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Inter- and intraspecies polymorphisms in the cholecystokinin-B/gastrin receptor alter drug efficacy

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ABSTRACT **The brain cholecystokinin-B/gastrin receptor (CCK-BR) is a major target for drug development because of its postulated role in modulating anxiety, memory, and the perception of pain. Drug discovery efforts have resulted in the identification of small synthetic molecules that can selectively activate this receptor subtype. These drugs include the peptide-derived compound PD135,158 as well as the nonpeptide benzodiazepine-based ligand, L-740,093 (S enantiomer). We now report that the maximal level of receptor-mediated second messenger signaling that can be achieved by these compounds (drug efficacy) markedly differs among species homologs of the CCK-BR. Further analysis reveals that the observed differences in drug efficacy are in large part explained by single or double aliphatic amino acid substitutions between respective species homologs. This interspecies variability in ligand efficacy introduces the possibility of species differences in receptor-mediated function, an important consideration when selecting animal models for preclinical drug testing. The finding that even single amino acid substitutions can significantly affect drug efficacy prompted us to examine ligand-induced signaling by a known naturally occurring human CCK-BR variant (glutamic acid replaced by lysine in position 288; $^{288}E \rightarrow K$). When examined using the $^{288}E \rightarrow K$ receptor, the efficacies of both PD135,158 and L-740,093 (S) were markedly increased compared with values obtained with the wild-type human protein. These observations suggest that functional variability resulting from human receptor polymorphisms may contribute to interindividual differences in drug effects.**

The cholecystokinin-B/gastrin receptor (CCK-BR) is a seven-transmembrane domain, G-protein coupled protein that is widely expressed in the central nervous system, as well as in a number of peripheral tissues including the pancreas and the stomach. *In vivo*, the CCK-BR is activated by endogenous neuro/enteroendocrine peptides including sulfated cholecystokinin octapeptide (CCK-8) and gastrin. Each of these peptides exerts a broad range of physiological functions (1, 2). In the central nervous system, CCK-BRs have been postulated to modulate anxiety, memory, and opiate-induced analgesia (3–7). In the periphery, receptor-mediated functions include gastric mucosal proliferation as well as acid secretion (1, 8).

Given the clinical importance of CCK-BR-mediated functions, there has been considerable interest in the identification of drugs that selectively activate or block this receptor subtype (9). Resulting from these efforts, it has recently been discovered that, in addition to peptide hormones, synthetic molecules have the potential to activate the CCK-BR. This was originally demonstrated for a limited number of "peptoid" compounds including PD135,158 (10, 11). These peptide-derived ligands were designed to retain several structural features of CCK-4, the C-terminal four amino acids of cholecystokinin (12), and are thus considered "peptoid" molecules. In addition, our laboratory has recently demonstrated that a nonpeptide compound, L-740,093 (S enantiomer), can mimic the activity of the endogenous peptide agonists cholecystokinin and gastrin. This benzodiazepine-based ligand (13) triggers a concentration-dependent increase in inositol phosphate production when stimulating the CCK-BR (M.B and A.S.K., unpublished data). L-740,093 (S enantiomer) therefore qualifies as the first synthetic CCK-BR agonist that is a "true" nonpeptide molecule.

Pharmacologic comparison of species homologs (dog, human, *Mastomys natalensis*, and rat) of the CCK-BR have revealed many conserved features (14–18). Agonist stimulation of all known CCK-BRs triggers phospholipase C mediated conversion of membrane phospholipids to inositol phosphates (IP) and diacylglycerol (1). Another feature common to all species variants of the receptor is subnanomolar affinity for both of the endogenous agonists, CCK-8 and gastrin. In contrast to these similarities, previous experiments in our laboratory revealed that the affinity of the CCK-BR for nonpeptide ligands may be highly species-dependent. Even single amino acid differences between species homologs of the CCK-BR can profoundly affect the binding affinities of nonpeptide antagonists (19).

The recent identification of peptoid and nonpeptide CCK-BR agonists prompted us to examine whether interspecies receptor polymorphisms may also affect the functional properties of these synthetic compounds. In this study we compared the abilities of L-740,093 (S), and PD135,158 to stimulate phospholipase C activity through the recombinant human, mouse, and dog CCK-BRs. Whereas the endogenous agonist, CCK-8, triggers comparable signaling with each of the CCK-BR homologs, marked species differences are observed in the ability of the synthetic agonists to trigger receptor-mediated IP production. Further analysis revealed that the observed differences in drug efficacy are the result of interspecies polymorphisms in transmembrane domain (TMD) VI of the CCK-BR.

EXPERIMENTAL PROCEDURES

Construction of Wild-Type, Chimeric, and Mutant Receptor cDNAs. Human (15), murine (GenBank accession no. AF019371), and canine (14) receptor cDNAs were subcloned into the expression vector pcDNA I (Invitrogen). To generate human/murine chimeras, *Mlu*I and *Hin*dIII sites (corresponding to human cDNA nucleotides 987–992 and 1116–1121, respectively) were introduced into the receptor cDNAs using

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Abbreviations: CCK-BR, cholecystokinin-B/gastrin receptor; CCK-8, cholecystokinin octapeptide; IP, inositol phosphates; TMD, transmembrane domain.
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oligonucleotide-directed mutagenesis (20). Naturally occurring *Pst*I sites, found in the same relative position in both the mouse and human receptors (corresponding to human cDNA nucleotides 607–612), were utilized to generate the *Pst*I chimera. In all chimeric proteins, wild-type human or wild-type mouse amino acid sequence was conserved in corresponding segments. Mutant receptor cDNAs were generated using oligonucleotide-directed mutagenesis (19). All receptor cDNA sequences were confirmed by the chain termination method (21).

Radioligand Binding Experiments. Wild-type, chimeric, and mutant CCK-BR cDNAs subcloned into pcDNA I were transiently expressed in COS-7 cells (14). Forty-eight hours after transfection, binding experiments were performed in 24-well plates using 20 pM ^{125}I CCK-8 (New England Nuclear) as the radioligand (19). Affinities for CCK-8 (Peninsula Laboratories), L-740,093 (S enantiomer) (Wyeth Research Laboratories), and PD135,158 (Parke–Davis Neuroscience Research Center) were determined by competition binding experiments with increasing concentrations of unlabeled ligand. The characterization of each recombinant receptor included three or more ($n \geq 3$) independent competition binding experiments for each of the ligands tested. The respective IC_{50} values were calculated by computerized nonlinear curve fitting (INPLOT 4.0; GraphPad, San Diego).

Measurement of Phospholipase C Activity. Transfected cells were labeled overnight with 3 μCi/ml [^{3}H]myo-inositol (40–60 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq) and then stimulated with ligand for 30 min at 37°C in the presence of 10 mM LiCl. Ligand concentrations utilized in the signaling assay were at least 25-fold higher than the corresponding IC_{50} values (Table 1); CCK-8 (0.1 μM), PD135,158 (1.0 μM), L-740,093 (S enantiomer) (0.5 μM). 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 maximal stimulation of receptor-mediated inositol phosphate production. Inositol metabolites were extracted with methanol/chloroform; the upper phase was analyzed for inositol phosphates by strong anion exchange chromatography. CCK-8-induced inositol phosphate production was expressed as a fraction of the total cellular tritium content which was incorporated during overnight exposure to [^{3}H]myo-inositol (tritiated inositol phosphates/total tritium incorporated).

Efficacy of a partial agonist is the magnitude of second messenger signaling relative to the response induced by a full agonist (e.g., CCK-8) (22). Therefore, in each experiment, CCK-8-stimulated inositol phosphate production was included as an internal standard, and the efficacy of each partial agonist [PD135,158 and L-740,093 (S)] was expressed as a percentage of the CCK-8 induced maximum.

RESULTS AND DISCUSSION

Comparable levels of inositol phosphate production were observed when recombinant human, murine, and canine CCK-BRs were expressed in COS-7 cells and stimulated with saturating concentrations (10^{-7} M) of the full agonist, CCK-8 (Fig. 1*A*). In contrast, considerable species-dependent variability in efficacy was observed for both partial agonists, PD135,158 and L-740,093 (S) (Fig. 1*B*). Saturating concentrations of PD135,158 activate the human receptor to only 16% of the CCK-8-induced maximum. In comparison, when tested with either the murine or the canine homologs, the efficacy of PD135,158 was found to be greater than 60% of the CCK-8-induced maximum. Species-dependent variability in drug efficacy was also observed with the partial agonist L-740,093 (S). Efficacy of this nonpeptide compound was 21% (human), 55% (mouse), and 17% (dog) of the CCK-8-induced value. To appreciate the biological significance of these differences in ligand efficacy, it should be emphasized that in a species in which drug efficacy is low, certain receptor-mediated effects may be unattainable even at high drug concentrations and full receptor occupancy. In contrast, in a species in which ligand efficacy is high, the drug may have significant effects even at relatively low concentrations. It is of note that, despite the observed interspecies differences in partial agonist efficacy, each of the receptor homologs (human, mouse, and canine) had similar binding affinities for PD135,158 and L-740,093 (S) (Table 1).

The species-dependence of drug efficacy was observed despite ≈90% amino acid sequence identity among the human, murine, and canine CCK-BRs (14–16). To determine the receptor domain underlying these efficacy differences, we generated a series of human-mouse chimeric proteins (Table

Table 1. Affinities for CCK-8, PD135,158, and L-740,093 (S) are comparable between wild-type and mutant CCK-Brs

	^{125}I-CCK-8 competition binding, pIC_{50} ± SEM		
	CCK-8	PD135,158	L-740,093 (S)
Mouse			
Wild type	10.04 ± 0.05	8.63 ± 0.04	7.61 ± 0.11
^{346}L → V (a)	10.07 ± 0.04	8.69 ± 0.09	ND
^{350}V → L (b)	9.89 ± 0.12	8.92 ± 0.13	ND
^{346}LLFFV → VLFFL (c)	10.06 ± 0.07	8.70 ± 0.09	7.71 ± 0.05
^{370}R → H	9.99 ± 0.07	ND	ND
^{374}A → S	10.08 ± 0.10	ND	ND
Human			
Wild type	10.10 ± 0.03	8.70 ± 0.07	7.71 ± 0.04
^{340}V → L (a)	10.26 ± 0.04	8.69 ± 0.04	ND
^{344}L → V (b)	10.17 ± 0.10	8.65 ± 0.06	ND
^{340}VLFFL → LLFFV (c)	10.11 ± 0.09	8.57 ± 0.05	7.52 ± 0.12
^{349}V → L (d)	10.02 ± 0.02	8.71 ± 0.05	ND
^{288}E → K	10.23 ± 0.09	8.89 ± 0.08	7.65 ± 0.02
Canine			
Wild type	10.12 ± 0.16	8.35 ± 0.13	7.18 ± 0.04
^{355}L → V (d)	10.17 ± 0.02	8.61 ± 0.08	ND

^{125}I-CCK-8 radioligand competition experiments were utilized to determine affinities for all constructs (20). Respective pIC_{50} ($-\log IC_{50}$) values were calculated using computerized nonlinear curve fitting (INPLOT 4.0; GraphPad). Data represent the means of at least three independent experiments ±SEM. ND, not determined; a, b, c, and d each represent a corresponding pair of mutant receptors.

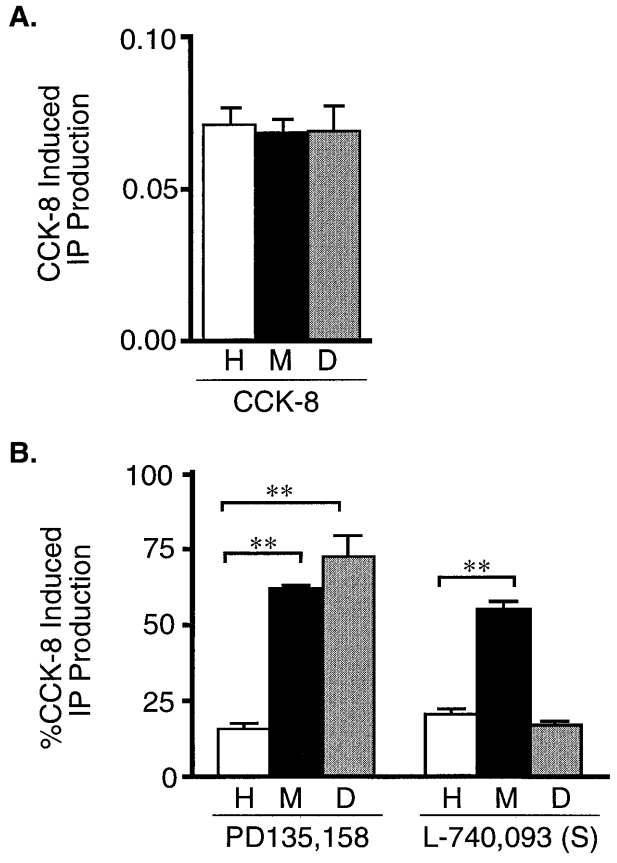

FIG. 1. Maximal CCK-8, PD135,158, and L-740,093 (S enantiomer) induced second messenger signaling. Ligand-induced inositol phosphate production was assessed in COS-7 cells expressing either the human (open column), murine (filled column), or canine (gray column) CCK-BR. Data represent means ± SEM of four independent experiments. Significance versus the human wild-type receptor, **, $P < 0.01$. H, human; M, mouse; D, dog. (*A*) CCK-8-induced inositol phosphate production is similar in cells expressing either the human, mouse, or canine CCK-BRs (no significant difference). CCK-8-induced IP production is expressed as a fraction of the total cellular tritium content which was incorporated during overnight exposure to [^{3}H]myo-inositol (tritiated inositol phosphates/total tritium incorporated). (*B*) There are marked differences in PD135,158- and L-740,093 (S)-induced second messenger signaling when assessed with cells expressing either the human, mouse, or canine CCK-BR receptors. Results are expressed relative to CCK-8 induced inositol phosphate production which was assigned an efficacy value of 100% in each species.

2). Replacement of the human receptor region spanning TMD VI and the third extracellular loop with the corresponding segment of the murine protein resulted in increased PD135,158 efficacy. Alignment of the human and murine sequences within this limited domain revealed only four amino acid differences, candidates to explain the interspecies variability of PD135,158-induced inositol phosphate formation. Each of these four murine residues, ^{346}L, ^{350}V, ^{370}R, and ^{374}A, was sequentially substituted with the corresponding amino acid from the human receptor. CCK-8-induced second messenger signaling was not significantly affected by any of these four mutations (shown only for ^{346}L → V and ^{350}V → L in Fig. 2*B*). In contrast, the efficacy of PD135,158 was significantly decreased when examined with two of the four mutant mouse receptors, ^{346}L → V or ^{350}V → L (Fig. 2*C*). Together, these two amino acid changes resulted in a cumulative reduction of murine receptor signaling to a level approaching the human wild-type value. Conversely, introduction of the corresponding murine amino acids into the human receptor, ^{340}V → L and

Table 2. Transfer of TMD VI and the third extracellular loop from the murine to the human receptor enhances PD135,158 efficacy

Receptor	PD135,158 Efficacy (% CCK-8 Induced IP Production)
Human wild type	20.3 ± 1.3 %
Hind III chimera	24.0 ± 1.5 %^{NS}
Mlu I chimera	53.0 ± 9.2 %**
Pst I chimera	63.7 ± 4.1 %**
Mouse wild type	62.5 ± 1.9 %**

Wild-type and human/murine chimeric CCK-BRs were expressed in COS-7 cells; PD135,158-stimulated inositol phosphate production was measured as described in *Experimental Procedures*. Results are expressed as a percent of the maximum CCK-8-induced inositol phosphate production triggered by each receptor. Data represent the means of at least three independent experiments ± SEM. Significance vs. the human wild type receptor: **, $P < 0.01$. NS, not significant.

^{344}L → V, each significantly enhanced the efficacy of PD135,158. The double amino acid substitution again resulted in a cumulative effect, increasing PD135,158 efficacy of the mutant human receptor to a level comparable with the mouse wild-type value.

The increase in PD135,158 efficacy observed with introduction of ^{340}V → L and ^{344}L → V into the human wild-type receptor is further highlighted in Fig. 3. Despite the marked increase in ligand efficacy seen with the double amino acid substitution, the potency of PD135,158 is almost identical when examined with either the mutant or the wild-type receptor.

The same two amino acids, human residues ^{340}V and ^{344}L, and their corresponding murine homologs, in large part explained the differences in L-740,093 (S) efficacy as well (Fig. 2*D*). The mouse double mutant (^{346}LLFFV → VLFFL) significantly decreased L-740,093 (S)-induced signaling whereas the converse human double mutant (^{340}VLFFL → LLFFV) markedly increased this ligand's efficacy. Consistent with results observed with the human and murine wild-type receptors, each of the tested amino acid substitutions that affected drug efficacy did so without altering the binding affinities of either PD135,158 or L-740,093 (S) (Table 1).

Based on the functional importance of TMD VI, we hypothesized that the enhanced efficacy of PD135,158 in activating the canine versus the human receptor (Fig. 1) could also be explained by amino acid variability within this region. Comparison between TMD VI of the canine and human CCK-BRs revealed only a single divergent amino acid. Substitution of this human residue, Val-349, with the corresponding canine amino acid, Leu-355, enhanced PD135,158 induced signaling by almost 3-fold from 20.3 ± 1.3 to 58.2 ± 3.6% of the CCK-8-induced values, respectively (mean ± SEM, $n = 3$, $P < 0.01$). The converse substitution in the dog receptor decreased PD135,158 efficacy to levels comparable with the human value, from 63.9 ± 5.8% to 28.7 ± 4.6% of the

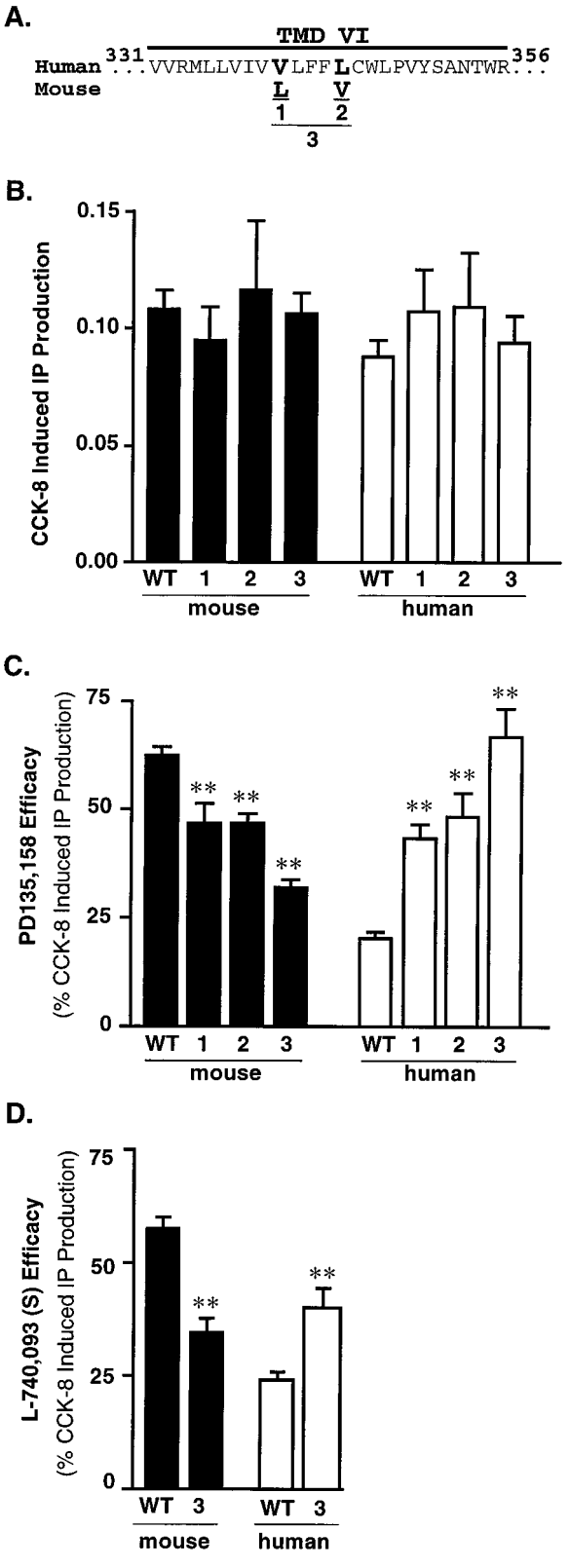

FIG. 2. Identification of CCK-BR amino acids that determine the efficacy of PD135,158 and L-740,093 (S). Using oligonucleotide-directed mutagenesis (20), the nucleotides encoding selected amino acids (see below) were exchanged between the mouse and human receptors. Wild-type and mutant receptors were transiently expressed in COS-7 cells; ligand-stimulated IP production was assayed as described in *Experimental Procedures.* Filled and open columns represent mouse and human receptors, respectively. Significance vs. corresponding wild-type receptors: **, $P < 0.01$; WT, wild type. (*A*) Amino acids 331–356 (single letter code) within TMD VI of the human CCK-BR are compared with the corresponding mouse sequence; only murine residues that diverge are shown. Mouse mutants 1, 2, and 3 correspond to ^{346}L→V, ^{350}V → L, and ^{346}LLFFV → VLFFL, respectively. Human mutants 1, 2, and 3 correspond to ^{340}V→L, ^{344}L→V, and ^{340}VLFFL → LLFFV, respectively. (*B*) CCK-8-induced IP production was not significantly different in cells expressing wild-type vs. mutant receptors. CCK-8-induced IP production is expressed as a fraction of the total cellular tritium content which was incorporated during overnight exposure to [^{3}H]myo-inositol (tritiated inositol phosphates/total tritium incorporated). (*C*) Exchange of human and mouse CCK-BR TMD VI amino acids results in marked alterations in PD135,158-induced second messenger signaling. Efficacy is expressed relative to the CCK-8-induced maximum (defined as 100%), means ± SEM from at least three independent experiments. (*D*) Exchange of human and mouse CCK-BR TMD VI amino acids results in marked alterations in L-740,093 (S)-induced second messenger signaling. Efficacy is expressed relative to the CCK-8 induced maximum (defined as 100%); means ± SEM from at least three independent experiments.

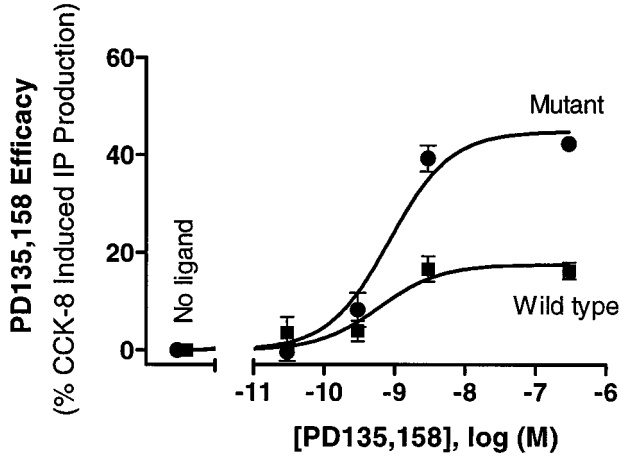

FIG. 3. A double amino acid substitution, ^{340}VLFFL → LLFFV, into the human CCK-BR markedly increases PD135,158 efficacy without significantly altering ligand potency. Human amino acids ^{340}V and ^{344}L were replaced with the corresponding mouse residues, L and V, respectively, using site-directed mutagenesis. The human wild-type and the mutant receptor were expressed in COS-7 cells and concentration-dependent PD135,158-induced inositol phosphate formation was assessed. Note that the tested ligand concentrations were 3.16 times the molar concentrations marked on the *x* axis (10^{-11}, 10^{-10}, 10^{-9}, and 10^{-7} M). Efficacy for each receptor is expressed relative to the CCK-8-induced maximum which was comparable for both constructs (Fig. 2*B*). The pEC_{50} values for PD135,158 were 9.08 ± 0.12 and 9.22 ± 0.25 for the wild-type and mutant human receptors, respectively (no significant difference). Data represent the means of three independent experiments ± SEM.

CCK-8-induced values, respectively (mean ± SEM, $n = 3$, $P < 0.01$). Again, neither of these mutations affected PD135,158 binding affinities (Table 1). We have previously reported that the same amino acid polymorphism (human vs. canine) that affects PD135,158 efficacy also determines affinity differences between these receptors for L-365,265, a nonpeptide ligand (19). Extending from this earlier observation, our present findings illustrate that aliphatic amino acid substitutions in TMD VI can selectively influence either affinity or efficacy, depending on the ligand.

To date, there is no precedent among either biogenic amine or peptide hormone receptors for polymorphisms which result in species-dependent variability in drug efficacy. We speculate that the observed species-specific activation by synthetic agonists is in part due to the lack of evolutionary pressure to conserve receptor interactions with such "artificial" ligands. The finding that differences in ligand-induced signaling exist with two structurally unrelated compounds, PD135,158 and L-740,093 (S), suggests that our observations are not fortu-

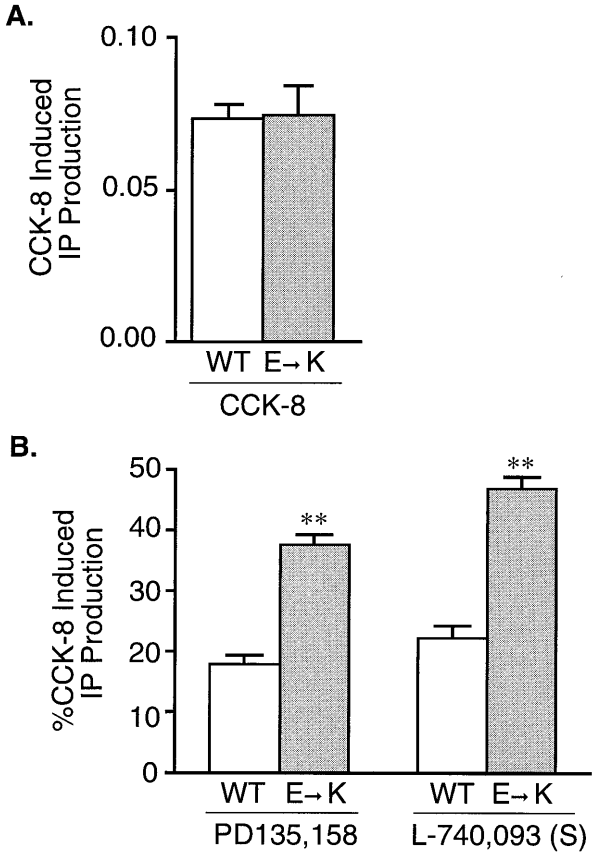

FIG. 4. A human CCK-BR polymorphism, $^{288}E \rightarrow K$, selectively increases the efficacy of PD135,158 and L-740,093 (S). The human wild-type and the $^{288}E \rightarrow K$ receptor were transiently expressed in COS-7 cells; ligand-stimulated IP production was assayed as described in *Experimental Procedures.* (*A*) CCK-8-induced signaling is comparable for the wild-type and the $^{288}E \rightarrow K$ receptor (no significant difference). CCK-8-induced IP production is expressed as a fraction of the total cellular tritium content that was incorporated during overnight exposure to [^{3}H]myo-inositol (tritiated inositol phosphates/total tritium incorporated). (*B*) PD135,158 and L-740,093 (S) efficacies are increased when assessed with the mutant vs. the wild-type receptor. Signaling is normalized to the CCK-8-induced maximum (assigned an efficacy value of 100%). Data represent the means of eight independent experiments ± SEM. Significance vs. corresponding wild-type receptors: **, $P < 0.01$.

itous. Rather, as more synthetic agonists for peptide hormone receptors are discovered, the occurrence of interspecies differences in drug efficacy will likely emerge as a common theme, and thus raise another important consideration in the selection of animal models for preclinical drug testing.

The differences in ligand efficacy observed with the human, murine, and canine CCK-BRs are in large part explained by species-dependent variability in TMD VI. It remains speculative how these minor structural changes in the receptor affect ligand function. The chemical nature of the relevant aliphatic amino acids, valine and leucine, makes these residues unlikely sites of direct ligand-receptor interaction. Furthermore, a rhodopsin-based model of G-protein coupled receptor TMD structure predicts that the residues corresponding to amino acids ^{340}V and ^{344}L in the human protein (residues ^{346}L and ^{350}V in the mouse) project away from a putative CCK-BR ligand binding pocket (20, 23). The orientation of these residues further suggests that at least two of the substituted amino acids that alter efficacy do not make direct contact with either PD135,158 or L-740,093 (S). In light of these findings, it is likely that the species-dependent changes in amino acid sequence lead to subtle conformational changes in the receptor which in turn either facilitate or inhibit ligand-induced second messenger signaling.

In contrast to the pharmacologically relevant interspecies polymorphisms between human and mouse, it is of note that the residue that confers efficacy differences between the human and canine receptors (^{349}V vs. ^{355}L, respectively) is modeled to project into the putative ligand binding pocket. It is possible that this species-dependent polymorphism alters ligand efficacy either by directly influencing ligand receptor interaction or by also inducing a subtle conformational change. Independent of the underlying mechanism, our data provide evidence that TMD polymorphisms, both within and outside of the putative ligand binding pocket (20, 23), have the potential to affect drug efficacy.

As a clinically important consideration, our observations raise the question of whether naturally occurring amino acid differences in the human receptor may similarly affect the ability of drugs to trigger second messenger signaling. Although to date no human polymorphisms have been found in TMD VI, there is a report of a single amino acid substitution within the third intracellular loop, $^{288}E \rightarrow K$ (24). When generated by site-directed mutagenesis and studied *in vitro*, this naturally occurring variant of the human CCK-BR doubled the efficacy of both PD135,158 and L-740,093 (S) (Fig. 4). Based on this finding, it is intriguing to speculate that human receptor polymorphisms may contribute to interindividual differences in receptor-mediated drug effects.

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