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CCK-B/Gastrin Receptor Transmembrane Domain Mutations Selectively Alter Synthetic Agonist Efficacy without Affecting the Activity of Endogenous Peptides

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ABSTRACT

Recent efforts have focused on identifying small nonpeptide molecules that can mimic the activity of endogenous peptide hormones. Understanding the molecular basis of ligand-induced receptor activation by these divergent classes of ligands should expedite the process of drug development. Using the cholecystokinin-B/gastrin receptor (CCK-BR) as a model system, we have recently shown that both affinity and efficacy of nonpeptide ligands are markedly affected by amino acid alterations within a putative transmembrane domain (TMD) ligand pocket. In this report, we examine whether residues projecting into the TMD pocket determine the pharmacologic properties of structurally diverse CCK-BR ligands, including peptides and synthetic peptide-derived partial agonists (peptoids). Nineteen mutant human CCK-BRs, each including a single TMD amino acid substitution, were transiently expressed in COS-7 cells and characterized. Binding affinities as well as ligand-induced inositol phosphate production at the mutant CCK-BRs were assessed for peptides (CCK-8 and CCK-4) and for peptoids (PD-135,158 and PD-136,450). Distinct as well as overlapping determinants of peptide and peptoid binding affinity were identified, supporting that both classes of ligands, at least in part, interact with the CCK-BR TMD ligand pocket. Eight point mutations resulted in marked increases or decreases in the functional activity of the synthetic peptoid ligands. In contrast, the functional activity of both peptides, CCK-8 and CCK-4, was not affected by any of the CCK-BR mutations. These findings suggest that the mechanisms underlying activation of G-protein-coupled receptors by endogenous peptide hormones versus synthetic ligands may markedly differ.

The cholecystokinin-B/gastrin receptor (CCK-BR) is a member of the G-protein-coupled seven transmembrane domain receptor superfamily. The CCK-BR is widely expressed both in the gastrointestinal tract and in the central nervous system. In the stomach this receptor regulates acid secretion and cellular proliferation (Nagata et al., 1996; Langhans et al., 1997). In the central nervous system the CCK-BR has been implicated in modulating anxiety and the perception of pain (Faris et al., 1983; Ravard and Dourish, 1990). Given its importance in regulating a wide range of physiologic functions, the CCK-BR has been a major target of drug development efforts.

The CCK-BR has high affinity for two endogenous peptides, gastrin and cholecystokinin octapeptide (CCK-8). Both ligands have similar efficacies in stimulating receptor-mediated activation of phospholipase C and subsequent generation of inositol phosphates (Beinborn et al., 1998). CCK-8 and gastrin share the same carboxyl-terminal four amino acids, CCK-4. This tetrapeptide has full biologic activity but considerably lower receptor affinity than CCK-8 and gastrin. The chemical structure of CCK-4 has been the template for the development of synthetic peptide-derived (peptoid) ligands (Hughes et al., 1990; Horwell, 1991). Two of these peptoid compounds, PD-135,158, and PD-136,450 (Fig. 1) have been shown to activate the CCK-BR as partial agonists either in vitro (Kopin et al., 1997) or in vivo (Schmassmann et al., 1994).

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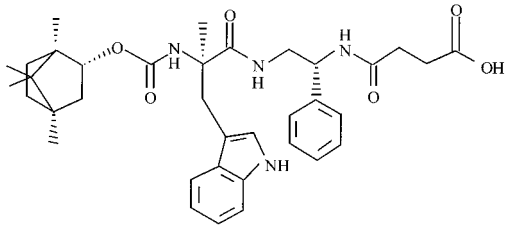
ABBREVIATIONS: CCK-BR, cholecystokinin-B/gastrin receptor; CCK, cholecystokinin; CCK-8, cholecystokinin-octapeptide; CCK-4, cholecystokinin-tetrapeptide; PD-135,158, 4-[[2-[[3-(1H-indol-3-yl)-2-methyl-1-oxo-2-[[(1.7.7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]carbonyl]amino]propyl]amino]-1-phenylethyl]amino-4-oxo-[1*S*-1α,2β[*S**(*S**)4α]]butanoate-*N*-methyl-D-glucamine; PD-136,450, [*R*-(*R**,*R**)]-4-[[2-[[3-(1H-indol-3-yl)-2methyl-1-oxo-2-[[(tricyclo[3.3.1.1]dec-2-oxy)carbonyl]amino]propyl]amino]-1-phenylethyl]amino]-4-oxo-2-butanoate *N*-methyl-D-glucamine; YM022, (*R*)-1–1[2,3,-dihydro-1-(2′-methylphenacyl)-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl]-3-(3-methylphenyl)urea; L-740,093*S*, [*N*-(3*S*)-5-(3-azabicyclo[3.2.2]nonan-3-yl)-2,3-dihydro-1-methyl-2-oxo-1H-1,4-benzodiazepin-3-yl]-*N*′-(3methylphenyl)urea].

A previous study from our laboratory resulted in the identification of a putative CCK-BR ligand pocket comprised of transmembrane domains (Kopin et al., 1995) (Figs. 2 and 3). A structurally similar pocket has been reported for biogenic amine receptors (Strader et al., 1989; Caron and Lefkowitz, 1993) as well as other peptide hormone receptors (Ji et al., 1994; Janagin et al., 1996). More recent work has demonstrated that, within the CCK-BR pocket, individual amino acids determine the affinity as well as the functional activity of a series of benzodiazepine-based nonpeptide ligands (Bläker et al., 1998). Single amino acid substitutions within the putative pocket are sufficient to convert nonpeptide antagonists into agonists and vice versa, raising the possibility that ligand receptor interactions within this domain of the CCK-BR play an important role in receptor activation by nonpeptide ligands (Bläker et al., 1998).

The aim of our current study was to examine to what extent transmembrane domain amino acids determine affinity and efficacy of a broader range of CCK-BR ligands, i.e., peptides and peptoids. We therefore characterized a series of mutant human CCK-BRs, each including the substitution of a single transmembrane domain amino acid (Figs. 2 and 3). Binding affinities as well as functional activities of peptide and peptoid ligands at the mutant receptors were assessed.

Within the CCK-BR ligand pocket, determinants of binding affinity were identified for both classes of compounds. As observed for nonpeptide ligands, the synthetic peptoid molecules were affected in their functional activity by multiple receptor mutations. In contrast, none of the mutations led to changes in the functional activity of either of the two peptide ligands, CCK-8 or CCK-4. These findings suggest that synthetic compounds and endogenous peptide hormones activate the CCK-BR by molecular mechanisms that are, at least in part, distinct.



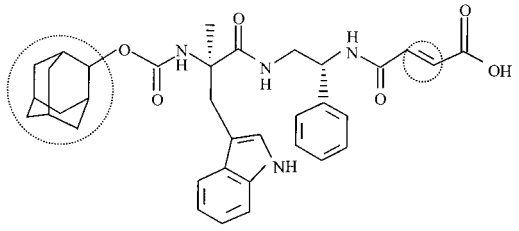


Fig. 1. Chemical structures of the peptide-derived (peptoid) ligands, PD-135,158 and PD-136,450. The two peptoid molecules were rationally designed to include structural features of CCK-4 (Hughes et al., 1990, Horwell, 1991). These ligands are distinguished by an endobornyloxycarbonyl group (PD-135,158) and an adamantyloxycarbonyl group (PD-136,450), respectively, as well as by an additional double bond in PD-136,450. Differences versus PD-135,158 are highlighted in the PD-136,450 molecule by dashed circles.

Materials and Methods

Generation of Mutant Receptors. Mutant human CCK-BR cDNAs were generated using oligonucleotide-directed site-specific mutagenesis as previously described (Beinborn et al., 1993; Bläker et al., 1998). Each cDNA construct encoded the CCK-BR with a single amino acid substitution. The nucleotide sequence of the protein-coding region of each mutant receptor cDNA was confirmed using an automated DNA sequencer (model 373; Applied Biosystems, Foster City, CA).

Radioligand Binding Experiments. 10^6 COS-7 cells were transfected with 5 μg of either wild type or mutant human CCK-BR cDNA subcloned into the expression vector pcDNAI (Invitrogen, San Diego, CA). Transfected cells were split into 24-well plates (4–50 $\times$ 10^3 cells/well), and binding experiments were performed using 20 pM ^{125}I-CCK-8 (NEN Life Science Products, Boston, MA) as the radioligand. Affinities for sulfated CCK-8 (Peninsula Laboratories, Inc., Belmont, CA), CCK-4 (Peninsula Laboratories, Inc.), PD-135,158, and PD-136,450 (Parke-Davis Neuroscience Research Center, Cambridge, UK) were determined by competition binding experiments with increasing concentrations of the unlabeled ligands. IC_{50} values were calculated by computerized nonlinear curve fitting (Inplot 4.0; GraphPad Software, San Diego, CA). Expression of wild type and mutant receptors was assessed by homologous competition experiments using ^{125}I-CCK-8 as the radioligand and CCK-8 as the unlabeled competitor. The CCK-8 binding capacity (B_{max}, fmol/10^4 transfected cells) was calculated using the MacLigand software package (McPherson, 1985).

Measurement of Inositol Phosphate Formation. Transfected COS-7 cells (1–2 $\times$ 10^5/well) were labeled overnight with 3 μCi/ml

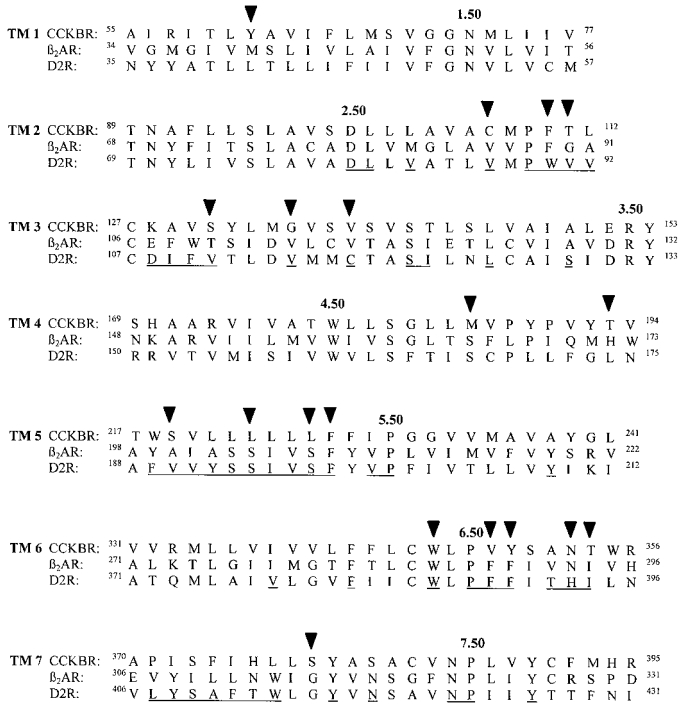


Fig. 2. Alignment of the human CCK-B/gastrin, β_2-adrenergic, and dopamine D2 receptor transmembrane domain amino acid sequences. Amino acids at the beginning and at the end of each line are numbered according to their position in the respective receptor. Boldface index numbers above each transmembrane domain alignment highlight residues that are conserved among members of the rhodopsin/adrenergic G-protein-coupled receptor family (Ballesteros and Weinstein, 1995). Amino acids in the CCK-BR, which were mutated in this study, are highlighted by arrowheads. Amino acids in the dopamine D2 receptor, which have been shown to be located in the binding site crevice, are underlined (see text). Note that analysis of residues in transmembrane domains 1 and 4 of the dopamine D2 receptor by this approach has not yet been reported. CCKBR, cholecystokinin-B/gastrin receptor; β_2AR, β_2-adrenergic receptor; D2R, dopamine D2 receptor

myo-[^{3}H]inositol (45–80 Ci/mmol; NEN Life Science Products) and then stimulated for 60 min at 37°C in the presence of 10 mM LiCl. Following ligand stimulation, inositol metabolites were extracted with methanol/chloroform; the upper phase was analyzed for inositol phosphates by strong anion exchange chromatography. [^{3}H]Inositol phosphate production was expressed as a percentage of the total cellular tritium content, which was incorporated during the overnight exposure to myo-[^{3}H]inositol (tritiated inositol phosphates/total tritium incorporated × 100).

The ligand concentrations utilized to assess efficacy were at least 50-fold higher than the corresponding IC_{50} values as determined by radioligand competition binding experiments (Table 1). As calculated according to the law of mass action (fractional receptor occupation = ligand concentration/[ligand concentration + IC_{50} value]), these ligand concentrations result in >95% receptor occupation, thus inducing the respective ligand's maximal effect on receptor-mediated inositol phosphate production (Ross, 1996; Kopin et al., 1997).

The relative efficacy of a ligand is the magnitude of ligand-induced second messenger signaling as a fraction of the response induced by a full agonist (e.g., CCK-8) (Ross, 1996; Kopin et al., 1997; Kenakin, 1999). As suggested by Kenakin, if the intracellular milieu remains constant and the receptor of interest lacks constitutive activity, relative efficacy provides a reasonable parameter to compare the functional properties of ligands. In each experiment, CCK-8-stimulated inositol phosphate production was included as an internal standard; activity of each ligand at different receptors was then expressed as a percentage of the CCK-8-induced maximum.

Statistical Analysis. Statistical analysis was performed by one-way ANOVA. Post tests were done by Tukey-Kramer multiple comparison analysis (InStat; GraphPad Software)

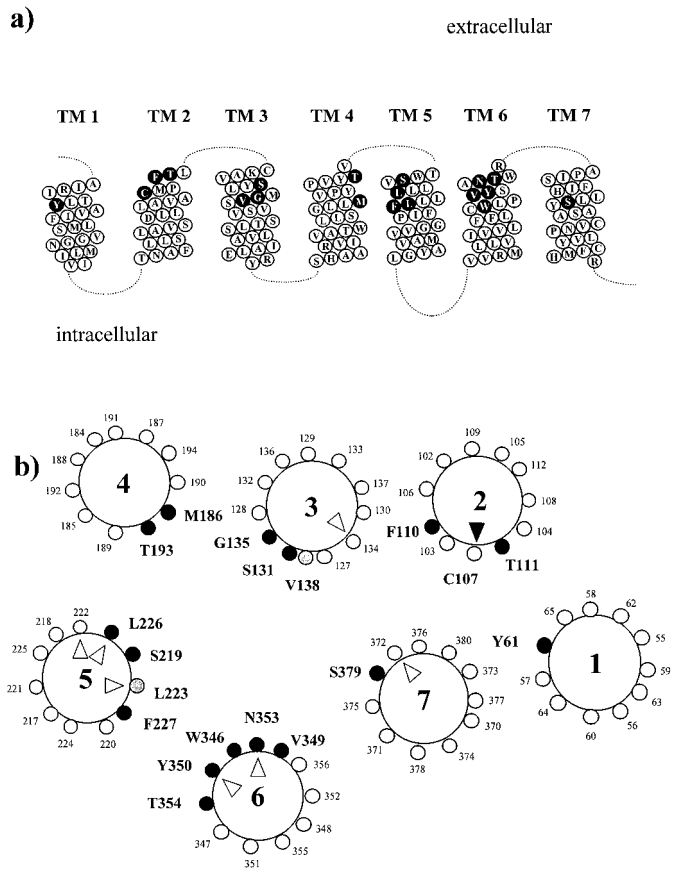


Fig. 3. Schematic representations of the human CCK-BR. a, two-dimensional model of the human CCK-BR, highlighting the transmembrane domains. Filled circles represent transmembrane domain amino acids that were mutated in this study. Intracellular and extracellular receptor domains are indicated by dashed lines. b, cross-sectional view of the seven transmembrane domains of the CCK-BR based on the Baldwin model of G-protein-coupled receptors (Baldwin, 1993). The depicted part of the receptor represents the outer third of the transmembrane domains adjacent to the extracellular space. The transmembrane domains (large circles 1 to 7) are arranged in an anticlockwise orientation, viewed from an outside-inward perspective. Together, these domains delineate the outer boundary of a putative ligand binding pocket. Small circles correspond to individual amino acids. Residues that were mutated are specified using the single-letter code and numbered according to their position in the receptor. Gray circles indicate that mutations resulted in constitutive receptor activation; black circles represent positions where amino acid substitutions did not affect the basal level of signaling. The closed arrowhead highlights the amino acid corresponding to glycine 90 in human rhodopsin. Mutation of glycine 90 to asparagine results in constitutive activation of rhodopsin (Rim and Oprian, 1995). Open arrowheads indicate amino acids that have been shown by mutagenesis studies to be important affinity determinants for ligands in the human β_2-adrenergic receptor (Cascieri et al., 1995; Wieland et al., 1996; Kurose et al., 1998; Sato et al., 1999); TM, transmembrane domain.

Results

A total of 19 mutant receptors were studied, each including an alanine or leucine substitution of a single CCK-BR pocket amino acid (Figs. 2 and 3). Each of these residues is postulated to project into the putative transmembrane domain ligand pocket. As demonstrated by the respective B_{max} values, the wild type and mutant receptors were all well expressed (Table 2). Furthermore, when stimulated with saturating concentrations of CCK-8, all 19 mutant receptors triggered inositol phosphate formation comparable to the wild type CCK-BR (Table 2). Three single amino acid substitutions (C107A, V138A, and L223A) resulted in constitutive receptor activation, indicated by a significant elevation in the basal levels of inositol phosphate production (Table 2). Activating mutations in the corresponding position (Cys-107) or in close proximity to these transmembrane domain residues have been previously reported for other G-protein-coupled receptors (Rim and Oprian, 1995; Pauwels and Wurch, 1998). It is well established that ligand-independent signaling due to a generalized shift toward active receptor conformations is a variable that may confound the comparison of mutation-induced changes in relative ligand efficacies (Kenakin, 1999). Because the objective of the present work was to explore more selective effects of single amino acid substitutions on the affinity and functional activity of individual ligands, the three constitutively active CCK-BRs were excluded from further study.

Analysis of the 16 remaining CCK-BR mutants revealed distinct as well as overlapping determinants of binding affinity for peptide and peptoid ligands. Three receptor point mutations, Y61A, T193A, and V349A, selectively decreased affinities for the two endogenous peptides, CCK-8 and CCK-4 (Table 1). The amino acid substitutions T111A, N353L, and T354A primarily reduced binding of the peptoid molecules, PD-135,158 and PD-136,450, with only slight effects on peptide affinity. Only one mutation, M186A, significantly decreased binding affinity for all tested peptide and peptoid molecules (Table 1).

To assess the effect of CCK-BR point mutations on the ability of individual ligands to trigger inositol phosphate production, COS-7 cells transiently expressing either the wild type or one of the mutant CCK-BRs were stimulated with saturating concentrations of ligand. For each receptor, relative ligand efficacies were calculated as a percentage of the maximal level of inositol phosphate production induced by the full agonist, CCK-8. Of the 16 CCK-BR mutations that were fully characterized, none led to significant changes in

peptide hormone (CCK-8 or CCK-4)-induced inositol phosphate production (Tables 2 and 3). In contrast, eight amino acid substitutions within the CCK-BR ligand pocket significantly altered the functional activity of one or both of the synthetic peptoid ligands (Table 3). PD-135,158 and PD-136,450 are partial agonists at the human wild type CCK-BR, stimulating inositol phosphate production to 20% and 42%, respectively, of the CCK-8-induced maximum (Table 3). Efficacy of at least one compound was significantly increased by two mutations, M186A and W346A, whereas peptoid efficacy was decreased by six other amino acid substitutions, Y61A, T111A, F227A, V349A, Y350A, and N353L (Table 3).

To examine the relationship between ligand affinity, relative efficacy, and potency in more detail, the pharmacologic properties of PD-136,450 at the wild type CCK-BR and at three selected mutant receptors were compared. These mutant CCK-BRs had either 1) normal affinity (Y61A), 2) decreased affinity (M186A), or 3) markedly decreased affinity (N353L) for the peptoid ligand. PD-136,450 induced a concentration-dependent increase in inositol phosphate production at the wild type receptor and at each of the three tested mutants (Fig. 4a). The respective EC_{50} values were 1.6 ± 0.2 nM (wild type CCK-BR), 2.5 ± 1.7 nM (Y61A), 4.9 ± 0.5 nM (M186A), and 11.2 ± 0.7 nM (N353L) (means ± S.E., $n = 3$). As illustrated in Fig. 4b, the EC_{50} values at the mutant receptors were shifted in the same direction as the corresponding IC_{50} values (Table 1), although the potency shifts were generally less pronounced than the affinity shifts.

To compare the effect of CCK-BR pocket mutations on a structurally different synthetic partial agonist, the functional activity of the benzodiazepine-based nonpeptide ligand L-740,093*S* (Beinborn et al., 1998) was assessed. At four of the mutant receptors (Y61A, T111A, Y350A, N353L) efficacy decreases were observed for both L-740,093*S* and at least one of the peptoids. In contrast, the F227A mutant selectively decreased peptoid efficacy without significantly altering L-740,093*S*-induced inositol phosphate production (Table 3). Two mutations (M186A, W346A) significantly increased the efficacy of PD-135,158 and/or PD-136,450 while decreasing the functional activity of L-740,093*S*. Conversely, alanine substitution of Val-349 decreased peptoid-induced signaling and at the same time increased L-740,093*S* efficacy.

In marked contrast to the mutation-induced efficacy changes of synthetic compounds, the functional activity of the endogenous peptides, CCK-8 and CCK-4, remained essentially unchanged by the 16 amino acid substitutions within the CCK-BR ligand pocket (Tables 2, 3).

Figure 5 provides an overview illustrating the mutation-induced changes in functional activity for each of three classes of molecules, peptides, peptoids, and nonpeptides. At each of the mutant receptors, CCK-8 and CCK-4 showed little or no deviation from the respective wild type values (=100%). In contrast, mutation-induced activity changes for the synthetic ligands cover a broad range, from complete loss of activity to more than a 100% increase in the respective value at the wild type receptor.

Discussion

Previous work from our laboratory resulted in the identification of a putative CCK-BR transmembrane domain ligand pocket (Kopin et al., 1995). More recently, we have shown that amino acids that project into this pocket affect

TABLE 1

Peptide and peptoid affinity at the wild type and mutant CCK-BRs

IC_{50} values (means ± S.E., $n \geq 3$) were determined by radioligand competition binding experiments. Affinity changes are expressed as ratios, mutant IC_{50}/wild type IC_{50}. Values > 1 represent decreases in affinity, and values < 1 represent increases in affinity. Affinity changes exceeding 3-fold (underlined) were statistically analyzed after conversion of respective IC_{50} values into pIC_{50} values; all of these were significantly different from corresponding wild type pIC_{50} values ($P < .05$).

	CCK-8		CCK-4		PD135,158		PD136,450	
	IC_{50}	mut/wt	IC_{50}	mut/wt	IC_{50}	mut/wt	IC_{50}	mut/wt
	nM		*nM*		*nM*		*nM*	
Wild type	0.09 ± 0.01		20.5 ± 3.5		1.9 ± 0.4		1.0 ± 0.2	
TM I								
Y61A	1.04 ± 0.13	11.6	262.0 ± 61.3	12.8	2.0 ± 0.7	1.1	1.2 ± 0.3	1.2
TM II								
F110A	0.12 ± 0.02	1.3	14.3 ± 4.8	0.7	1.5 ± 0.4	0.8	1.7 ± 0.4	1.7
T111A	0.17 ± 0.03[a]	1.9	94.7 ± 9.1	4.6	12.0 ± 3.2	6.3	6.3 ± 0.2	6.3
TM III								
S131A	0.09 ± 0.01[a]	1.0	24.2 ± 3.2	1.2	2.5 ± 0.7	1.3	1.4 ± 0.4	1.4
G135A	0.08 ± 0.01	0.9	25.3 ± 1.9	1.2	1.7 ± 0.6	0.9	1.5 ± 0.3	1.5
TM IV								
M186A	0.92 ± 0.17	10.2	160.3 ± 12.4	7.8	53.6 ± 17.3	28.2	12.8 ± 1.9	12.8
T193A	4.00 ± 0.64	44.4	1186.1 ± 328.6	57.9	1.5 ± 0.6	0.8	1.9 ± 0.6	1.9
TM V								
S219A	0.06 ± 0.01[a]	0.7	22.0 ± 1.6	1.1	1.0 ± 0.5	0.5	0.5 ± 0.2	0.5
L226A	0.13 ± 0.02	1.4	26.7 ± 1.9	1.3	3.4 ± 1.4	1.8	2.4 ± 0.6	2.4
F227A	0.10 ± 0.03	1.1	21.7 ± 3.1	1.1	1.1 ± 0.1	0.6	0.5 ± 0.1	0.5
TM VI								
W346A	0.16 ± 0.03[a]	1.8	31.6 ± 2.4	1.5	2.3 ± 0.6	1.2	0.8 ± 0.1	0.8
V349A	0.33 ± 0.10[a]	3.7	239.3 ± 25.4	11.7	1.3 ± 0.3	0.7	0.8 ± 0.1	0.8
Y350A	0.12 ± 0.03[a]	1.3	63.7 ± 9.1	3.1	0.7 ± 0.2	0.4	0.5 ± 0.0	0.5
N353L	0.15 ± 0.04[a]	1.7	74.6 ± 8.8	3.6	242 ± 78.1	127.3	41.3 ± 7.2	41.3
T354A	0.24 ± 0.05	2.7	66.5 ± 4.8	3.2	26.8 ± 5.8	14.1	7.3 ± 1.2	7.3
TM VII								
S379A	0.09 ± 0.03[a]	1.0	35.8 ± 6.9	1.7	1.1 ± 0.4	0.6	0.4 ± 0.1	0.4

TM, transmembrane domain; mut, mutant; wt, wild type.
[a] Previously reported data (Bläker et al., 1998).

affinity and/or signaling properties of benzodiazepine-based nonpeptide CCK-BR ligands (Bläker et al., 1998).

In the present study we examined whether pocket residues also confer ligand affinity and functional activity to two additional classes of molecules, peptides and peptide-derived synthetic agonists (peptoids). This analysis utilized a panel of 16 mutant human CCK-BRs, each including substitution of a single amino acid that is predicted to project into the previously established CCK-BR transmembrane domain binding pocket. The majority of these amino acids are found in positions that correspond to the binding site crevice of the human dopamine D2 receptor as established by the cysteine accessibility method (Fig. 2) (Javitch et al., 1995a,b, 1998, 1999; Fu et al., 1996).

Each of the 16 CCK-BR mutants share a number of pharmacologic properties with the human wild type receptor, including a similar magnitude of CCK-8-induced signaling and a lack of constitutive (ligand-independent) activity (Table 2). Further analysis of the CCK-BR mutants revealed that four amino acid substitutions, Y61A, M186A, T193A, and V349A, significantly decreased binding affinity for both CCK-8 and CCK-4. It is apparent that the magnitude of affinity changes for the Y61A and T193A mutants exceeded 10-fold (versus the wild type value) without altering peptoid binding. These observations suggest that endogenous peptides interact with the putative CCK-BR transmembrane domain binding pocket. At the same time, it is apparent that the determinants of peptide affinity within this domain appear, at least in part, to be distinct from those amino acids that determine the affinity for small nonpeptide molecules (Beinborn et al., 1993; Mantamadiotis and Baldwin, 1994; Kopin et al., 1995). Previously, CCK-BR peptide affinity determinants have been reported both within the extracellular loops (Silvente-Poirot and Wank, 1996) and the transmembrane domains (Kopin et al., 1995). The presence of additional peptide affinity determinants within the transmembrane domains is consistent with reports on other G-protein-coupled receptors. For the neurokinin$_1$, bradykinin B_2, and the angiotensin AT_1 receptors, sites of potential peptide interaction have been localized within the upper portion of the transmembrane domain pocket (Schwartz et al., 1995). As with the CCK-BR, these sites of putative peptide interactions may act in concert with those that have been found in the extracellular loops.

A total of four amino acid substitutions (T111A, M186A, N353L, and T354A) significantly decreased affinity for PD-135,158 and PD-136,450. One of these mutations, M186A, also reduced peptide (CCK-8 and CCK-4) affinities. Given the structural similarities between peptides and peptoids, it is possible that M186 represents a site that either defines a critical microenvironment or itself interacts with structural motifs common to these ligands. Such shared affinity determinants in the ligand may be the aromatic ring structures of

TABLE 2

Comparison of receptor number (B_{max}) as well as capacity to trigger inositol phosphate production of the wild-type and mutant CCK-BRs when expressed in COS-7 cells

Mutated amino acids are numbered according to their positions in the CCK-BR. In addition, index numbers refer to the respective positions relative to the most conserved residue (indexed as X.50, see Fig. 2) in corresponding transmembrane domains (Ballesteros and Weinstein, 1995).

	Index no.	B_{max}[a]	n	Inositol Phosphate Production		n
				Basal	+ CCK-8	
		fmol / 10^4 cells		*% H^3 incorporation*		
Wild type		6.12 ± 0.8	16	3.85 ± 0.35	29.71 ± 0.41	11
TM I						
Y61A[b]	1.39	4.06 ± 1.8	3	3.52 ± 0.27	29.03 ± 2.60	7
TM II						
C107A	2.57	7.00 ± 0.3	3	7.87 ± 0.27*	37.74 ± 2.29	3
F110A	2.60	3.21 ± 1.6	3	3.94 ± 0.34	29.40 ± 2.44	8
T111A	2.61	5.71 ± 1.1	3	4.10 ± 0.19	32.17 ± 2.84	3
TM III						
S131A	3.29	9.90 ± 2.4	3	3.99 ± 0.92	30.04 ± 1.99	3
G135A	3.33	5.50 ± 1.2	3	3.81 ± 0.26	31.39 ± 1.84	3
V138A	3.36	8.98 ± 2.1	3	8.81 ± 0.85*	33.84 ± 2.29	3
TM IV						
M186A	4.57	5.41 ± 1.4	3	4.51 ± 0.39	30.79 ± 2.57	7
T193A	4.64	5.88 ± 1.0	3	3.36 ± 0.16	30.37 ± 2.02	6
TM V						
S219A	5.39	7.34 ± 0.5	3	3.68 ± 0.46	31.53 ± 1.58	3
L223A	5.43	7.36 ± 0.6	3	7.16 ± 1.29*	31.65 ± 1.07	3
L226A	5.46	5.37 ± 1.0	3	4.24 ± 0.39	30.49 ± 1.41	8
F227A	5.47	3.60 ± 0.7	3	4.22 ± 0.33	27.41 ± 2.73	8
TM VI						
W346A	6.48	4.54 ± 0.7	3	3.95 ± 1.82	28.81 ± 1.96	3
V349A	6.51	4.84 ± 0.8	3	3.13 ± 0.32	33.89 ± 1.92	3
Y350A	6.52	6.17 ± 0.5	3	3.92 ± 0.25	33.76 ± 0.68	3
N353L	6.55	3.91 ± 0.5	3	3.52 ± 0.87	33.75 ± 3.64	3
T354A	6.56	9.59 ± 5.0	3	3.83 ± 0.64	34.32 ± 1.25	3
TM VII						
S379A	7.42	7.42 ± 0.9	3	5.11 ± 0.21	35.57 ± 1.33	3

TM, transmembrane domain.

[a] B_{max} was determined by homologous competition binding experiments (^{125}I-CCK-8 versus unlabeled CCK-8).

[b] For each mutant, CCK-8 (3×10^{-7} M)-induced inositol phosphate production was comparable to the control value assessed at the wild-type receptor. In contrast, the basal (ligand-independent) signaling of the C107A, V138A, and L223A mutant receptors (underlined) was significantly increased when compared to the wild-type CCK-BR. These mutant receptors were excluded from further study (see text). Basal signaling by the wild-type CCK-BR and by all other mutants was virtually identical to the level of inositol phosphate production measured in cells transfected with the empty expression vector, pcDNAI (not shown). Inositol phosphate production is expressed as inositol [^{3}H]phosphates/total cellular ^{3}H × 100 (see *Materials and Methods*).

* $P < .05$ versus wild type.

tryptophan and/or phanylalanine, at the carboxyl terminus of CCK-8, which are conserved in CCK-4 and separated by a spacer in peptoid ligands (Horwell, 1991). Alternatively, it must be considered whether substitution of M186 nonspecifically disrupts the overall tertiary structure of the receptor and thus decreases affinity for both peptides and peptoids. To address the latter concern, additional binding experiments with two different benzodiazepine-based nonpeptide ligands, YM022 and L-740,093*S* (Beinborn et al., 1998) were performed. Affinities for these nonpeptides at the M186A receptor were not significantly different from respective wild type values (data not shown), suggesting that the tertiary structure of the mutant has not been grossly disrupted. It is therefore plausible to conclude that the mutated amino acid (M186) may, in fact, be a selective affinity determinant, which is shared by peptide and peptoid, but not by benzodiazepine-based nonpeptide ligands.

The most pronounced affinity shifts at any of the mutant receptors were observed for the peptoid compounds at the N353L mutant (Table 1). At the same time, the N353L mutation only slightly decreased CCK-4 affinity and did not significantly affect CCK-8 binding. In contrast to these findings, leucine substitution of the corresponding residue in the human β_2-adrenergic receptor (Asn-293) leads to a marked affinity decrease for full agonists while altering the affinity of partial agonists to a much lesser extent (Wieland et al., 1996) (Fig. 3). These converse observations indicate that, although biogenic amine receptors and peptide hormone receptors share structural features such as the transmembrane domain ligand pocket, the interactions with endogenous ligands that occur within this pocket may differ significantly. Consistent with the notion that the role of individual positions in the binding pocket may be highly receptor- and/or ligand-dependent, we found that Leu-226 and Tyr-350, which correspond to important affinity determinants in the β_2-adrenergic receptor (Ser-207, Phe-290) (Cascieri et al., 1995) (Fig. 3), do not appear to be equally important for ligand binding to the CCK-BR.

Measurement of ligand efficacy revealed that the functional activity of PD-135,158 and/or PD-136,450 was either increased or decreased by eight amino acid substitutions within the CCK-BR pocket. There appeared to be no general correlation between CCK-BR mutations that affected the relative efficacies of these peptoid compounds and those that influenced binding affinities. These findings are consistent with our earlier report, which demonstrated that CCK-BR pocket mutations may selectively affect affinity or relative efficacy of benzodiazepine-based nonpeptide ligands (Bläker et al., 1998). In this previous study, several pocket amino acid substitutions were identified, which converted nonpeptide agonists to antagonists and vice versa without necessarily affecting the respective binding affinities. Similar observations have recently been reported for the angiotensin AT_1 receptor. Mutations within the putative transmembrane domain pocket of this receptor result in decreases in the efficacy of nonpeptide partial agonists without affecting binding affinities of these compounds (Perlman et al., 1997).

More detailed analysis of PD-136,450-induced inositol phosphate production at selected CCK-BR pocket mutations (Y61A, M186A, N353L) further supports the notion that alterations in apparent affinity and relative efficacy may vary in opposite directions (Fig. 4). According to current theories, ligand-induced receptor activation has been attributed to

TABLE 3

CCK-4, peptoid, and nonpeptide efficacy at respective wild-type and mutant CCK-BRs

Multiple CCK-BR pocket mutations significantly alter efficacy of the synthetic peptoid (PD-135,158, PD-136,450) and nonpeptide (L-740,093*S*) ligands (values are underlined). Mutant CCK-BRs were stimulated with saturating ligand concentrations which were at least 50-fold higher than their respective IC_{50} values (Table 1). IC_{50} values for L-740,093*S* at the mutant receptors (not shown) were not significantly different from the wild-type value (25 ± 3.7 nM) except for the T111A (84 ± 10.9 nM) and S379A mutants (8.8 ± 1.6 nM). Data represent means ± S.E. of at least three independent experiments.

	Ligand efficacy			
	CCK-4	PD-135,158	PD-136,450	L-740,093*S*
	% CCK-8-induced inositol phosphate production			
Wild type	99 ± 1	20 ± 1	42 ± 2	30 ± 2[a]
TM I				
Y61A	99 ± 6	6 ± 1	16 ± 3*	12 ± 2*
TM II				
F110A	95 ± 5	25 ± 3	47 ± 3	26 ± 4
T111A	109 ± 3	10 ± 3	16 ± 4*	6 ± 2*[a]
TM III				
S131A	103 ± 9	21 ± 2	38 ± 4	27 ± 5[a]
G135A	103 ± 3	27 ± 7	46 ± 10	24 ± 2
TM IV				
M186A	92 ± 8	41 ± 8*	67 ± 7*	9 ± 2*
T193A	95 ± 5	29 ± 3	44 ± 3	25 ± 2
TM V				
S219A	106 ± 10	19 ± 3	43 ± 2	42 ± 6[a]
L226A	100 ± 3	26 ± 3	48 ± 2	25 ± 1
F227A	104 ± 6	0 ± 0*	8 ± 3*	41 ± 4
TM VI				
W346A	99 ± 5	43 ± 5*	57 ± 3	12 ± 3*[a]
V349A	103 ± 5	3 ± 2*	3 ± 3*	72 ± 8*[a]
Y350A	98 ± 3	0 ± 2*	0 ± 2*	0 ± 3*[a]
N353L	85 ± 4	9 ± 2	15 ± 2*	12 ± 1*[a]
T354A	96 ± 8	34 ± 5	53 ± 7	5 ± 2*
TM VII				
S379A	101 ± 2	31 ± 5	51 ± 1	44 ± 6[a]

TM, transmembrane domain.
[a] Previously reported data (Bläker et al., 1998).
* $P < 0.05$ versus wild type.

changes in the ratios between multiple receptor conformations (Kenakin, 1999). In interpreting our data, it is important to acknowledge that radioligand binding studies with any given ligand may only detect a fraction of the receptor conformations. On this basis, the observed dissociation between affinity and efficacy can be attributed to CCK-BR conformations, which are detectable with ^{125}I-CCK-8. The existence of multiple other receptor conformations can not be excluded.

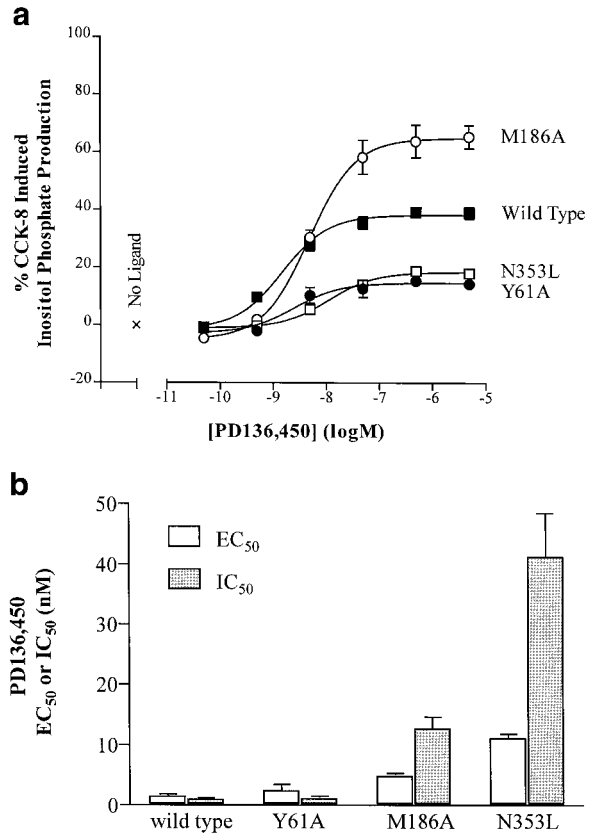


Fig. 4. Point mutations within the CCK-BR transmembrane domain pocket alter affinity and potency of PD-136,450 in parallel. a, PD-136,450 stimulates a concentration-dependent increase in inositol phosphate production in COS-7 cells expressing the human wild type CCK-BR (■), or any one of the mutant receptors, Y61A (●), M186A (○) or N353L (□). Inositol phosphate production is expressed as a percentage of the CCK-8 (3×10^{-7} M)-induced maximal value (=100%). x indicates inositol phosphate production in the absence of ligand (defined as 0% for each tested receptor). Symbols represent means ± S.E. of three independent experiments. b, comparison of PD-136,450 EC_{50} and IC_{50} values at the wild type CCK-BR and at the Y61A, M186A, and N353L receptor mutants.

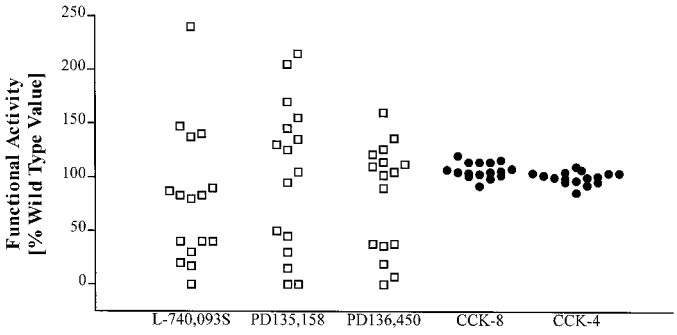


Fig. 5. CCK-BR mutations markedly affect the efficacies of synthetic nonpeptide and peptoid ligands without altering the efficacies of endogenous peptides. Each symbol (□, synthetic peptoids, PD-135,158 and PD-136,450, and the benzodiazepine-based nonpeptide ligand L-740,093*S*; ●, peptide ligands CCK-8 and CCK-4) represents functional activity of the individual ligands at one of the 16 CCK-BR mutants at which ligand efficacies were assessed. The ligand-induced inositol phosphate production of each compound at the human wild type receptor was defined as 100% (see CCK-4, peptoid, and L-740,093*S* efficacies in Table 3, and CCK-8-induced inositol phosphate production in Table 2).

The observed shifts in PD-136,450 potency at the mutant receptors largely reflect the corresponding alterations in peptoid affinity (Fig. 4). The receptor mutation that did not alter affinity (Y61A) had no significant effect on the potency of PD-136,450, whereas the mutations that decreased PD-136,450 affinity to different degrees (M186A, N353L) also resulted in concomitant decreases in potency (Fig. 4b). These parallel changes in peptoid affinity and potency are consistent with the well established pharmacologic principle that ligand-induced receptor stimulation is a function of fractional receptor occupation, which in turn is dependent on ligand affinity (Ross, 1996).

It is of note that increases or decreases in PD-136,450 efficacy at any of the mutant CCK-BRs were paralleled by similar alterations in PD-135,158-induced signaling (although respective changes did not in all cases reach statistical significance). To examine whether the shifts in the signaling properties of both peptoid compounds reflected a systematic amplification or reduction of partial agonist function at the respective receptor mutants, we examined changes in relative efficacy with a structurally different ligand. Efficacy of the benzodiazepine-based nonpeptide partial agonist L-740,093*S* was also affected by multiple point mutations within the CCK-BR pocket (Table 3). However, four of the mutations that significantly alter functional activities of the peptoid ligands either shift efficacy of L-740,093*S* in the opposite direction or leave its efficacy unchanged. These differential effects on ligand function suggest that CCK-BR pocket mutations do not cause systematic shifts in partial agonist activity. Rather, the observed mutation-induced changes in relative efficacy are suggestive of more selective alterations in ligand receptor interactions.

In marked contrast to the findings for the peptoid molecules and the nonpeptide agonist L-740,093*S*, the efficacy of the two peptides, CCK-8 and CCK-4, was not significantly affected by any of the CCK-BR mutations that were examined in this study. For example, alanine substitution of Met-186 leads to a 2-fold increase in peptoid efficacy and simultaneously decreases L-740,093*S* efficacy more than 3-fold, yet at the same time, it does not alter efficacy of either CCK-4 or CCK-8. Also, mutation of Val-349 abolishes peptoid activity while increasing efficacy of L-740,093*S* more than 2-fold. As with M186A, the V349A mutation does not influence signaling induced by either of the two peptide molecules, CCK-4 or CCK-8. These observations suggest considerable differences in the mechanisms underlying ligand-induced receptor activation by endogenous peptide hormones versus synthetic ligands. Consistent findings with the angiotensin AT_1 receptor support that this conclusion may be applicable to a wider range of G-protein-coupled peptide hormone receptors. It has recently been shown, for the angiotensin AT_1 receptor, that single amino acid substitutions within the putative ligand pocket markedly decrease efficacy of nonpeptide molecules without altering the function of the endogenous peptide, angiotensin II (Perlman et al., 1997).

Accumulating experimental evidence indicates that G-protein-coupled peptide hormone receptors can be activated by

molecular interactions (e.g., with antibodies, synthetic ligands), which differ from the receptor's interactions with peptide hormones (Schwartz and Rosenkilde, 1996). For the CCK-BR, we hypothesize that synthetic compounds induce receptor activation by ligand receptor interactions within the transmembrane domain ligand pocket, whereas peptide hormones stabilize one or more active CCK-BR conformations by interactions with different domains, e.g., both transmembrane domains and extracellular loops. In addition, it appears that activation of the CCK-BR by peptides is less sensitive to minor receptor alterations (i.e., mutations). Under evolutionary pressure, both the endogenous ligands and the receptor may have adapted structures, which ensure optimal receptor activation, independent of single amino acid differences. This would explain why different species homologs of the CCK-BR, which share 90% amino acid identity (mouse, dog, human), show an equal response to CCK-8 yet demonstrate marked differences in the response to synthetic drugs (Kopin et al., 1997).

Several reports have demonstrated that polymorphisms in G-protein-coupled receptors, including the CCK-BR, are found within the human population (Green et al., 1993; Reihsaus et al., 1993; Herget et al., 1994; Kato et al., 1996). Our present work suggests that the activity of synthetic ligands may be highly sensitive to these receptor polymorphisms (Kopin et al., 1997). This variability in drug-induced response may become of increasing clinical relevance with the continued development of receptor specific drugs. With better understanding of the interactions that confer functional activity, it may be possible to structurally modify candidate compounds to reduce the ligand's sensitivity to sporadic mutations or polymorphisms in the targeted receptor.

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