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Ligand Function at Constitutively Active Receptor Mutants Is Affected by Two Distinct Yet Interacting Mechanisms

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ABSTRACT

It has been proposed that mutations that induce constitutive activity in G-protein-coupled receptors (GPCRs) concomitantly enhance the ability of partial agonists to trigger second-messenger signaling. Using the cholecystokinin type 2 receptor (CCK-2R) as a model system, we have explored whether this association applies to a diverse set of activating mutations. Consistent with established principles, constitutively active CCK-2Rs resulting from amino acid substitutions within the third intracellular loop each systematically increased partial agonist activities versus corresponding wild-type values. In contrast, activating mutations within transmembrane domain segments near the extracellular loops led to an increase in efficacy of only a subset of compounds but decreased or did not change the function of others. When transmembrane domain amino acid substitutions were introduced in combination with intracellular amplifying mutations, observed changes in ligand activity were defined by the product of two discernible factors 1) systematic amplification caused by an equilibrium shift from the inactive to the active receptor conformation and 2) ligand-specific alterations in signaling, which probably result from mutation-induced changes in the putative binding pocket. These findings illustrate functional heterogeneity among GPCR mutants with ligand-independent signaling. A subgroup of activating mutations facilitates receptor isomerization to the active state and in parallel perturbs ligand receptor interactions. These mutants do not adhere to the previously proposed "hallmark criteria" of constitutive activity.

G-protein-coupled receptors (GPCRs) are seven transmembrane domain proteins that regulate a broad range of cellular functions. It is now well established that GPCR activation may result from either the binding of an agonist or as a consequence of mutations within the receptor molecule (Gether, 2000). The identification of mutations that trigger ligand-independent signaling has supported the concept that receptor stimulation reflects a change in the equilibrium between putative inactive and active protein conformations (Kenakin, 2002). Experimental findings, as well as mathematical models that accommodate these observations, suggest that mutations that trigger ligand-independent signaling simultaneously induce predictable alterations in ligand receptor interactions (Samama et al., 1993). In fact, mutation-induced pharmacological changes follow defined rules that are considered hallmark features of constitutively active receptor variants (Tiberi and Caron, 1994). These features include 1) increased affinity of agonists, and 2) enhanced efficacy of partial agonists, when respective values at mutant versus corresponding wild-type GPCRs are compared.

In addition to constitutively active GPCR mutants that are generated by in vitro engineering, such variants also occur in nature as a cause of human disease (Seifert and Wenzel-Seifert, 2002). It has been proposed that disorders resulting from activating mutations may be treated by a new class of therapeutic agents, termed inverse agonists, which can attenuate ligand-independent signaling (Milligan et al., 1995; Strange, 2002). In addition to their potential to cause disease, constitutively active GPCRs may be expected to alter drug response because of the increase in agonist affinity and efficacy that is predicted at such mutants. With the emerging field of pharmacogenomics, it is of considerable interest to

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ABBREVIATIONS: GPCR, G protein-coupled receptor; CCK-2R, cholecystokinin type 2 receptor; CCK-8, cholecystokinin octapeptide; L-365,260, 3*R*-(+)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl)-*N*1-(3-methylphenyl)urea; L-740,093, [*N*-(3*R* or 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]; PD-135,158,4-[[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*-1α,2β[*S**(*S**)4α]]butanoate-*N*-methyl-D-glucamine; YM022, (*R*)-1-[2,3-dihydro-1-(2′-methylphenacyl)-2-oxo-5-phenyl-1*H*-1,4-benzodiazepin-3-yl]-3- (3-methylphenyl)urea; L-364,718, *N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1H-1,4-benzodiazepin-3-yl)1H-2-carboxamide.

define themes that underlie polymorphism-induced alterations in drug effects (Kopin et al., 2000). The current study was designed to assess whether the presumed "hallmark" features of constitutively active GPCR mutants, as outlined above, are generally applicable to a variety of receptors which demonstrate ligand-independent signaling.

To investigate the pharmacological changes that are associated with constitutive activity, we used the cholecystokinin-2 receptor (CCK-2R) as a model system. The CCK-2R is endogenously stimulated by either of two peptide agonists, cholecystokinin octapeptide (CCK-8) or gastrin (Noble et al., 1999). This receptor regulates important physiological functions in both the gastrointestinal tract and the central nervous system. CCK-2R–modulated processes include gastric acid secretion and mucosal growth, as well as the perception of pain. Given the longstanding interest in the development of drugs that target the CCK-2R, a broad range of nonpeptide ligands, including agonists, antagonists, and inverse agonists, have been identified (Beinborn et al., 1998b; de Tullio et al., 2000; Kopin et al., 2003). Using selected compounds, we have previously demonstrated that a prototype constitutively active mutant of the CCK-2R (L325E) shows a systematic increase in ligand affinities and efficacies versus respective values at the wild-type receptor (Beinborn et al., 1998b). The pharmacological characteristics of the L325E variant were thus consistent with the classic hallmark features as established for nonpeptidergic constitutively active GPCRs (Tiberi and Caron, 1994).

Herein, we report the identification and characterization of a series of additional constitutively active CCK-2R mutants that result from amino acid substitutions in different receptor domains. Comparative analysis versus the wild-type CCK-2R revealed that in certain constitutively active variants, compound-dependent changes in receptor-ligand interactions occur in parallel with mutation-induced shifts in the equilibrium between inactive and active receptor conformations. Consequently, overall alterations in ligand properties at these mutants do not fully adhere to the systematic pattern that has been defined as a feature typical of previously characterized constitutively active receptors.

Materials and Methods

Materials. Cell culture media and fetal calf serum were obtained from Invitrogen (Carlsbad, CA) and from Intergen (Purchase, NY), respectively. Bolton-Hunter conjugated ^{125}I-CCK-8 (2200 Ci/mmol) and [myo-^{3}H]inositol (45–80 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Unlabeled CCK-8 (sulfated form) and gastrin heptadecapeptide (unsulfated form) were obtained from Bachem (Torrance, CA). Compounds L-365,260, L-740,093 (*R* and *S* forms), YM022, and L-364,718 (Beinborn et al., 1998b) were generously provided by Merck Sharp and Dohme Research Laboratories (Harlow, UK). The 'peptoid' compound PD-135,158 (Beinborn et al., 1998a) was a gift from Parke-Davis Research Center (Cambridge, UK).

Generation of Mutant Receptors. Constitutively active receptor variants were identified in the course of mutagenesis studies aimed at defining the role of amino acids that either project into the putative CCK-2R binding pocket (Kopin et al., 1995) or reside in the carboxyl-terminal portion of the third intracellular loop, a domain that seems to play an important role in receptor activation (Beinborn et al., 1998b). Each of the mutants was generated by modifying a pcDNA 1.1 construct encoding the wild-type human CCK-2R (Lee et al., 1993). CCK-2R variants were generated by oligonucleotide-directed, site-specific mutagenesis, as described previously (Beinborn et al., 1993). Oligonucleotides of interest were synthesized at the Tufts University DNA Synthesis Core Facility (Boston, MA). Introduction of desired mutations was confirmed by restriction enzyme analyses followed by dideoxynucleotide sequencing of the entire protein-coding region using an automated ABI 37X DNA sequencer (Applied Biosystems, Foster City, CA).

Binding Experiments. COS-7 cells (1×10^6) were plated onto 10-cm culture dishes (Costar, Cambridge, MA) and grown overnight in Dulbecco's modified Eagle's medium/10% fetal calf serum at 37°C. The cells were transfected with 5 μg of wild-type or mutant CCK-2R cDNA. The following day, COS-7 cells were seeded into 24-well dishes (Costar) at a density of 5 to 25×10^3/well. After an additional 24 h, competition binding experiments were performed in Hank's balanced salt solution supplemented with 25 mM HEPES, pH 7.3, 0.2% bovine serum albumin, and 0.15 mM phenylmethylsulfonyl fluoride, using 20 pM ^{125}I-CCK-8 as the radioligand. After an 80-min incubation in the absence or presence of unlabeled ligands, cell monolayers were washed three times with Hank's balanced salt solution and then hydrolyzed in 1 N NaOH for γ-counting. Binding affinities were calculated by nonlinear curve fitting using Prism software version 3.0 (GraphPad, San Diego, CA). Receptor densities were calculated from homologous ^{125}I-CCK-8 competition binding experiments (versus unlabeled CCK-8) using the MacLigand software package (Kell for Windows Version 6; Biosoft, Cambridge, UK).

Measurement of Inositol Phosphate Accumulation. Twenty-four hours after transfection, COS-7 cells were split into 12-well plates (2×10^5 /well) (Nalge Nunc, Naperville, IL). The cells were then prelabeled overnight with 3 μCi/ml [myo-^{3}H]inositol in serum-free Dulbecco's modified Eagle's medium. To assess ligand-induced inositol phosphate production, the medium was replaced with phosphate-buffered saline containing 10 mM LiCl, and cells were incubated with the respective ligands for 60 min at 37°C. Ligands were assessed at saturating concentrations (i.e., 3×10^{-7} M for CCK-8, 10^{-6} M for gastrin, or 10^{-5} M for nonpeptide compounds). These concentrations were adjusted to exceed the lowest corresponding affinity at any of the tested receptors (see Fig. 3) by at least 15-fold. After incubation, cells were lysed and extracted with methanol/chloroform; the upper phase was analyzed for inositol phosphates by strong anion exchange chromatography. Inositol phosphate production was expressed as a fraction of the total cellular tritium content that was incorporated during an overnight exposure to [myo-^{3}H]inositol.

Statistics. In experiments assessing the function of multiple compounds, ligand affinities/efficacies at different receptor variants were compared by analysis of variance and Tukey-Kramer post tests. Dunnett post tests were used for comparing a single function (basal activity) at mutant CCK-2Rs versus the wild-type protein (control). Calculations were performed using the Instat software package (GraphPad).

Results

The current study examines how different activating mutations in the CCK-2R alter drug-induced signaling. When expressed in COS-7 cells, each of the mutants shown in Fig. 1 triggered comparable inositol phosphate production when stimulated with saturating concentrations of either of the endogenous full agonists, CCK-8 or gastrin (Fig. 2, right). Observed levels of peptide-stimulated signaling at the mutant receptors fell within a narrow range (i.e., between 1.0- and 1.3-fold of the wild-type level). Consistent with this finding, the mutant receptors also showed similar expression levels, ranging between 0.6- and 1.5-fold of the wild-type value (see Fig. 3). In contrast to the wild-type CCK-2R, each of the mutants showed some degree of ligand-independent (i.e., constitutive) activity (Fig. 2, left). Respective levels of

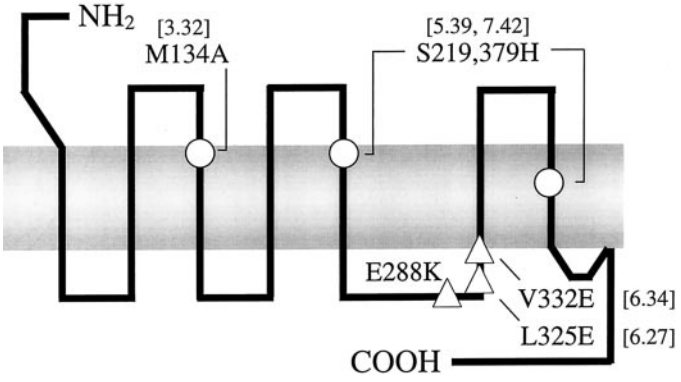


Fig. 1. Two-dimensional diagram of the CCK-2 receptor illustrating the positions of amino acid substitutions which confer constitutive activity. The black line represents the receptor protein; the cell membrane is shown as a shaded horizontal bar. ○, activating mutations within the receptor transmembrane domains; △, activating mutations located within the third intracellular loop. Note that one of the constitutively active receptor variants results from a double amino acid substitution, S219H in transmembrane domain 5 and S379H in transmembrane domain 7. The positions of mutations that project either within or adjacent to transmembrane domains are also defined (in square brackets) using the Ballesteros and Weinstein (1995) nomenclature. NH_2, amino terminus; COOH, carboxyl terminus.

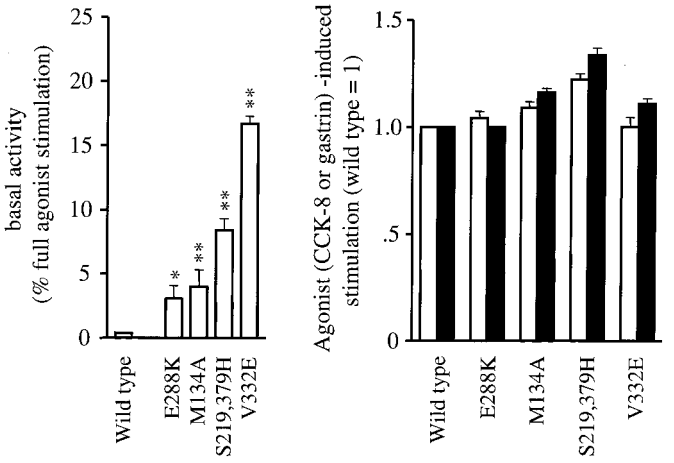


Fig. 2. Mutant CCK-2 receptors trigger constitutive activity yet have comparable agonist-induced function as the wild-type protein. Basal and ligand-induced production of inositol phosphates was assessed in COS-7 cells expressing recombinant receptors. Inositol phosphate production ([^{3}H]inositol phosphate/total incorporated tritium), before normalization (see below), was as follows: vector-transfected cells (no receptor expression), 0.025 ± 0.001); wild-type CCK-2R, basal, 0.027 ± 0.002; CCK-8 (3×10^{-7} M)-stimulated, 0.270 ± 0.009; gastrin (10^{-6} M)-stimulated, 0.283 ± 0.013; no significant difference between CCK-8 and gastrin stimulation by two-sided t test. Left, in contrast to the wild-type CCK-2 receptor, mutant proteins show significant levels of ligand-independent signaling. Values were corrected for signaling observed in vector-transfected cells and normalized to the agonist-induced maximum at the corresponding receptor (100%; see right). Significance of basal signaling versus wild-type, *, $p < 0.05$; **, $p < 0.01$. Right: the full agonists CCK-8 and gastrin stimulate the wild-type and mutant receptors to a similar extent. There is no significant difference, at any receptor, between respective efficacies of these two peptides. CCK-8- and gastrin-induced signaling (□ and ■, respectively) were normalized to the corresponding value at the wild-type CCK-2 receptor. Data represent means ± S.E.M. of three or more independent experiments.

basal signaling by individual receptor variants ranged from 3% (E288K) to 17% (V332E) of the hormone-induced maximum.

Compared with the wild-type CCK-2R, assessment of small molecule activity at either mutant V332E or E288K resulted in a systematically enhanced response (i.e., one that correlated with the level of ligand activity at the wild-type receptor). The amplification of ligand efficacy at the mutant receptors is illustrated in Fig. 4 using a series of CCK-2R ligands that had different activities at the wild-type receptor. With each of the compounds, L-364,718, L-365,260, PD-135,158, and (*S*)-L-740,093, a systematic increase in ligand activities was observed at the mutant receptors versus corresponding values at the wild-type CCK-2R. In addition, a significant reduction in basal activity was evident with compound (*R*)-L-740,093 at mutant V332E, consistent with the classification of this ligand as a weak inverse agonist (Beinborn et al., 1998b). In contrast to the mutation-induced changes observed with other compounds, YM022 showed no intrinsic activity at either the wild-type or at any of the constitutively active receptors, consistent with the prior classification of this ligand as a neutral antagonist (Beinborn et al., 1998b).

A systematic amplification of ligand efficacies is considered one of the hallmark features of constitutively active GPCRs (Tiberi and Caron, 1994). However, in contrast to findings with V332E and E288K or with a previously described constitutively active CCK-2R mutant, L325E (Beinborn et al., 1998b), we noted that this principle did not apply to two other receptor variants with ligand-independent signaling, M134A and S219,379H. Rather than amplifying ligand function, these mutants (location is shown in Fig. 1) induced differential effects on the signaling properties of individual compounds, most notably those of PD-135,158, and (*S*)-L-740,093 (Fig. 5A). Mutant M134A increased the activity of (*S*)-L-740,093 (versus activity of this compound at the wild-type receptor) but decreased the function of PD-135,158. In contrast, mutant S219,379H selectively amplified the function of PD-135,158 (versus wild-type) but markedly reduced (*S*)-L-740,093–induced signaling.

The differential changes in ligand function observed at the constitutively active mutants M134A and S219,379H were reminiscent of the pattern of pharmacological changes that occur with a different CCK-2R mutant, N353L. The N353L substitution [transmembrane domain location is 6.55 according to the commonly used indexing system (Ballesteros and Weinstein, 1995)] does not concomitantly induce ligand-independent signaling but probably affects ligand interactions within the putative CCK-2R transmembrane domain binding pocket (Bläker et al., 1998). In the current study, the N353L mutant was assessed using the same panel of ligands as examined with the M134A and S219,379H mutants (Fig. 5B). Whereas substitution of asparagine 353 with a leucine residue led to parallel efficacy decreases of compounds PD-135,158 and (*S*)-L-740,093 versus corresponding values at the wild-type CCK-2R, this mutation markedly increased the efficacy of another ligand, L-365,260.

We hypothesized that the differential alterations in ligand function by a subset of constitutively active receptors may reflect the composite of two mutation-induced effects. Observed changes may result from 1) a generally enhanced tendency of receptor variants to undergo ligand-induced activation (which should uniformly affect all ligands) in conjunction with 2) mutation-induced alterations in receptor domains that variably influence the function of individual compounds. To explore this possibility, we generated and

	CCK-8				Gastrin				PD-135,450				L-365,260			
	pK_i	n	K_i	Ratio	pK_i	n	K_i	Ratio	pK_i	n	K_i	Ratio	pK_i	n	K_i	Ratio
Wild type	10.14 ± 0.05	24	0.073	1	9.15 ± 0.06	18	0.71	1	8.71 ± 0.05	14	1.9	1	8.42 ± 0.05	20	3.8	1
V332E	10.67 ± 0.17	9	0.021	**3.4**	9.89 ± 0.11	4	0.13	**5.5**	9.53 ± 0.11	6	0.30	**6.6**	8.51 ± 0.05	7	3.1	1.2
E288K	10.46 ± 0.06	3	0.035	**2.1**	9.60 ± 0.08	3	0.25	**2.8**	9.04 ± 0.06	3	0.92	**2.1**	8.75 ± 0.33	3	1.8	**2.1**
M134A	10.06 ± 0.02	3	0.088	0.8	8.78 ± 0.06	3	1.6	0.4	7.84 ± 0.04	3	14	**0.1**	6.80 ± 0.05	3	159	**0.02**
S219,379H	9.17 ± 0.17	3	0.67	**0.1**	7.90 ± 0.08	3	13	**0.06**	7.67 + 0.07	7	21	**0.1**	6.54 ± 0.13	3	290	**0.01**
S219,379H+L325E	9.61 ± 0.14	3	0.25	**0.3**	8.50 ± 0.13	3	3.2	**0.2**	8.18 ± 0.03	7	6.6	**0.3**	6.45 ± 0.14	3	360	**0.01**
N353L	9.87 ± 0.13	3	0.14	0.5	8.44 ± 0.14	4	3.6	**0.2**	6.64 ± 0.13	3	230	**0.01**	7.01 ± 0.03	3	97	**0.04**
N353+L325E	10.26 ± 0.04	3	0.054	1.3	9.51 ± 0.18	4	0.31	2.3	6.93 ± 0.02	3	120	**0.02**	7.46 ± 0.32	3	35	**0.1**

	(*S*)-L-740,093				(*R*)-L-740,093				L-364,718				YM022			
	pK_i	n	K_i	Ratio	pK_i	n	K_i	Ratio	pK_i	n	K_i	Ratio	pK_i	n	K_i	Ratio
Wild type	7.76 ± 0.06	14	18	1	9.82 ± 0.07	10	0.15	1	6.92 ± 0.09	13	120	1	10.07 ± 0.10	8	0.086	1
V332E	7.96 ± 0.06	7	11	1.6	9.88 ± 0.05	6	0.13	1.1	6.97 ± 0.07	6	110	1.1	10.10 ± 0.11	6	0.079	1.1
E288K	7.80 ± 0.08	3	16	1.1	9.87 ± 0.18	3	0.13	0.9	7.25 ± 0.26	5	57	2.1	9.92 ± 0.04	3	0.12	0.7
M134A	7.24 ± 0.14	3	58	**0.3**	N.D.				N.D.				N.D.			
S219,379H	6.39 ± 0.05	6	400	**0.04**	8.37 ± 0.19	3	4	**0.04**	7.56 ± 0.11	4	27	**4.4**	8.65 ± 0.08	5	2.2	**0.04**
S219,379H+L325E	6.20 ± 0.09	5	630	**0.03**	7.95 ± 0.06	5	11	**0.01**	6.99 ± 0.09	5	100	1.2	8.78 ± 0.16	5	1.6	**0.05**
N353L	7.09 ± 0.04	3	82	**0.2**	7.34 ± 0.15	5	46	**0.003**	6.78 ± 0.05	3	170	0.7	8.66 ± 0.05	4	2.2	**0.04**
N353L+L325E	7.53 ± 0.03	3	29	0.6	7.36 ± 0.11	3	44	**0.003**	6.65 ± 0.14	5	220	0.5	8.57 ± 0.10	4	2.7	**0.03**

Fig. 3 Ligand affinities at the wild type and mutant CCK-2Rs. pK_i values are given as means ± S.E.M. K_i values are given in nanomolar. Under Ratio, affinity versus wild-type receptor, bolded and underlined values indicate that respective pK_i values are significantly different from wild type ($p < 0.05$ by analysis of variance). N.D., not determined. Receptor expression levels as calculated from homologous ^{125}I-CCK-8 competition binding experiments were as follows (all values in femtomoles per 10^4 cells): wild-type, 6.3 ± 0.2; V332E, 5.7 ± 0.1; E288K, 5.4 ± 0.8; M134A, 9.7 ± 1.9; S219,379H, 5.9 ± 0.9; S219,379H+L325E, 7.5 ± 0.8; N353L, 5.7 ± 2.0; N353L+L325E, 4.0 ± 0.6.

characterized two additional CCK-2R variants. In these receptors, an activating mutation that results in systematic amplification of ligand function (L325E) was introduced in parallel with a second alteration that modifies the ligand activity profile in a compound-dependent manner. Two different examples for the second type of mutation were included in these studies, one that does concomitantly induce ligand-independent signaling (S219,379H; Fig. 5A) and one that does not (N353L; Fig. 5B). Based on the assumption that two distinct yet interacting mechanisms work in concert to effect ligand-induced receptor stimulation, one would anticipate that in composite receptor constructs that include the specified combinations of amino acid substitutions, the amplifying mutation (L325E) will systematically enhance function but preserve the ligand activity profile that results from a concurrent mutation. This prediction was assessed by comparing mutation-induced functional changes that applied to two nonpeptide ligands, (*R*)-L-740,093 and L-364,718. These compounds were selected for further comparison because each had opposite effects at the N353L mutant (trace agonism) versus the S219,379H variant (trace inverse agonism). The introduction of an additional L325E substitution in either of the N353L or S219,379H templates enabled us to test the prediction that the opposite ligand activities at either of these receptors would be qualitatively conserved but quantitatively enhanced because of systematic amplification.

Data supporting the composite profile hypothesis are shown in Fig. 6. For compound (*R*)-L-740,093, introduction of L325E substitution into mutants N353L or S219,379H leads to markedly enhanced partial agonist or inverse agonist function, respectively (Fig. 6A). Amplification of (*R*)-L-740,093 function at the mutant receptors thus paralleled the increase in inverse agonist activity of this compound when the L325E substitution was introduced into the wild-type CCK-2R template. A similar pattern of systematic amplification at different receptor templates was also observed for compound L-364,718 (Fig. 6B). Again, comparison of the activities of this compound at different CCK-2R variants which either lack (Fig. 6B, ■) or include the L325E substitution (Fig. 6B, ▨) reveals that the latter mutation enhances the function of L-364,718 but does not alter its classification as either a trace partial or inverse agonist at respective receptor templates.

The finding that systematic amplification of ligand function by certain activating mutations can occur independently of the receptor template on which such mutations are introduced is further illustrated in Fig. 7. This analysis was performed with an extended series of functionally distinct compounds; each was assessed at respective mutants using saturating concentrations, as described in Fig. 4. For these ligands, activity coordinates were defined by the signaling induced at a given receptor template (x-axis) versus the signaling induced at the respective template when combined with a systematically amplifying mutation (y-axis). This analysis was first performed with the wild-type CCK-2R template, where ligand activities (x-axis) were plotted versus corresponding activities at the systematically amplifying L325E mutant (y-axis). The resulting data points follow a rectangular hyperbolic curve (Fig. 7, dashed line). When the activities of the same ligands were compared at the wild-type CCK-2R (x-axis) versus corresponding values at the V332E

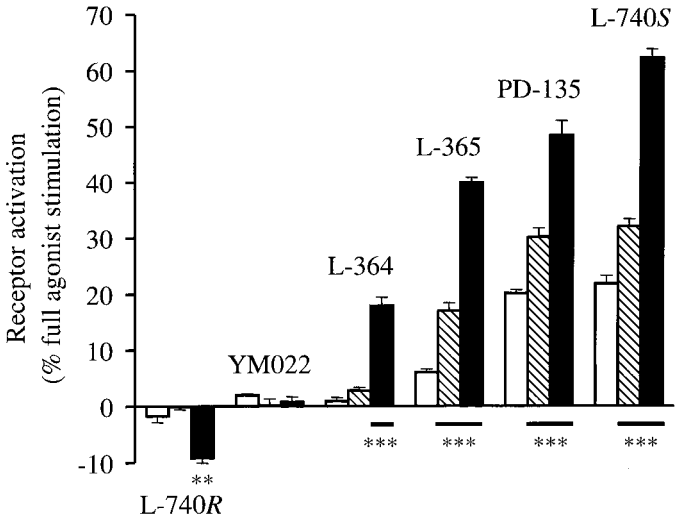


Fig. 4. The constitutively active mutants E288K and V332E systematically amplify ligand efficacies versus corresponding values at the wild-type CCK-2 receptor. Receptors were expressed in COS-7 cells. Ligand-induced production of inositol phosphates was compared at the wild-type receptor (□) versus the E288K (▨) and V332E mutants (■). At each receptor, ligand-induced signaling at saturating concentrations (10^{-5} M) was normalized relative to the corresponding basal activity (0%) and to the peptide-induced maximum (100%; see Fig. 2 for respective basal and CCK-8- or gastrin-stimulated values). Data represent means ± S.E.M. of three or more independent experiments. Significance of ligand function versus the corresponding value at the wild-type receptor, **, $p < 0.01$; ***, $p < 0.001$.

mutant (*y*-axis), respective data points (Fig. 7, ■) fell close to the previously defined curve. This observation suggests that the amplifying mutations L325E (Beinborn et al., 1998b) and V332E (current study) enhance ligand function to a similar extent.

Furthermore, we noted that when ligand activities at either of two mutant receptor templates (N353L or S219,379H) were plotted versus corresponding activities at combination constructs in which an additional L325E substitution was introduced (Fig. 7, ○ and ●, respectively), respective data points were also superimposable on the previously defined curve. The predictable mathematical relationship between ligand activities at different template receptors (wild-type, N353L, S219,379H) versus activities with addition of a mutation that results in systematic amplification of ligand-induced signaling supports the view that the latter component enhances but does not otherwise interfere with the compound-dependent mechanisms of receptor stimulation.

Assessment of CCK-2R ligand binding (Fig. 3) revealed a trend toward higher agonist affinities (up to 6.6-fold) when comparing the dissociation constants at the two systematically amplifying mutants (i.e., V332E and E288K) with those at the wild-type receptor. It is of note that the affinities of compounds with lowest intrinsic activities [(*R*)-L-740,093, L-364,718, YM022; see Fig. 4] were not significantly changed at either of the mutant receptors (versus wild-type values). The trend toward higher agonist affinities observed with the V332E and E288K receptors was not found with any of the other constitutively active CCK-2R mutants that were analyzed. In the latter group of mutants (i.e., M134A and S219,379H), most ligand affinities were lower compared with corresponding wild-type values; however, the magnitudes of observed shifts differed widely among individual compounds and the receptor variants at which they were studied. Furthermore, within the latter set of data, no correlation between affinity shifts and functional ligand activities was apparent. For composite mutants (S219,379H + L325E and N353L + L325E), there was a trend toward higher agonist affinities (i.e., for CCK-8, gastrin, and PD-135,450) when the dissociation constants of individual ligands were compared with corresponding values at respective 'template' receptors that did not include the L325E substitution (i.e., S219,379H and N353L).

Discussion

Mutation-induced signaling of G-protein coupled receptors has been ascribed to a shift from an 'inactive' to an 'active' conformation (Gether, 2000). Comprehensive pharmacological analysis of a constitutively active β-adrenoceptor mutant, one of the earliest examples in which ligand-independent signaling was observed, suggested an 'extended ternary model' of interactions between the receptor, ligand, and G-protein (Lefkowitz et al., 1993). Several hallmark features

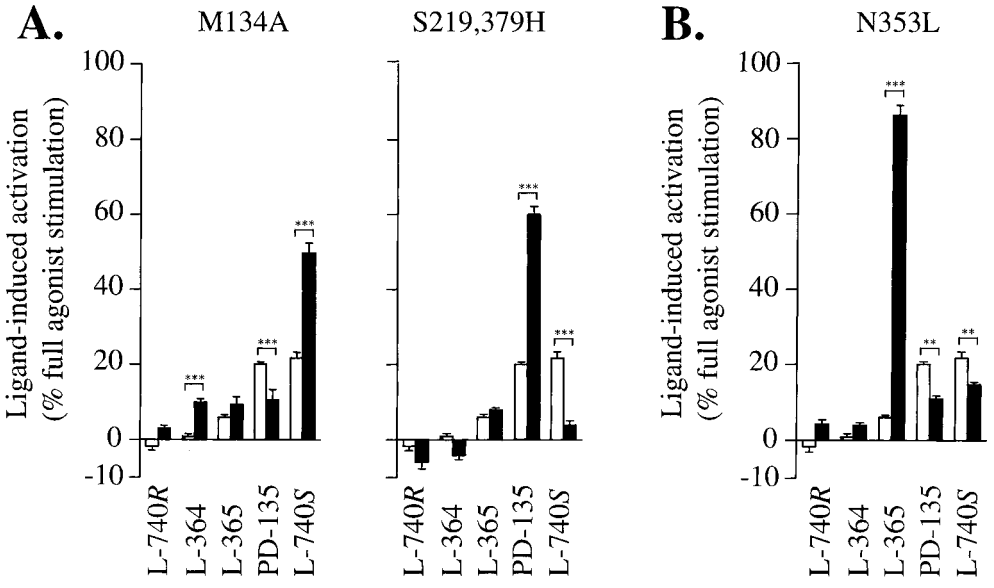
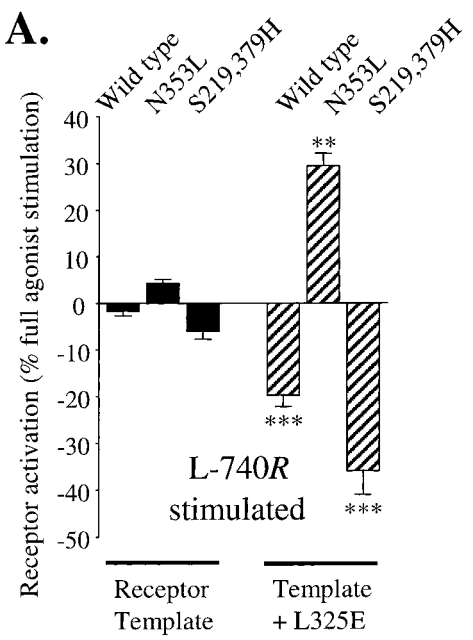


Fig. 5. The constitutively active mutants M134A and S219,379H, as well as a mutant that lacks constitutive activity, differentially alter ligand efficacies versus corresponding values at the wild-type CCK-2 receptor. Ligand-induced production of inositol phosphates was assessed in COS-7 cells expressing recombinant receptors. A and B, signaling in the presence of the indicated ligand was compared between the wild-type receptor (□) and the specified mutant (■). At each receptor, ligand-induced signaling was assessed at saturating concentrations (10^{-5} M) and normalized relative to the corresponding basal activity (0%) and to the peptide-induced maximum (100%; see Fig. 2 for respective basal and CCK-8 or gastrin-stimulated values). Ligand-induced signaling was assessed at two constitutively active mutants (A) as well as at CCK-2R mutant N353L (B), which lacks constitutive activity (Blaker et al., 2000). Data represent means ± S.E.M. of three or more independent experiments. Significance of ligand function versus the corresponding value at the wild-type receptor, ***, $p < 0.001$.



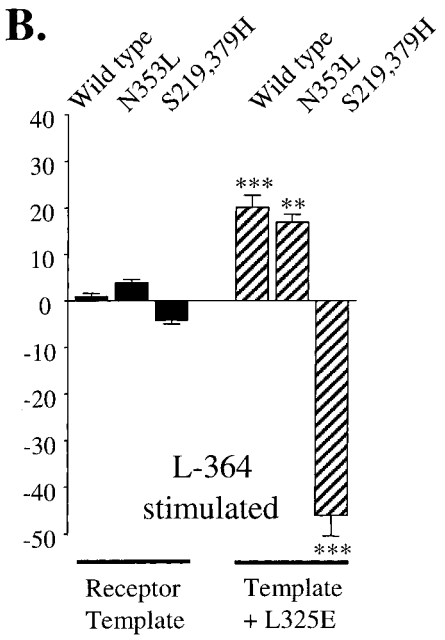


Fig. 6. Addition of the L325E substitution results in systematic amplification of ligand efficacies at different CCK-2 receptor variants. Receptors were expressed in COS-7 cells. Ligand-induced inositol phosphate production was compared at CCK-2 receptor variants, either without (■) or with addition of an L325E substitution (▨). At each receptor, either (*R*)-L-740,093- (A) or L-364,718–induced signaling (B) was assessed at saturating ligand concentrations (10^{-5} M). Ligand function at each receptor was normalized relative to the corresponding basal activity (0%) and to the peptide-induced maximum (100%). Mutant and wild-type CCK-2Rs had comparable levels of CCK-8 (3×10^{-7} M)- and gastrin (10^{-6} M)-induced signaling (not shown). Data represent means ± S.E.M. of three or more independent experiments. Significance of ligand function at combination mutants versus the corresponding value at the indicated template receptor, **, $p < 0.01$; ***, $p < 0.001$. WT, wild type.

were predicted based on this model that distinguish constitutively active mutants from corresponding wild-type receptors. These characteristics include 1) an increase in ligand affinity that correlates with compound function (i.e., the largest shifts occur with full agonists, whereas antagonists maintain comparable affinities at the mutant versus wild-type receptors), and 2) a systematic amplification of partial agonist activity at the mutant versus the corresponding wild-type receptors (Tiberi and Caron, 1994). After the original description of these features in the adrenoreceptor system, applicability of these principles to a limited number of other constitutively active G-protein coupled receptor mutants including the CCK-2R L325E variant has been demonstrated (Herrick-Davis et al., 1997; Beinborn et al., 1998b; Egan et al., 1998; Morin et al., 1998).

The current study sought to address the extent to which these hallmark pharmacological features apply to other constitutively active receptors. Our analysis of CCK-2R mutants suggests that only a subset of receptors with ligand-independent signaling (discussed here as type I) strictly adheres to the expected criteria, whereas other mutants (classified as type II) result in compound-dependent changes in ligand activity/affinity. Consistent with established principles, mutants V332E and E288K lead to an amplification of partial agonist activity versus wild-type values and on this basis were categorized as class I. In contrast, mutants assigned to class II (i.e., M134A and S219,379H) despite being constitutively active, either increase or decrease the function of individual ligands in an unpredictable, compound-dependent manner.

The different pharmacological characteristics of class I versus class II mutations may be attributed to the location of respective amino acid substitutions within the CCK-2R protein. The type I mutations explored in this work result from substitutions of amino acids that cluster in the carboxy-terminal portion of the third intracellular loop receptor domain. There is precedent from several other GPCRs that sequence alterations in this region, adjacent to transmembrane domain 6, may either attenuate agonist-induced signaling (Kosugi et al., 1992; Shapiro et al., 1993; Parent et al., 1996; Wang, 1997a,b) or result in constitutive activity (reviewed by (Pauwels and Wurch, 1998). Together, these findings suggest that the carboxyl-terminal portion of intracellu-

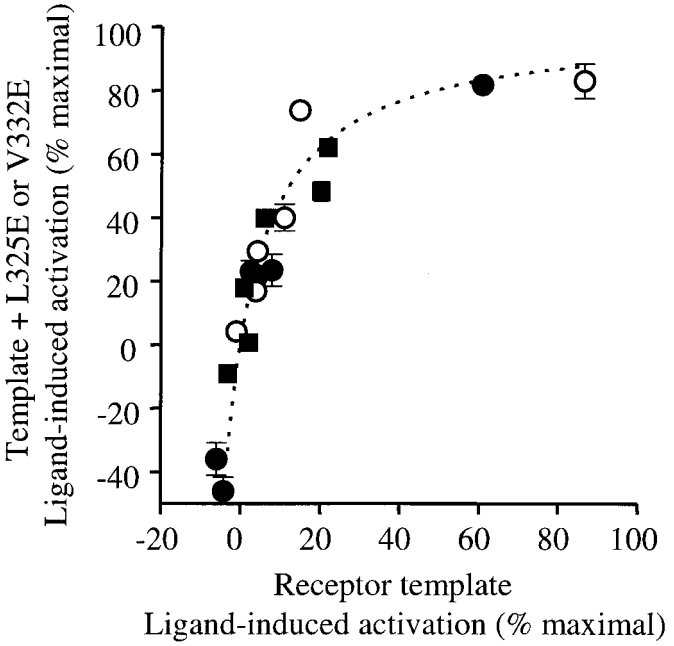


Fig. 7. Amplifying mutations enhance ligand activity independent of the CCK-2 receptor template. Ligand-induced production of inositol phosphates was assessed in COS-7 cells expressing recombinant receptors. Signaling induced by selected ligands when assessed at saturating concentrations (10^{-5} M; see Fig. 4) was compared at CCK-2 receptor variants without (*x*-axis) or with (*y*-axis) the addition of a third intracellular loop amplifying mutation (L325E or V332E). Symbols represent values found with individual ligands at the following receptors: ■, wild-type CCK-2 receptor versus V332E; ○, N353L versus N353L+L325E; ●, S219,379H versus S219,379H+L325E. The dashed line indicates a previously reported rectangular hyperbolic correlation between compound activities at the wild-type receptor versus the L325E mutant (Beinborn et al., 1998b). Data represent means ± S.E.M. of three or more independent experiments with each compound at each receptor.

lar loop 3 plays an important role in receptor activation and in many GPCRs may be part of the trigger that enables receptor-mediated signaling. Several third intracellular loop mutations that lead to constitutive activity have been characterized with regard to changes in the activities and affinities of multiple ligands. Retrospectively, in the cases where such study has been pursued (Samama et al., 1993; Herrick-Davis et al., 1997; Hwa et al., 1997; Beinborn et al., 1998b; Egan et al., 1998; Wilson et al., 2001), the findings seem to be consistent with our definition of class I, systematic amplifiers.

In contrast to the shared intracellular location of class I mutants, all of the constitutively active CCK-2R variants that fall into class II result from substitution of transmembrane domain residues that are in close proximity of the extracellular receptor surface (Fig. 1). Furthermore, based on a three-dimensional model of transmembrane domain structure, the side chains of the latter amino acids project into a putative CCK-2R ligand pocket (Kopin et al., 1995). Residues Met134, Ser219, and Ser379, located in positions 3.32, 5.39, and 7.42 when mapped relative to highly conserved 'fingerprint' amino acids that are shared among rhodopsin-type G-protein coupled receptors (Ballesteros and Weinstein, 1995), fall on the same or immediately adjacent locations within the helical transmembrane domains that are well established affinity determinants for ligands in the human β2-adrenoceptor (Cascieri et al., 1995; Kurose et al., 1998; Sato et al., 1999). The latter correlation provides a plausible framework for understanding the diverse and compound-dependent changes that occur with amino acid substitutions of respective residues in the CCK-2R. Mutations in positions 3.32 (M134A), 5.39 (S219H), and 7.42 (S379H) are likely to affect receptor-ligand interactions in a manner that depends largely on structural elements of individual compounds. These changes may be superimposed on the tendency toward enhanced ligand binding and function that results from facilitated receptor isomerization toward the active state.

The above interpretation of class II mutants is supported by an analysis of receptor variants in which amino acid substitutions within the putative transmembrane domain binding pocket were introduced in parallel with a type I mutation. Comparison of individual versus combined mutations suggests that systematic amplification of ligand function by a type I mutation (i.e., L325E) occurs regardless of the 'pocket template' on which L325E was introduced (respective templates being either the wild-type, N353L, or S219,379H receptors). As a result, whatever the classification of an individual ligand (e.g., inverse agonist, partial agonist) as determined by the configuration of the transmembrane domain pocket, amplification of the resultant activity is observed with addition of a type I mutation (i.e., L325E; Figs. 6 and 7).

Our findings suggest that transmembrane domain mutations may affect receptor-ligand interactions by two concurrent mechanisms, changes in intermolecular receptor/ligand interactions and intramolecular shifts between different receptor conformations. Although both of these distinct elements are well established in the literature, we are not aware of prior reports in which a combination of these components has been demonstrated to determine ligand activity/affinity at certain constitutively active GPCRs. It is of note that a recent observation with the β2-adrenoceptor can be viewed as complementary evidence for the proposed interpretation. In the work by Del Carmine et al. (2002), it was found that mutations of residues within the putative binding pocket of the β2-adrenoceptor (S204,207A, predicted to project adjacent to our S219H mutation in the CCK-2R) decreased ligand affinities by shifting the receptor toward a less active conformation. For a subset of compounds that was investigated, the latter effect (i.e., loss of affinity secondary to a shift in receptor conformation) seemed to be superimposed with a mutation-induced disruption of direct receptor-ligand interactions. These composite effects were demonstrated with a β2-adrenoceptor variant that was shifted toward a less active state, and thus provide a complementary perspective of the mechanisms that apply to type II constitutively active CCK-2R mutants in the current study.

The principle of type II activating mutations, although illustrated here specifically on the basis of two CCK-2R mutants (M134A and S219,379H), is likely to have broader applicability. We are aware of a few reported examples of GPCR mutants with ligand-independent signaling in which the function of individual ligands was altered in a manner that did not conform with the typical "hallmark criteria" of constitutive activity. Two inverse agonists at the wild-type human bradykinin B2 receptor, which has slight yet detectable basal activity when expressed in COS-7 cells, were converted to partial agonists with introduction of an activating mutation in either transmembrane domain 3 or 6 (Marie et al., 1999). Furthermore, there is precedent in the literature in which mutation-induced constitutive activation of GPCRs led to affinity decreases of certain agonist ligands compared with wild-type values (Spalding et al., 1998; Zhang et al., 2002), rather than increases, as might have been predicted with receptor variants in a more active conformation. Again, the reported amino acid substitutions were introduced within the TMDs (i.e., outside the intracellular receptor portions). Although the range of compounds with which these mutants were analyzed and the scope of pharmacological characterization was generally limited, the apparent ligand-dependent nature of observed pharmacological changes suggests the retrospective classification of these constitutively active receptors as type II mutants.

Distinguishing subgroups of constitutively active receptors may be of practical significance for the recently proposed use of such mutants as tools for in vitro drug discovery (Chen et al., 2000). As originally demonstrated for the CCK-2R L325E mutant, constitutively active receptors provide a magnifying glass of ligand function (Beinborn et al., 1998b) and therefore have considerable utility in discovering agonist lead compounds (Kopin and Beinborn, 1998; Kopin, 2003). Once identified, these hits can then be further optimized into clinically useful drugs. In addition, constitutively active mutants facilitate the identification and optimization of 'neutral' antagonists that are functionally inert, as well as enable the discovery of inverse agonists that can attenuate the level of ligand-independent signaling. The current study suggests that constitutively active receptors that are classified as type I (e.g., L325E) will provide the best prediction of drug function at corresponding wild-type receptor isoforms. Type II constitutively active mutants, although in principle also facilitating ligand binding and ligand-induced receptor activation, have the potential to introduce unpredictable additional effects on a subset of selected compounds. One needs to be aware that these additional effects may either enhance or

offset the desired amplification of ligand activity, depending on the compound being assessed. Given this caveat, mutations that are spatially remote from likely sites of receptor-ligand interaction are therefore preferable for use in drug discovery.

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