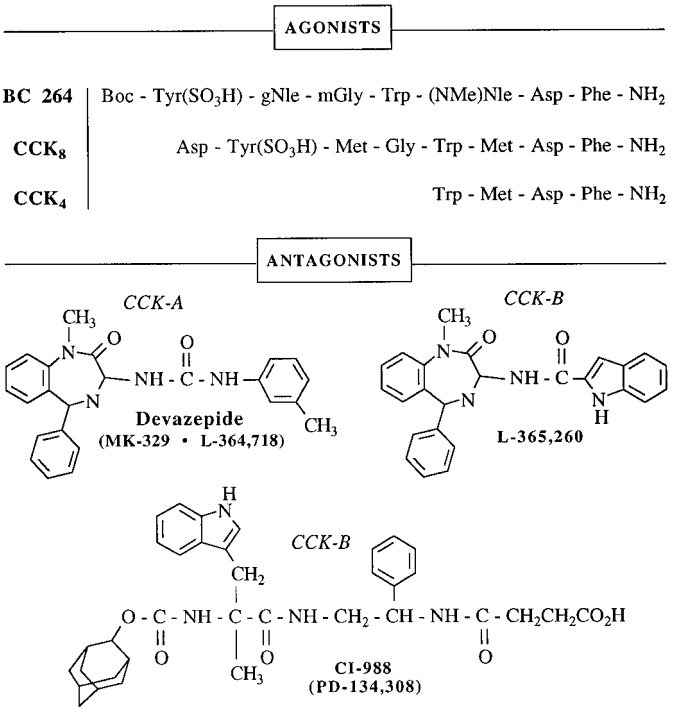

Fig. 2. Structures of CCK agonists and antagonists.

TABLE 2

Binding parameters of WT and mutated CCK_B receptors expressed in Cos-7 cells

The values reported were calculated from saturation binding experiments using [^{3}H]pCCK_8 or [^{3}H]pBC264 as radioligands as described in the text. Data are expressed as mean ± standard error of three or four experiments, each performed in triplicate.

cDNA transfected	CCK_B receptor	[^{3}H]pCCK_8		[^{3}H]pBC264	
		K_d	B_{max}	K_d	B_{max}
$\mu g/10^5$ cells		nM	fmol/10^6 cells	nM	fmol/10^6 cells
0.5	WT	1.03 ± 0.30	37 ± 10		
0.5	F227A	0.55 ± 0.17	32 ± 4		
0.5	F347A	1.20 ± 0.18	13 ± 1		
0.5	WT			0.78 ± 0.16	35 ± 8
0.5	W351A			1.22 ± 0.20	5 ± 1
8	WT	1.09 ± 0.40	788 ± 153		
8	Δ1,53	2.88 ± 0.63	80 ± 12		

viously shown (Table 2), F347A and W351A mutants have a lower binding capacity than the WT. Therefore, preliminary studies were carried out with the WT CCK_B receptor to determine the effect of receptor density on CCK_8-mediated PI hydrolysis. As shown in Table 5, an increase in amounts of transfected DNA into Cos-7 cells induced a proportional increase in B_{max} values and led to an enhanced level of the total IP produced after CCK_8 stimulation without modification of the EC_{50} values (Table 5, Fig. 5). On the other hand, in a previous study, we have shown that the B_{max} value was optimal when the WT CCK_B receptor was transfected in Cos-7 cells with 8 μg of DNA/10^5 cells (Jagerschmidt *et al.*, 1995). Thus, in the current study, we used this amount of DNA for transfection of the mutant receptors in Cos-7 cells. In cells expressing the F227A receptor, the accumulation of inositol phosphates induced by CCK_8 was in the same range (~7-fold) as that observed in Cos-7 cells transfected with the WT receptor, and the concentrations giving half-maximal responses were similar (EC_{50} = 1.41 and 1.3 nM for F227A and WT, respectively). The exchange of Trp351 for an alanine decreased the efficacy of CCK_8 to stimulate PI hydrolysis (EC_{50} = 8.7 nM) compared with WT, with a slight decrease in potency (Table 5, Fig. 5). In contrast, cells expressing the F347A mutant receptor were almost completely unable to produce IP in response to CCK_8 stimulation (Figs. 4 and 5), although the binding affinity for this ligand was found to be unaffected by the mutation (Table 2). This result cannot be attributed to the decrease in expression level of the mutated receptor (B_{max} = 166 ± 15 fmol/10^6 cells) because cells expressing similar levels of WT CCK_B receptor (B_{max} = 180 ± 12 fmol/10^6 cells) produced large amounts of IP (Table 5, Fig. 5).

TABLE 3

Compared pharmacological properties of WT and mutated CCK_B receptors expressed in Cos-7 cells

K_i values were calculated from competition binding experiments using [^{3}H]pCCK_8 (0.5 nM) as radioligand as described in the text. Data are expressed as the mean ± standard error of three or four experiments, each performed in triplicate.

Ligand	K_i			
	WT	F227A	F347A	Δ1,53
	nM			
Agonist				
pBC264	0.58 ± 0.11	0.39 ± 0.01	0.20 ± 0.02	0.30 ± 0.004
Antagonist				
PD-134,308	4.39 ± 2.03	1.62 ± 0.29	6.83 ± 1.90	2.77 ± 0.64
L-365,260	5.96 ± 0.14	3.73 ± 0.52	1.91 ± 0.14[a]	3.17 ± 0.44
L-364,718	68 ± 15	207 ± 30[a]	133 ± 48	101 ± 10

[a] $p < .01$, Student's t test.

TABLE 4

Compared pharmacological properties of WT and mutated CCK_B receptors expressed in Cos-7 cells

K_i values were calculated from competition binding experiments using [^{3}H]pBC264 (0.5 nM) as radioligand as described in the text. Data are expressed as the mean ± standard error of three or four experiments, each performed in triplicate.

Ligand	K_i	
	WT	W351A
	nM	
Agonist		
CCK_8	1.40 ± 0.16	8.14 ± 2.05[a]
pBC264	1.21 ± 0.20	1.61 ± 0.35
CCK_4	30.3 ± 3.50	359 ± 41[a]
Antagonist		
L-365,260	10.9 ± 2.00	11.8 ± 0.1
PD-134,308	2.60 ± 0.10	24.9 ± 4.7[a]
L-364,718	276 ± 85	960 ± 269[a]

[a] $p < .01$, Student's t test.

Discussion

Numerous studies have examined peptide ligand binding domains of GPCRs showing a considerable diversity in both the number and location of ligand interacting determinants. Several aromatic residues located in the TM helices are conserved among most of the GPCRs (Probst *et al.*, 1992). This is the case for the Ar-X_2-Pro-X_7-Ar motif (where Ar is aromatic and X is any residue), which is very often found in the TM-V domain, and for Ar-X_3-Ar-X-Pro, which is found in the TM-VI helix of most of the GPCRs (Underwood *et al.*, 1994). These motifs also were found in the CCK_B receptor, and the high conservation of these constituting amino acids (Table 1) suggests that they could have a critical role in the structural organization and/or in the functioning of GPCRs. Furthermore, the importance of the external amino-terminal region of several GPCRs for the binding of peptide ligands has been

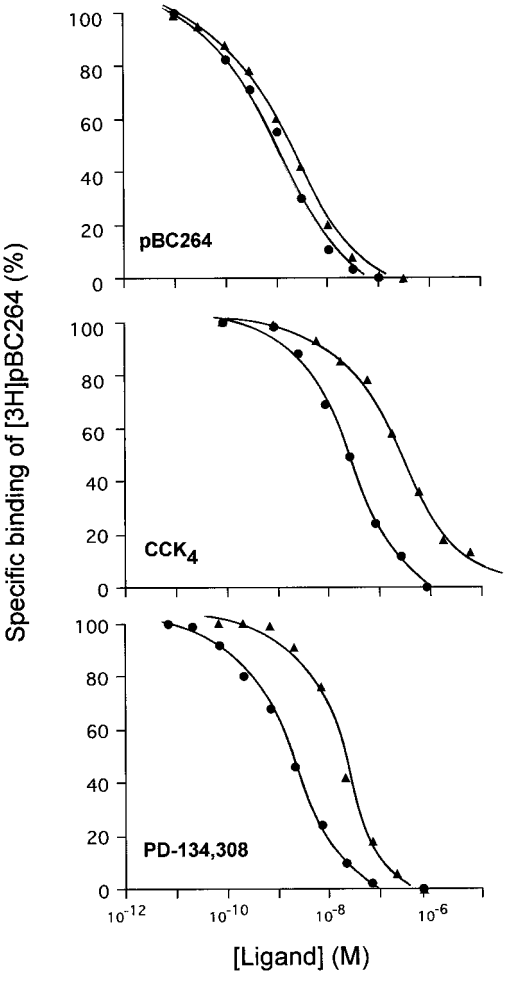

Fig. 3. Competition of CCK ligands toward the binding of [^{3}H]pBC264 to WT and W351A rat CCK_B receptors. Cos-7 cells transiently expressing WT (●) or W351A mutant (▲) receptors were tested for competition experiments using pBC 264, CCK_4 and PD-134,308 as described in Experimental Procedures. Each point is the average of triplicate determinations. Data, which are representative of three or four separate experiments, are depicted with standard error values in Table 4.

reported (Hjorth *et al*., 1994; Hong *et al*., 1994). Thus, Miyake (1995) has shown that a splice variant of the CCK_B receptor, in which the amino-terminal extracellular domain and almost all the residues of the first transmembrane helix were absent, displayed altered binding and functional properties. Therefore, in the current study, we evaluated the involvement in ligand binding and signal transduction of three aromatic residues belonging to the highly conserved domains (Phe227 in TM-V, Phe347 and Trp351 in TM-VI) and of the external amino-terminal region of the CCK_B receptor (Fig. 1).

Exchange of Phe227 for alanine produced only a slight decrease in the affinity of the selective CCK_A antagonist L-364,718, without effects on CCK_8 and CCK_B antagonists affinities and second messenger production. A recent study on the human NK_1 receptor demonstrated that the exchange of a tyrosine localized in the same TM-V domain (Tyr205) (Table 1) for alanine resulted in a decrease of substance P

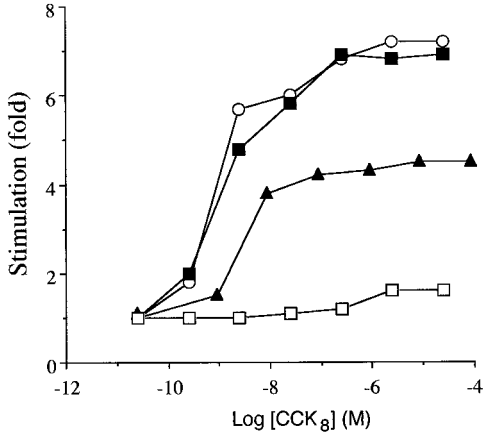

Fig. 4. CCK8 stimulation of IP formation in Cos-7 cells expressing WT (○), F227A (■), F347A (□), and W351A (▲) CCK_B receptors. Cells were transfected using 8 μg of DNA/10^5 cells, and assays were performed as described in Experimental Procedures. Data points represent mean values of triplicate determinations in three experiments. There was no CCK_8-stimulated IP formation in nontransfected Cos-7 cells. For comparison, the maximum amount of [^{3}H]IP accumulation induced by CCK_8 activation of WT CCK_B receptor, in a typical experiment, was 1700 cpm, with a basal accumulation of 250 cpm.

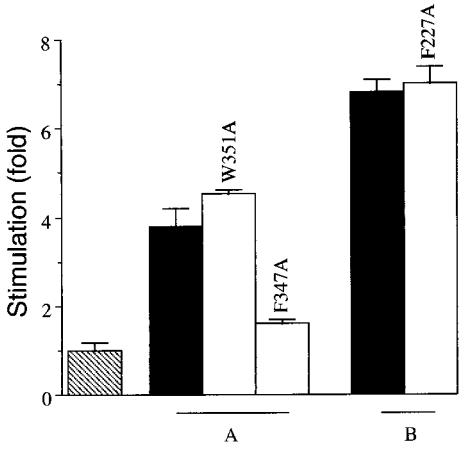

Fig. 5. CCK_8 stimulation of IP formation in Cos-7 cells expressing WT (■) and mutated (□) CCK_B receptors. After transfection, Cos-7 cells were stimulated using 0.1 μM CCK_8, and assays were performed as described in Experimental Procedures. *Histograms*, mean values of triplicate determinations in three experiments for cells expressing 100–200 (A) and 700–800 (B) fmol receptor/10^6 cells. *Hatched bars*, CCK_8 stimulation of IP formation in nontransfected Cos-7 cells.

affinity, associated with a similar loss in the biological potency illustrated by a parallel reduction in IP production (Huang *et al*., 1994). Together, these results emphasize the diversity of GPCRs binding sites.

The mutation of Phe347 for alanine in the rat CCK_B receptor did not alter the binding of CCK agonists. This result suggests that in the rat CCK_B receptor, the removal of the aromatic ring of residue 347 located in the TM-VI does not affect the binding site for agonists. Furthermore, the mutant rat CCK_B receptor (F347A) exhibits a slightly improved affinity for the CCK_B antagonist L-365,260, whereas affinity for the benzodiazepine-derived antagonist L-364,718 remains unchanged (Table 3). This could be explained by differences in the spatial orientation of the two antagonists within the receptor binding site. Accordingly, in a previous study, we have shown that the two structurally close benzodiazepine-derived compounds L-365,260 and L-364,718 recognize some specific residues into the ligand binding pocket of the CCK_B receptor (Jagerschmidt *et al*., 1996). Moreover, mutation F347A of the CCK_B receptor, while slightly affecting the binding of CCK antagonists, did not affect the binding of CCK agonists, suggesting that both classes of molecules do not interact with the same region of the receptor. Differences between chemical determinants for agonists and antagonists binding has already been shown for peptide receptors, including the CCK_B receptor (Beinborn *et al*., 1993; Mantamadiotis and Baldwin, 1994).

Studies on the functionality of the CCK_B receptor showed a different pattern of responses in the case of F347A and F227A mutants. Thus, mutation of Phe347 disrupts PI signaling, indicating a complete loss of the transduction process associated with agonists binding. In contrast, at comparable expression levels, both efficacy and potency values were found to be very close for the WT and F227A receptors, indicating that the Phe227 residue of the rat CCK_B receptor is not involved in the signal transduction mechanism. The loss in pharmacological activity of CCK_8 with the F347A mutant receptor was not accompanied by a similar loss in affinity for CCK_8. Moreover, the observed lack of functional activity cannot be attributed to differences in receptor expression level; for similar amounts of F347A and WT receptors, only the latter induced phosphatidylinositol hydrolysis (Fig. 4). This observation suggests that mutation of Phe347 affects a region involved in the agonist-induced second messenger production without affecting the overall integrity of the receptor. It is interesting to observe that the amino acids involved in G protein activation and located in TM VI and VII domains of GPCRs are very often tyrosine (Wess *et al*., 1992; Marie *et al*., 1994), threonine (Wess *et al*., 1992), or proline (Wess *et al*., 1993) residues. Nevertheless in the 5-hydroxytryptamine$_2$ receptor, a phenylalanine residue also has been shown to be implicated in second messenger production (Choudhary *et al*., 1993). Recently, we have shown that the Asp100 residue of the rat CCK_B receptor is involved in signal transduction. It was hypothesized that Asp100 points in a direction of a cluster of basic amino acids (Lys333/Lys334/Arg335) located in the third intracellular loop of the receptor at the bottom of the TM-VI domain (Jagerschmidt *et al*., 1995). This was confirmed by results reported by Wang (1997) showing that these three basic amino acids play a critical role in CCK_B receptor activation of G_q proteins. Thus, although not directly involved in the binding of CCK_B ligands

as shown by the lack of change in binding affinities after its replacement by alanine, Phe347, which belongs to the TM-VI domain, could be a residue implicated in transduction processes by playing a key role in agonist-induced changes in receptor conformation triggering G protein stimulation. The same explanation could be proposed if we take into account the allosteric model of receptor. In both cases, the exchange of Phe347 by alanine could produce a conformational change in the sequence containing the basic triplet Lys333/Lys334/Arg335, located just beneath TM VI, in the third intracellular loop, known to be involved in G protein coupling (Schwartz and Rosenkilde, 1996).

To further explore which amino acids are critical for ligand binding, we have shown by site-directed mutagenesis that Trp351, another conserved aromatic amino acid, when mutated to alanine, leads to a loss in affinity for CCK_8 and CCK_4 and for the CCK_4-based antagonist, PD-134,308 (6-, 10-, and 10-fold, respectively). These results indicate that the Trp351 residue seems to be involved in the agonist binding site of the rat CCK_B receptor. This observation and the fact that the binding of the pseudopeptide agonist pBC264 and of nonpeptide ligands, such as benzodiazepine-derived antagonists L-365,260 and L-364,718, was unaffected by the mutation, suggest that replacement of Trp351 by alanine directly affects a region of the binding site for CCK on the receptor without affecting the overall integrity of the receptor. These results emphasize previous results obtained with the NK_1 (Fong *et al.*, 1994), CCK_A (Kennedy *et al.*, 1997), and CCK_B (Jagerschmidt *et al.*, 1996) receptors, suggesting that within the receptor binding site, the residues involved in ligand recognition are different, with molecules belonging to different chemical classes, a result also found in δ-opioid receptors (Befort *et al.*, 1996). A Trp residue, located at an equivalent position in the TM-VI domain of M_3 muscarinic receptor, also was shown to be involved in both agonist and antagonist binding (Wess *et al.*, 1993), demonstrating the critical role of this highly conserved Trp residue. In good agreement with the current experimental data, molecular modeling studies have suggested that this Trp residue, in concert with several other aromatic amino acids, may be directly involved in ligand/receptor recognition (Hibert *et al.*, 1991; Underwood *et al.*, 1994). Furthermore, the mutation of the Trp351 residue in the rat CCK_B receptor, while strongly affecting the binding of CCK_4 and CCK_4-derived ligand (PD-134, 308), affects to a lesser extent the binding of CCK_8, suggesting the existence of specific interaction or interactions between the carboxyl-terminal part of CCK_8 [Asp-Tyr(SO_3H)-Met-Gly] and the CCK_B receptor, stabilizing the ligand/receptor complex. On the other hand, the reduction in affinity for CCK_8 of the mutant W351A receptor resulted in a loss in the biological efficacy of CCK_8 to produce IP. However, as for the WT receptor, the efficacy of CCK_8 to stimulate PI hydrolysis (EC_{50} = 8.7 nM) was in the same range as its affinity (K_i = 8.14 nM) for the sulfated octapeptide. Moreover, in the absence of stimulation, no IP release was observed, with the W351A mutant showing that this receptor is not constitutively activated.

TABLE 5

Expression levels and functional properties of WT and mutant CCK_B receptors expressed in Cos-7 cells

Cells were transfected using the indicated DNA concentrations and assays realized as described in the text. The results (mean ± standard error) represent measurements obtained from two to four experiments, each performed in triplicate.

Receptor	DNA transfected	Receptor number	CCK_8 EC_{50}	Maximal increase of IP production
	μg / 10⁵ cells	*fmol / 10⁶ cells*	*nM*	*fold basal*
CCKB (WT)	0.5	37 ± 10		1.3 ± 0.2
CCKB (WT)	2	180 ± 12	1.6 ± 0.3	3.8 ± 0.4
CCKB (WT)	8	788 ± 153	1.3 ± 0.2	6.8 ± 0.3
F227A	8	742 ± 112	1.41 ± 0.18	7.0 ± 0.4
F347A	8	166 ± 15		1.6 ± 0.1
W351A	8	126 ± 13	8.70 ± 0.46[a]	4.52 ± 0.1

[a] $p < .01$, Student's t test compared with WT (2 μg).

The importance of the amino-terminal region of the CCK_B receptor was demonstrated by the discovery of a splice variant that was deleted in the amino-terminal extracellular region (Δ1,63) and the upper part of the first transmembrane domain and had different binding properties than the WT CCK_B receptor (Miyake, 1995). Moreover, a segment of five amino acids in the second extracellular loop of the CCK_B receptor was shown to be essential for the high affinity of this receptor for gastrin, suggesting that determinants of the binding site of the CCK_B receptor could be located within the extracellular domains (Silvente-Poirot and Wank, 1996). To further explore this hypothesis, we evaluated the affinity of several CCK ligands for a mutant receptor in which only the external amino-terminal domain has been deleted (Δ1,53). The results obtained show that this truncation did not affect the binding of the CCK ligand, which is in contrast to the 5-fold reduction in affinity observed with the splice variant (Δ1,63) (see above). Thus, it seems that the extracellular amino-terminal region of the CCK_B receptor is not important for ligand/receptor complex formation. In the case of the CCK_A receptor, Kennedy *et al.* (1997) identified two amino acids located in the amino-terminal region that play a role in agonist binding. The difference between the two CCK receptor types is reflected by the low sequence homology between their amino-terminal domains. The importance of this amino-terminal region has been investigated in several GPCRs, and the results show divergent results. Thus, ligands have been shown to bind to the external amino-terminal domain of several receptors, such as κ-opioid (Kong *et al.*, 1994) or AT_1 (Hjorth *et al.*, 1994) receptors. In contrast, deletion of the 64 amino-terminal amino acids of the μ-opioid receptor did not affect binding of agonists and antagonists, indicating that the amino-terminal domain does not contribute to ligand binding (Surratt *et al.*, 1994). This also seems to be the case for the CCK_B receptor. Nevertheless, this amino-terminal domain that possesses glycosylation sites seems to be important for the expression of the receptor at the membrane levels, as the truncated mutant shows an expression level 10-fold lower than that of the CCK_B WT receptor.

In conclusion, using site-directed mutagenesis of the CCK_B receptor and analysis of the binding affinity and biological potency of CCK ligands, we have shown that the amino-terminal region is not involved in the formation of the ligand/receptor complex. Moreover, we demonstrated that the highly conserved aromatic amino acid residues in GPCRs, Phe227 and Phe347, do not play an important role in the recognition of the agonists, whereas a loss in the affinity of the antagonists to the mutated receptors was observed. We also have identified, for the first time, one amino acid (Trp351) in the agonist binding site of the receptor that is involved in the binding of the carboxyl-terminal sequence of CCK_8 as illustrated by the similar reduction in affinity for both CCK_8 and CCK_4. With regard to signal transduction, it

seems that the Phe347 residue plays a key role in CCK_8-stimulated IP production. The results obtained with the W351A mutant also seem to demonstrate that the Trp351 residue could be involved in receptor/G protein coupling. These findings represent an important step toward the complete delineation of the agonist and antagonist binding sites, as well as the determination of the regions involved in the specificity of receptor/G protein coupling.

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