

# Mutations within the Cholecystokinin-B/Gastrin Receptor Ligand 'Pocket' Interconvert the Functions of Nonpeptide Agonists and Antagonists

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## ABSTRACT

We have reported previously that the transmembrane domains of the cholecystokinin-B/gastrin receptor (CCK-BR) comprise a putative ligand binding pocket. In the present study, we examined whether amino acid substitutions within the CCK-BR pocket altered the affinities and/or functional activities of L-365,260 (the prototypical nonpeptide CCK-BR antagonist) and two structural derivatives, YM022 (a higher affinity antagonist) and L-740,093S (a partial agonist). Eight amino acids that project into the CCK-BR pocket were individually replaced by alanine, using site-directed mutagenesis. Affinities for the nonpeptide molecules, as well as ligand-induced inositol phosphate production, were assessed with the wild-type and mutant receptors. For each of the nonpeptide ligands examined, a

distinct series of mutations altered the affinity, suggesting that each ligand possessed a characteristic pattern of interactions within the CCK-BR pocket. Basal signaling levels and inositol phosphate formation induced by the full agonist CCK octapeptide were comparable for the wild-type receptor and all of the mutant CCK-BR forms. In contrast to the peptide agonist CCK octapeptide, the functional activities of the nonpeptide molecules were selectively altered by single point mutations within the CCK-BR pocket, resulting in interconversion of agonists and antagonists. These findings suggest that interactions between nonpeptide molecules and transmembrane domain amino acids of the CCK-BR can determine the functional activity and affinity of the ligands.

The CCK-BR is a seven-transmembrane domain, G protein-coupled protein that is expressed both in the gastrointestinal tract and in the central nervous system. Two peptides, CCK and the structurally related enteroendocrine hormone gastrin, share subnanomolar affinity for the CCK-BR. It is well established that stimulation of the CCK-BR by either of these peptides triggers activation of phospholipase C, with subsequent generation of IPs and elevation of the intracellular calcium concentration (Wank, 1995). In the stomach, activation of the CCK-BR stimulates gastric acid secretion as well as mucosal proliferation (Nagata *et al.*, 1996; Langhans *et al.*, 1997). Within the central nervous system, this receptor subtype has been implicated in the modulation of anxiety, panic attacks, and pain perception (Faris *et al.*, 1983; Ravard and Dourish, 1990; Singh *et al.*, 1991; Wiertelak *et al.*, 1992).

Given its potential importance in human health and dis-

ease, the CCK-BR has been a major target for drug development. These efforts have led to the identification of high affinity synthetic antagonists that are significantly smaller molecules than the endogenous peptides. The prototype among these ligands is L-365,260 [(3*R*)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl)-*N'*-(3-methylphenyl)urea], a benzodiazepine-based compound with 200-fold selectivity for the human CCK-BR, relative to the structurally related CCK-A receptor subtype (Kopin *et al.*, 1995).

A previous study completed in our laboratory demonstrated that high affinity for L-365,260 is in large part determined by amino acids that are located near the extracellular surface of the CCK-BR transmembrane domains (Kopin *et al.*, 1995). When oriented according to the G protein-coupled receptor transmembrane domain structural model proposed by Baldwin (1993), these residues appear to outline a putative ligand binding pocket, similar to the one that is well established for biogenic amine receptors (Strader *et al.*, 1989b; Caron and Lefkowitz, 1993). Based on this work, we postulated that a series of L-365,260 derivatives might occupy the CCK-BR transmembrane domain ligand binding

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**ABBREVIATIONS:** CCK-BR, cholecystokinin-B/gastrin receptor; CCK, cholecystokinin; CCK-8, cholecystokinin octapeptide (sulfated form); IP, inositol phosphate.

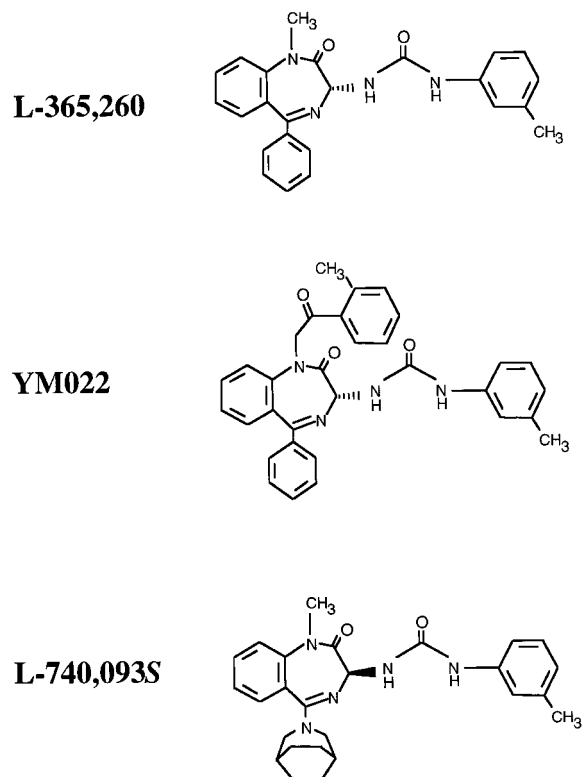
pocket in a way analogous to that in which biogenic amines occupy their respective receptors.

The goal of the present study was to investigate whether ligand-receptor interactions within the putative CCK-BR pocket influence the affinity and/or functional activity of three benzodiazepine-based molecules, L-365,260 and its derivatives YM022 [(*R*)-1,1-[2,3-dihydro-1-(2'-methylphenacyl)-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl)urea] (a higher affinity antagonist) (Nishida *et al.*, 1994) and L-740,093S [*N*-[(3*S*)-5-(3-azabicyclo[3.2.2]nonan-3-yl)-2,3-dihydro-1-methyl-2-oxo-1*H*-1,4-benzodiazepin-3-yl]-*N*-(3-methylphenyl)urea] (a partial agonist) (Kopin *et al.*, 1997; Beinborn *et al.*, 1998) (Fig. 1). Amino acids that were postulated to project into the binding pocket (Baldwin, 1993; Kopin *et al.*, 1995) were considered candidate sites for ligand interactions. These residues were replaced by alanine (Fig. 2), thus minimizing the size of the amino acid side chain and the potential of the targeted amino acid to interact with the ligand (Schwartz *et al.*, 1995). Alterations in affinity and efficacy for the three nonpeptide ligands, L-365,260, YM022, and L-740,093S, were then assessed.

Using this approach, we demonstrate that CCK-BR transmembrane domain amino acids are important determinants of both the binding affinity and functional activity of nonpeptide ligands. In addition, our studies reveal that single amino acid substitutions in the CCK-BR can interconvert the functional activities of agonists and antagonists.

## Materials and Methods

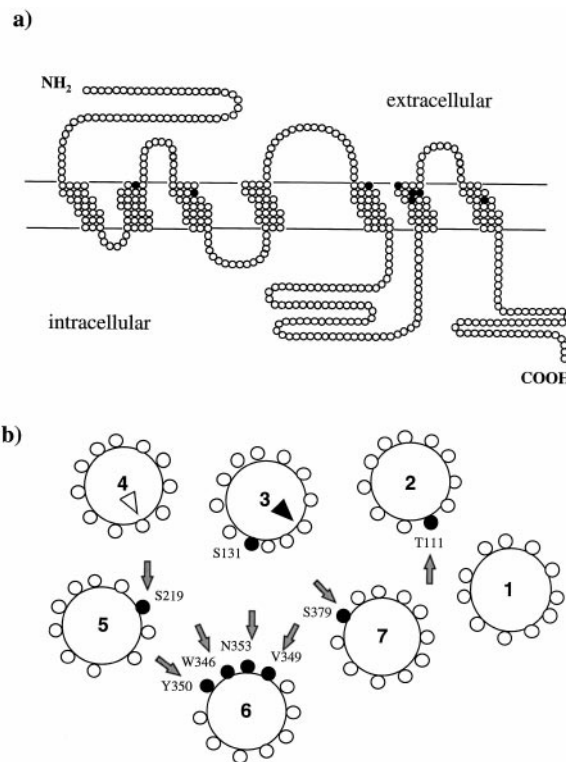
**Generation of mutant receptors.** Mutant human CCK-BR cDNAs were generated using oligonucleotide-directed, site-specific



**Fig. 1.** Comparison of L-365,260 and two related, benzodiazepine-based, nonpeptide ligands. YM022 (a high affinity antagonist) and L-740,093S (a partial agonist at the wild-type CCK-BR) are distinguished from L-365,260 by minor structural differences. *Shaded triangle* (*R*-enantiomer) or *black triangle* (*S*-enantiomer).

mutagenesis, as previously described (Beinborn *et al.*, 1993). The complete protein coding region of each mutant receptor cDNA was confirmed using an Applied Biosystems 373 automated DNA sequencer.

**Radioligand binding experiments.** COS-7 cells were transfected with 5  $\mu$ g of either wild-type or mutant human CCK-BR cDNA subcloned into the expression vector pcDNA1 (Invitrogen). Forty-eight hours after transfection, competition binding experiments were performed in 24-well plates ( $4-5 \times 10^3$  cells/well), using 20 pM [ $^{125}$ I]-CCK-8 (New England Nuclear) as the radioligand. Affinities for sulfated CCK-8 (Peninsula Laboratories) and L-740,093S, L-365,260, and YM022 (generously provided by Wyeth Research Laboratories) were determined by competition binding experiments with increasing concentrations of the unlabeled ligands. The respective  $IC_{50}$  values were calculated by computerized nonlinear curve-fitting (Inplot 4.0; GraphPad). Expression of wild-type and mutant receptors was assessed in homologous competition experiments, using [ $^{125}$ I]-CCK-8 as the radioligand and CCK-8 as an unlabeled competitor. The CCK-8 binding capacity (in femtomoles per 1000 transfected cells) was calculated using the MacLigand software package.



**Fig. 2.** Schematic representations of the human CCK-BR. **a**, Proposed membrane topology of the CCK-BR, based on hydropathy analysis. *Black circles*, transmembrane domain amino acids close to the extracellular space that were mutated in this study. **b**, Cross-sectional view of the seven transmembrane domains of the CCK-BR. This diagram is based on a model of G protein-coupled receptors (Baldwin, 1993) derived from the crystalline structure of rhodopsin (Schertler *et al.*, 1993). The depicted part of the receptor represents the outer third of the transmembrane domains, adjacent to the extracellular space. The seven transmembrane domains (arranged in a counterclockwise orientation, viewed from an outside-in perspective) surround a putative binding pocket of the receptor. *Large circles*, helices 1–7; *small circles*, individual amino acids. *●*, residues that were mutated in this study. *Gray arrows*, amino acids that were found in this study to be pharmacologically important for the binding affinity and/or efficacy of the three investigated nonpeptide molecules (L-365,260, YM022, and L-740,093S). *Arrowhead*, the amino acid corresponding to Asp113 of the  $\beta_2$ -adrenergic receptor (Strader *et al.*, 1989a, 1991) (see text). *Open arrowhead*, the residue corresponding to a highly conserved serine in transmembrane domain 4 of the  $\mu$ - and  $\delta$ -opioid receptors (Claude *et al.*, 1996) (see text) is located one helical turn below the amino acid indicated.

**Measurement of IP formation.** Transfected cells were labeled overnight with 3  $\mu\text{Ci/ml}$  *myo*-[ $^3\text{H}$ ]inositol (New England Nuclear) and then stimulated for 30 min at 37° with different ligands, in the presence of 10 mM LiCl. Concentration-response curves for CCK-8 and the nonpeptide ligands were assessed after stimulation of the cells for 60 min. After ligand stimulation, inositol metabolites were extracted with methanol/chloroform; the upper phase was analyzed for IPs by strong anion exchange chromatography. IP production was expressed as a fraction of the total cellular tritium content that was incorporated during overnight exposure to *myo*-[ $^3\text{H}$ ]inositol (tritiated IPs/total tritium incorporated) (Beinborn *et al.*, 1998).

Ligand concentrations used in the signaling assay were at least 50-fold higher than the corresponding  $\text{IC}_{50}$  values (see Tables 1 and 3). As calculated according to the law of mass action [fractional receptor occupation = ligand concentration/(ligand concentration +  $\text{IC}_{50}$  value)], these ligand concentrations result in >95% receptor occupation. To confirm that receptor-mediated IP production was maximally stimulated under these conditions, full concentration-response curves were measured for selected mutants with markedly altered ligand efficacies (see Fig. 4).

The efficacy (or intrinsic activity) of a given ligand is the magnitude of ligand-induced second messenger signaling relative to the response induced by a full agonist (e.g., CCK-8) (Goodman and Gilman, 1990). Therefore, in each experiment, CCK-8-stimulated IP production was determined as an internal standard, and the efficacy of each ligand was expressed as a percentage of the CCK-8-induced maximum.

**Statistical analysis.** Analysis of variance was performed using the InStat software program (GraphPad). *Post hoc* analysis was performed using the Dunnett multiple-comparisons test.

## Results

To enhance the likelihood that the overall tertiary structure of each of the mutant receptors would be conserved, the following two inclusion criteria were established: 1) affinity for CCK-8 in the subnanomolar range and 2) second messenger responses to saturating concentrations of CCK-8 comparable to those of the wild-type receptor. Receptor expression levels, as determined in  $^{125}\text{I}$ -CCK-8 competition binding experiments, were similar for all of the recombinant receptors (Table 1). Only one mutant did not fulfill inclusion criterion 1 for this study. The N353A receptor demonstrated 36-fold decreased binding affinity for CCK-8 (Table 1). As an alternative construct, we replaced Asn353 with leucine, thus substituting the original polar amino acid with an aliphatic

TABLE 1

Comparison of receptor expression levels and CCK-8 affinities for wild-type and mutant CCK-BRs

Wild-type and mutant receptors were expressed at comparable densities and shared subnanomolar affinities for CCK-8, with the exception of the N353A mutant (see text). Data represent means  $\pm$  standard errors from the number of independent experiments indicated in parentheses.

Receptor	$B_{\text{max}}$ <i>fmol / 10<sup>4</sup> cells</i>	CCK-8 binding affinity, $\text{IC}_{50}$ <i>nM</i>
Wild-type	6.1 $\pm$ 0.8 (17)	0.086 $\pm$ 0.01 (7)
T111A	5.7 $\pm$ 1.1 (3)	0.17 $\pm$ 0.03 (4)
S131A	9.9 $\pm$ 2.4 (3)	0.086 $\pm$ 0.01 (4)
S219A	7.3 $\pm$ 0.5 (3)	0.055 $\pm$ 0.01 (4)
W346A	4.5 $\pm$ 0.7 (3)	0.16 $\pm$ 0.03 (4)
V349A	4.8 $\pm$ 0.8 (3)	0.33 $\pm$ 0.10 <sup>a</sup> (5)
Y350A	6.2 $\pm$ 0.5 (3)	0.12 $\pm$ 0.03 (4)
N353A	6.3 $\pm$ 0.7 (3)	3.10 $\pm$ 0.60 <sup>a</sup> (6)
N353L	3.9 $\pm$ 0.5 (3)	0.15 $\pm$ 0.04 (3)
S379A	7.4 $\pm$ 0.9 (3)	0.085 $\pm$ 0.03 (4)

<sup>a</sup>  $p < 0.01$ , compared with corresponding wild-type values.

residue of similar size. The N353L receptor, as well as all other mutants that were studied, showed subnanomolar affinity for CCK-8 (Table 1). In addition, the second messenger responses to saturating concentrations of CCK-8 were comparable for the wild-type receptor and all of the mutant receptors, thus fulfilling inclusion criterion 2 (Table 2).

Radioligand competition experiments revealed that, among the tested benzodiazepine-based ligands (L-740,093S, L-365,260, and YM022) (Fig. 1), the affinity for each compound was affected by a distinct subset of receptor mutations (Table 3). Several of the observed affinity changes were statistically significant; however, the magnitude of most of these shifts was relatively minor (in the 2.5–5-fold range). Only the T111A and N353L mutations resulted in affinity shifts of >10-fold, compared with the wild-type receptor.

In the absence of ligand, COS-7 cells expressing either wild-type receptors or any one of the mutant CCK-BRs exhibited similar levels of IP formation. This basal level of signaling was not significantly different from that of control cells transfected with the empty expression vector pcDNA1 (Table 2). All of the mutant receptors showed comparable responses to stimulation with the full agonist CCK-8. In contrast, the CCK-BR mutations induced marked alterations in the efficacies of L-365,260, L-740,093S, and YM022, sufficient to interconvert the agonist and antagonist properties of these molecules (Fig. 3). Despite the high degree of structural similarity among the tested nonpeptide compounds, efficacy for each of the ligands was altered by a distinct subset of receptor mutations.

Among the three ligands tested, YM022 was the least susceptible to mutation-induced efficacy changes. When examined with the wild-type receptor, this nonpeptide compound did not trigger IP production. Of the CCK-BR binding pocket mutations that were tested, only one significantly altered YM022 efficacy. The amino acid substitution W346A markedly increased the functional activity of YM022, converting this ligand into a partial agonist with an efficacy of 24%, compared with the CCK-8-induced value (100%).

In contrast to YM022, L-740,093S is a partial agonist at the wild-type CCK-BR, stimulating IP production to 30% of

TABLE 2

Basal and CCK-8-induced signaling in COS-7 cells expressing wild-type and mutant CCK-BRs

Basal (in the absence of ligand) and CCK-8-induced IP production levels were comparable for wild-type and mutant receptors. Signaling is expressed as [ $^3\text{H}$ ]IPs/total cellular  $^3\text{H} \times 100$  (see Materials and Methods). Basal signaling levels were not significantly different from those measured in cells transfected with the empty expression vector pcDNA1 (1.10  $\pm$  0.07% of  $^3\text{H}$  incorporation, mean  $\pm$  standard error from 29 experiments). In addition, CCK-8-induced IP production by mutant receptors was not significantly different from the corresponding value of the wild-type CCK-BR. Data represent means  $\pm$  standard errors from the number of independent experiments indicated in parentheses.

Receptor	IP production	
	Basal	CCK-8-induced
	% of $^3\text{H}$ incorporation	
Wild-type	1.18 $\pm$ 0.07	8.71 $\pm$ 0.42 (22)
T111A	1.28 $\pm$ 0.35	10.56 $\pm$ 1.95 (6)
S131A	0.65 $\pm$ 0.08	7.21 $\pm$ 1.46 (3)
S219A	0.96 $\pm$ 0.21	8.86 $\pm$ 0.97 (4)
W346A	1.07 $\pm$ 0.41	7.32 $\pm$ 1.11 (4)
V349A	1.45 $\pm$ 0.43	8.64 $\pm$ 1.50 (4)
Y350A	1.26 $\pm$ 0.65	10.33 $\pm$ 2.66 (4)
N353L	1.06 $\pm$ 0.16	10.20 $\pm$ 0.78 (5)
S379A	1.28 $\pm$ 0.35	8.23 $\pm$ 0.87 (4)



the CCK-8-induced maximum (Kopin *et al.*, 1997; Beinborn *et al.*, 1998). Point mutations within the putative binding pocket of the receptor could result in either an increase or a decrease in L-740,093S efficacy. The S219A, V349A, and S379A mutations increased cellular IP production in response to L-740,093S from 30% (wild-type receptor) to 42, 72, and 44% of the CCK-8-induced value, respectively. In contrast, the T111A, W346A, and N353L mutations significantly reduced the efficacy of L-740,093S. The transmembrane domain 6 mutation Y350A, which is adjacent to Trp346 (Fig. 2), abolished the ability of L-740,093S to induce second messenger formation, thus converting this compound into an antagonist.

Four mutations (S219A, V349A, N353L, and S379A) led to significant increases in L-365,260 efficacy, each producing a different level of agonist activity. The most pronounced shift in ligand efficacy among the tested compounds was observed with L-365,260 when Asn353 was replaced with leucine. This mutation increased the efficacy of L-365,260 close to the value observed with the full agonist CCK-8 (Fig. 3).

For each of the nonpeptide compounds, full concentration-response curves were assessed with selected mutant CCK-BRs. YM022 triggered a concentration-dependent increase in IP production in COS-7 cells expressing the W346A mutant ( $EC_{50} = 1.1$  nM) but not in cells expressing the wild-type receptor (Fig. 4A). For L-740,093S, a sigmoidal concentration-response curve was observed with both the wild-type receptor ( $EC_{50} = 4.7$  nM) and the V349A mutant ( $EC_{50} = 13.7$  nM), whereas no clear concentration-dependent signaling was found with the T111A mutant (Fig. 4B). L-365,260 was converted into a partial or nearly full agonist by the S379A and N353L mutations, respectively. In each case, a concentration-dependent increase in IP production was triggered by L-365,260. Corresponding  $EC_{50}$  values were 0.6 nM for the S379A mutant and 19.5 nM for the N353L mutant (Fig. 4C). For each ligand and receptor for which full concentration-response curves were generated, ligand efficacies derived from this more detailed analysis approximated the values that were independently determined with a single, supra-maximal, ligand concentration (compare maximal effects in Fig. 4 and data shown in Fig. 3).

In contrast to the nonpeptide compounds, CCK-8-induced IP production by the T111A, W346A, V349A, N353L, and S379A mutants was not significantly different from the cor-

responding value for the wild-type receptor (Table 2). Additional experiments (data not shown) revealed that CCK-8 concentration-response curves for all of these mutant receptors had sigmoidal shapes, resembling the corresponding curve for the wild-type receptor (two experiments). There was no significant difference in  $EC_{50}$  values between the mutants (range, 0.22–0.45 nM) and the wild-type receptor ( $EC_{50} = 0.30$  nM).

## Discussion

Previous work from our laboratory (Kopin *et al.*, 1995) suggested that the nonpeptide ligand L-365,260 occupies a binding pocket composed of CCK-BR transmembrane domain amino acids, analogous to the one that has been well established for biogenic amine receptors (Strader *et al.*, 1989b; Caron and Lefkowitz, 1993). Based on this observation, we hypothesized that other, structurally related, nonpeptide ligands (L-740,093S and YM022) might interact with the same region of the CCK-BR. To address this possibility, we substituted amino acids projecting into this putative pocket with alanine residues and examined the effects on ligand affinity and efficacy.

Consistent with the hypothesis that the structurally related, benzodiazepine-based, nonpeptide compounds interact with transmembrane domain amino acids, six of the eight mutant CCK-BRs that were studied showed altered binding affinity for at least one of the tested compounds. Although the affinity for each compound was affected by a distinct subset of receptor mutations, the data also suggest two candidate sites for receptor interactions that are common to all three ligands. Substitution of Thr111 led to 3.3-, 13.7-, and 11.6-fold decreases in affinity for L-740,093S, L-365,260, and YM022, respectively. It is possible that binding of a structural element common to all of these molecules is disrupted when the side chain of Thr111 (which includes an hydroxyl substituent) is replaced by the methyl group of alanine. Similarly, affinity for each of the three nonpeptide compounds is affected by the N353L substitution in transmembrane domain 6, which removes the amide substituent of asparagine. These results suggest that the affinity determinants for the three structurally related, but functionally different, compounds within the CCK-BR transmembrane domains partially overlap. It remains to be established whether these

TABLE 3

CCK-BR pocket mutations alter ligand affinity for each of the three nonpeptide molecules examined

For the three tested nonpeptide ligands,  $pIC_{50}$  values for different CCK-BR constructs and  $IC_{50}$  ratios (mutant receptors vs. simultaneously tested wild-type receptors) are shown.  $IC_{50}$  ratios of  $>1$  represent decreases in binding affinity, whereas values of  $<1$  reflect increases in binding affinity. Values in parentheses represent the number of experiments.

Receptor	L-740,093S		L-365,260		YM022	
	$pIC_{50}$	$IC_{50}$ ratio	$pIC_{50}$	$IC_{50}$ ratio	$pIC_{50}$	$IC_{50}$ ratio
Wild-type	$7.63 \pm 0.07$	1 (7)	$8.48 \pm 0.07$	1 (9)	$9.73 \pm 0.03$	1 (6)
T111A	$7.07 \pm 0.05^a$	3.3 (3)	$7.29 \pm 0.05^a$	13.7 (4)	$8.68 \pm 0.01^a$	11.6 (3)
S131A	$7.66 \pm 0.15$	1.0 (3)	$8.34 \pm 0.08$	1.4 (3)	$9.60 \pm 0.01$	1.4 (3)
S219A	$7.74 \pm 0.12$	0.9 (4)	$8.36 \pm 0.10$	1.4 (3)	$9.86 \pm 0.15$	0.9 (4)
W346A	$7.76 \pm 0.09$	1.1 (4)	$8.71 \pm 0.09$	0.7 (3)	$9.31 \pm 0.03^a$	3.1 (3)
V349A	$7.19 \pm 0.09^a$	2.7 (3)	$7.80 \pm 0.15^a$	5.0 (4)	$9.62 \pm 0.06$	1.3 (3)
Y350A	$8.05 \pm 0.04^a$	0.4 (4)	$8.11 \pm 0.15$	2.7 (3)	$9.66 \pm 0.03$	1.2 (3)
N353L	$7.21 \pm 0.10^b$	2.6 (3)	$7.16 \pm 0.12^a$	30.0 (3)	$8.84 \pm 0.09^a$	8.7 (4)
S379A	$8.05 \pm 0.07^a$	0.3 (3)	$8.75 \pm 0.07$	0.5 (4)	$9.63 \pm 0.06$	1.3 (3)

<sup>a</sup>  $p < 0.01$ , significant difference versus the corresponding wild-type affinity.

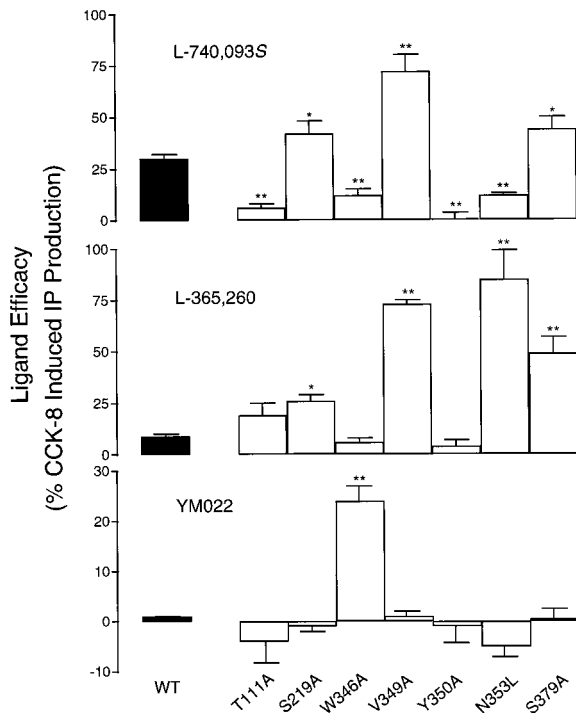
<sup>b</sup>  $p < 0.05$ , significant difference versus the corresponding wild-type affinity.

affinity determinants represent sites of direct ligand-receptor interactions (e.g., hydrogen bonding).

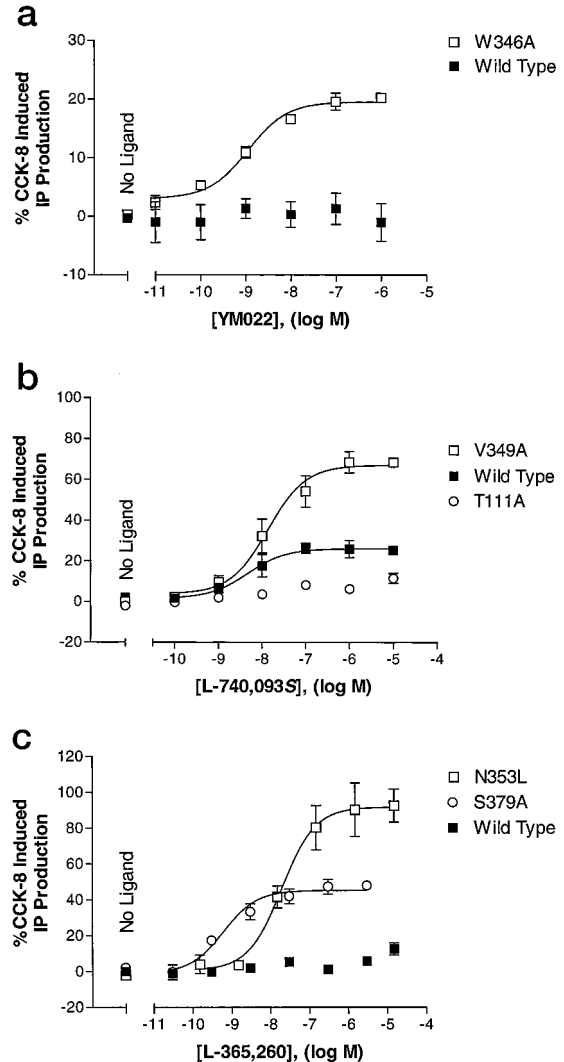
We previously determined that the minor structural alterations that distinguish L-365,260 from YM022 and L-740,093S (Fig. 1) result in different functional properties at the wild-type CCK-BR (Beinborn *et al.*, 1998). It is likely that the observed functional differences among these ligands are the result of slight modifications in ligand-receptor interactions. Given the evidence for a ligand binding pocket, where interactions between the CCK-BR and these small nonpeptide molecules occur, we postulated that minor alterations of receptor residues that project into this putative pocket might mimic the effects of modification of the ligands and therefore change the functional activities of L-365,260 derivatives. In fact, the ligand-induced signaling data are consistent with this hypothesis and suggest that specific ligand-receptor interactions within the putative CCK-BR binding pocket influence the efficacy of small nonpeptide molecules. The specificity of these interactions is underlined by the distinct pattern of receptor mutations that alter efficacy for each of the nonpeptide compounds (Fig. 3).

For a variety of G protein-coupled receptors, single amino acid changes in transmembrane domains have been demonstrated to result in constitutive receptor activation (Dryja *et al.*, 1993; Robbins *et al.*, 1993; Shenker *et al.*, 1993; Balmforth *et al.*, 1997; Groblewski *et al.*, 1997; Ishii *et al.*, 1997). In some cases, the constitutive activity of these mutant recep-

tors leads to amplification of the responses to endogenous and synthetic ligands (Groblewski *et al.*, 1997; Ishii *et al.*, 1997). The alteration in ligand efficacy in these cases may be attributed to mutation-induced shifts between the inactive and active forms of the receptors. It is therefore important to emphasize that none of the mutant receptors examined in



**Fig. 3.** Point mutations within the CCK-BR transmembrane domain pocket result in a specific pattern of efficacy changes for each of the three nonpeptide ligands examined. Ligand efficacies were compared for the wild-type (WT) and mutant CCK-BRs. The efficacies of L-740,093S (*top*), L-365,260 (*middle*), and YM022 (*bottom*) are expressed relative to the CCK-8-induced maximal level of IP production (defined as 100%). The efficacies of L-740,093S and L-365,260 were affected by multiple receptor mutations, whereas only one amino acid substitution, Trp346, significantly altered YM022-induced signaling. Data represent means  $\pm$  standard errors from at least three independent experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ , compared with ligand efficacy at the wild-type CCK-BR.



**Fig. 4.** Single amino acid substitutions in the putative CCK-BR ligand pocket markedly alter the activity of nonpeptide ligands. Data points represent means  $\pm$  standard errors of three independent experiments. A, YM022 induced a concentration-dependent increase in IP production with the W346A mutant ( $EC_{50}$ , 1.1 nM; 95% confidence interval, 0.6–2.0 nM; maximal effect,  $19.6 \pm 0.6\%$  of CCK-8-induced IP production). In contrast, YM022 had no appreciable effect on IP production with the wild-type receptor. B, The efficacy of L-740,093S was either increased or decreased by single point mutations in the CCK-BR. L-740,093S induced concentration-dependent IP production in COS-7 cells expressing either the wild-type CCK-BR ( $EC_{50}$ , 4.7 nM; 95% confidence interval, 1.2–17.8 nM; maximal effect,  $26.2 \pm 1.8\%$  of CCK-8-induced IP production) or the V349A mutant ( $EC_{50}$ , 13.7 nM; 95% confidence interval, 6.1–31.2 nM; maximal effect,  $67.2 \pm 3.3\%$  of CCK-8-induced IP production). Only minimal L-740,093S activity was detectable with the T111A mutant. C, L-365,260 was a partial agonist with the S379A mutant and approximated a full agonist with the N353L receptor. L-365,260 stimulated a concentration-dependent increase in IP production in COS-7 cells expressing either the S379A mutant ( $EC_{50}$ , 0.6 nM; 95% confidence interval, 0.2–1.5 nM; maximal effect,  $45.3 \pm 2.1\%$  of CCK-8-induced IP production) or the N353L mutant ( $EC_{50}$ , 19.5 nM; 95% confidence interval, 7.5–30.8 nM; maximal effect,  $91.9 \pm 6.3\%$  of CCK-8-induced IP production). With the wild-type receptor, only minimal L-365,260 activity was detectable.

this study were constitutively active. Rather, the changes in L-740,093S, L-365,260, and YM022 efficacy resulting from CCK-BR binding pocket mutations were observed without concomitant alterations in basal receptor signaling. Moreover, two of the investigated mutations (W346A and N353L) specifically increased the efficacy of one ligand while decreasing the functional activity of another, confirming that the observed changes in ligand efficacy are not the result of a shift toward a generally more active receptor state. The specificity of the observed efficacy changes is consistent with the concept that multiple agonist-specific receptor conformations can activate G proteins (Kenakin, 1997). It is possible that the mutations that were introduced within the CCK-BR transmembrane domains differentially affect the receptor conformations that are relevant for agonist-induced signaling by the respective ligands.

The alterations in ligand efficacy observed with pocket mutations were limited to small nonpeptide molecules. CCK-8-induced signaling was not altered by the amino acid substitutions examined. This suggests that the molecular mechanisms that underlie ligand-receptor interactions at the CCK-BR differ for synthetic nonpeptide compounds and the endogenous peptide agonist CCK-8. For the small molecules that are the focus of this study, it is possible that ligand-receptor interactions are restricted to the transmembrane domains. The binding pattern for the larger peptide ligands is likely to involve multiple receptor regions (Schmitz *et al.*, 1996; Silvente-Poirot and Wank, 1996), which may result in reduced functional sensitivity to point mutations within any single domain of the CCK-BR. Within the amino terminus of the structurally related CCK-A receptor, a distinct site of interaction with CCK was recently identified by mutational analysis (Kennedy *et al.*, 1997) and by photoaffinity labeling (Ji *et al.*, 1997). It remains to be determined whether the amino-terminal domain of the CCK-BR is equally important for interactions with CCK.

The selection of amino acids to be mutated in this study was largely based on our previous findings, which established the putative binding pocket of the CCK-BR (Kopin *et al.*, 1995). Those initial studies identified three amino acid residues in transmembrane domain 6 as affinity determinants, suggesting that this transmembrane domain is involved in ligand-receptor interactions. It was, however, surprising to find that all four mutated amino acids in transmembrane domain 6 were important in determining the functional properties of the benzodiazepine-based ligands.

The functional importance of the sixth transmembrane domain in the photoreceptor rhodopsin has been demonstrated by proximity measurements and experiments involving disulfide cross-linking between transmembrane domains 3 and 6 (Farrens *et al.*, 1996), as well as construction of zinc binding sites that link these two helices (Sheikh *et al.*, 1996). It was concluded from those studies that the movement of transmembrane domains 3 and 6, relative to one another, is required for photoreceptor activation. By analogy with rhodopsin, it is possible that the movement of transmembrane domain 6, relative to other transmembrane domains, is an important factor for activation of the CCK-BR and that this movement can be facilitated or prevented by ligand-receptor interactions within the CCK-BR binding pocket. The observed mutation-induced alterations in ligand efficacy could thus be a result of changes in the ability of the ligand to

influence the movement of transmembrane domain 6, relative to other transmembrane domains.

A model of receptor-mediated signaling that includes distinct equilibrium constants for receptor-ligand association and receptor activation has been proposed (Scheer *et al.*, 1996). Consistent with this concept, our study suggests that there is no generalizable correlation between mutation-induced affinity and efficacy changes for CCK-BR nonpeptide ligands. Despite this observation, it cannot be excluded that, in certain domains within the CCK-BR ligand binding pocket and/or for certain ligands, the determinants of affinity and efficacy may overlap.

There have been two previously reported examples of the conversion of nonpeptide antagonists to agonists by point mutations within the transmembrane domains of G protein-coupled receptors. In the binding pocket of the  $\beta_2$ -adrenergic receptor, substitution of Asp113 leads to receptor mutants that are activated by ligands that are antagonists at the wild-type  $\beta_2$ -adrenergic receptor (Strader *et al.*, 1989a, 1991). Similarly, mutation of a transmembrane domain 4 serine residue that is conserved among opioid receptor subtypes converts well established antagonists to agonists (Claude *et al.*, 1996). Although the location of this serine residue was not originally considered with respect to the Baldwin model of transmembrane domain structure, retrospective analysis of the data suggests that this opioid receptor residue projects into a ligand pocket similar to the one postulated for the CCK-BR (Fig. 2). These additional examples support the idea that ligand interactions within the putative transmembrane domain pocket may be important determinants of the functional activity of both biogenic amine and peptide hormone receptor ligands.

The observation that single amino acid differences are sufficient to change the functional properties of ligands raises the possibility that receptor polymorphisms among humans could influence the functional activities of receptor-specific drugs. Naturally occurring receptor variants are well established to occur among G protein-coupled receptors (Reihnsaus *et al.*, 1993). One of the reported variants of the  $\beta_2$ -adrenergic receptor exhibits altered ligand binding and functional responses to the full agonist epinephrine (Green *et al.*, 1993). For the CCK-BR, there are two reports of naturally occurring polymorphisms (Herget *et al.*, 1994; Kato *et al.*, 1996). One of these amino acid substitutions (E288K) (Herget *et al.*, 1994) has been functionally characterized and demonstrated to increase the efficacy of both L-740,093S and PD135,158 [4-[[2-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[[(1.7.7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]carbonyl]amino]propyl]amino]-1-phenylethyl]amino-4-oxo-[1*S*-1 $\alpha$ ,2 $\beta$ [(*S'*)-4 $\alpha$ ]-butanoate-*N*-methyl-*D*-glucamine], a peptide-derived or 'peptoid' ligand (Kopin *et al.*, 1997). These examples, taken together with the findings presented in this study, suggest that interindividual receptor polymorphisms may lead to differences in drug efficacy among humans.

In summary, our data illustrate that the side chains of amino acids that project into the putative CCK-BR binding pocket influence both the affinity and efficacy of nonpeptide ligands. It remains to be determined which of the amino acids projecting into the pocket are in direct contact with nonpeptide molecules and which residues indirectly contribute to ligand binding and signaling by maintaining a given conformation of the transmembrane domains. Understanding the



mechanisms that underlie ligand-receptor interactions may eventually enhance our ability to predict the functional properties of candidate ligands, bringing us closer to the ultimate goal of rational drug design.

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