Minor Modifications of a Cholecystokinin-B/Gastrin Receptor Non-peptide Antagonist Confer a Broad Spectrum of Functional Properties*  

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The development of non-peptide agonists for peptide hormone receptors would markedly expand the treatment options for a large number of diseases. However, difficulty in identifying non-peptide molecules which possess intrinsic activity has been a major obstacle in achieving this goal. At present, most of the known non-peptide ligands for peptide hormone receptors appear in standard functional assays to be antagonists. Here, we report that a constitutively active mutant of the human cholecystokinin-B/gastrin receptor, Leu325→Glu, offers the potential to detect even trace agonist activity of ligands which, at the wild type receptor isoform, appear to lack efficacy. The enhanced functional sensitivity of the mutant receptor enabled us to detect intrinsic activity of L-365,260, an established non-peptide antagonist for the cholecystokinin-B/gastrin receptor. Extending from this observation, we were able to demonstrate that minor structural modifications could convert L-365,260 into either: (i) an agonist or (ii) an inverse agonist (attenuates ligand-independent signaling). The ability to confer functional activity to small non-peptide ligands suggests that the properties of endogenous peptide hormones can be mimicked, and even extended, by considerably less complex molecules.

Current understanding of G-protein-coupled receptor activation has in large part been based on the study of biogenic amine receptors (1). The corresponding endogenous ligands, together with synthetic derivatives of these small molecules, cover a spectrum of functional activities ranging from full agonists to antagonists. With the discovery of constitutively active receptor mutants, this range has been further extended to include inverse agonists, distinguished by their ability to attenuate ligand-independent signaling (2).

Another major group of G-protein-coupled receptors is activated by endogenous peptide molecules. Compared with biogenic amines, these peptide agonists are significantly larger and structurally more complex. Since endogenous peptides exert important hormone and neurotransmitter functions, there is considerable interest in whether their function can be mimicked by non-peptide drugs (3). This possibility is suggested by the opioid receptor system. Naturally occurring opioid receptor non-peptide agonists (e.g. morphine) as well as synthetic derivatives have been utilized since the early 19th century (4). Over the last 10 years, numerous non-peptide compounds have been identified which recognize specific peptide hormone receptor subtypes with high affinity. Unlike the corresponding endogenous peptide agonists, the vast majority of these new ligands appear to lack intrinsic activity and have been pharmacologically classified as antagonists (5).

The difficulty in generating non-peptide agonists is exemplified by the extensive efforts which have focused on the identification of ligands for the human cholecystokinin-B/gastrin receptor (CCK-BR).1 This receptor has been implicated in modulating memory, anxiety, and pain perception, as well as in regulating gastrointestinal mucosal growth and secretion (6–8). With the exception of some peptide-derived compounds (9, 10), all of the synthetic CCK-BR non-peptide ligands which have been discovered to date are reported to be antagonists and thus appear unable to satisfy the structural requirements for receptor activation.

The prototype of such non-peptide, selective CCK-BR antagonists is L-365,260, a benzodiazepine-based ligand which was discovered in 1989 (11). Widely tested both in vivo and in vitro, this compound has become a cornerstone in the characterization and pharmacological classification of CCK receptors. We now report that L-365,260 has unexpected residual intrinsic activity, which we were able to detect using a constitutively active CCK-BR mutant. Extending from this finding, we demonstrate that slight structural modifications of L-365,260 convert this ligand into either: (i) a non-peptide agonist for the wild type CCK-BR, or (ii) an inverse agonist. These findings illustrate that existing non-peptide ligands may have considerably higher potential to activate peptide hormone receptors than was previously appreciated, and suggest that constitutively active receptor mutants provide promising tools to detect and optimize such functional properties.

EXPERIMENTAL PROCEDURES  

Materials—Cell culture media and fetal calf serum were obtained from Life Technologies, Inc. (Gaithersburg, MD) and from InterGen (Purchase, NY), respectively. 125I-CCK-8 (2,200 Ci/mmol) and myo-[3H]inositol (45–80 Ci/mmol) were purchased from NEN Life Science Products (Boston, MA). Unlabeled gastrin heptadecapeptide (G-17, unsulfated form), CCK-8 (sulfated form), CCK-8US (unsulfated form), and CCK-4 were obtained from Peninsula Laboratories (Belmont, CA). L-365,260, L-740,093 (R and S forms), YM022, and L-364,718 were generously provided by Wyeth Research Ltd. (Taplow, United Kingdom). The “peptoid” compounds PD-135,158 and PD-136,450 were a gift from Parke-Davis Research Center (Cambridge, UK). Measurement of Inositol Phosphate Accumulation—COS-7 cells (1 × 10⁶) were plated onto 10-cm culture dishes (Costar, Cambridge, MA) and grown overnight in Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, and 10% antibiotic–antimycotic solution. After 24 h, cells were stimulated for 30 min with either 10 nM CCK-8 (sulfated form) or 10 μM L-365,260 (both from Parke-Davis Research Center). Inositol phosphates were extracted and assayed as described previously (12).

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1 The abbreviations used are: CCK-BR, cholecystokinin-B/gastrin receptor; G-17, gastrin heptadecapeptide (unsulfated form); CCK-8, cholecystokinin octapeptide (sulfated form); CCK-8US, cholecystokinin octapeptide (unsulfated form).
calf serum at 37 °C. The cells were transfected with 5 μg of wild type or mutant CCK-BR cDNA (12), subcloned into the expression vector pcDNA3 (Invitrogen). The following day, cells were split into 6-well plates (3 × 10^4/well) (Nunc). The cells were then prelabeled overnight with 3 μCi/ml myo-[3H]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 30 min at 37 °C. Each ligand was tested at a concentration which was at least 25-fold higher than the corresponding dissociation constant (K, value) determined in radioligand binding experiments (see below, Table I). According to the simple Langmuir isotherm (fractional receptor occupancy = ligand concentration/Ligand concentration + K), the ligand concentrations which were utilized result in >95% receptor occupancy (13), and will thus induce near maximal receptor stimulation and inositol phosphate production. After incubation, cells were lysed and extracted with methanol/chloroform; the upper phase was analyzed for inositol phosphates by strong anion exchange chromatography (14). Inositol phosphate production was expressed as a percentage of the total cellular tritium content which was incorporated during an overnight exposure to myo-[3H]inositol. Concentration-response curves were calculated using the GraphPad Prizm software (GraphPad, San Diego, CA).

**Results and Discussion**

Molecular characterization of the third intracellular loop of the human CCK-BR led to the identification of a point mutation (Leu^325 → Glu) which results in constitutive receptor activity (17). When transiently expressed in COS-7 cells, the Leu^325 → Glu mutant triggers agonist independent production of inositol phosphates, to levels exceeding cells expressing the wild type receptor at similar densities (Fig. 1A). Despite this difference in ligand-independent signaling, the wild type and the mutant receptors increase inositol phosphate production to comparable levels when stimulated with saturating concentrations of either CCK-BR or G-17, two of the principal endogenous agonists for the CCK-BR (6, 7, 18). In addition, both CCK-8 and G-17 have similar potencies and binding affinities when respective values at the wild type and the mutant receptor isoforms are compared (Fig. 1B, Table I).

Contrary to expectation, we found that the non-peptide ligand, L-365,260, has significant partial agonist activity when stimulating the mutant (versus the wild type) receptor (Fig. 2A). L-365,260, previously considered a prototype CCK-BR antagonist (5, 11), is able to activate the Leu^325 → Glu receptor mutant with approximately half the efficacy of the full agonist, G-17. On closer examination, it became apparent that L-365,260 also induces a small yet significant (p < 0.01) increase of inositol phosphate production in cells expressing the wild type CCK-BR (Fig. 2A, left panel).

These findings prompted us to investigate the functional properties of compounds which are structurally related to L-365,260, including YM022 (19) and (R)-L-740,093, as well as its stereoisomer, (S)-L-740,093 (20). All of these molecules share a 1,4-benzodiazepine backbone (Fig. 2B), and are reported to function as antagonists. However, when assessed with the constitutively active receptor mutant (Fig. 2A), it became readily apparent that these compounds cover a broad range of intrinsic activities. The amplification of second messenger signaling observed with the Leu^325 → Glu receptor allowed us to demonstrate that minor structural changes of L-365,260 result in considerable functional alterations. Replacing the C5-phenyl moiety of the core benzodiazepine structure with an azabicyclo[3.2.2]nonane substituent, combined with changing the C3 stereochemistry, converts L-365,260 into (S)-

**Fig. 1.** A point mutation in the CCK-B/gastrin receptor (CCK-BR) triggers ligand-independent inositol phosphate formation without altering peptide agonist potencies. The human wild type CCK-BR and the constitutively active mutant, Leu^325 → Glu, were transiently expressed in COS-7 cells. Control cells (no receptor) were transfected with the empty expression vector, pcDNA3. Cells were prelabeled overnight with myo-[3H]inositol and then stimulated with ligand for 30 min in the presence of 10 mM LiCl. In parallel with the second messenger signaling assays, receptor densities in COS-7 cells were measured by homologous 125I-CCK-8 competition experiments. Maximal binding was 1.95 ± 0.46 fmol/10^4 cells for the wild type CCK-BR and 2.60 ± 0.57 fmol/10^4 cells for the Leu^325 → Glu mutant, respectively (means ± S.E. of 10 independent experiments, no significant difference, p > 0.05). A, the constitutively active CCK-BR mutant (right panel) is distinguished from the wild type receptor isoform (left panel) by its ability to trigger inositol phosphate production in the absence of agonists. The endogenous peptide agonists CCK-8 and G-17 further stimulates both receptors to a similar maximum. Both agonists were tested at concentrations which result in >95% receptor occupancy (see “Experimental Procedures” and Table I); respective concentrations were 1 μM for CCK-8 and 0.1 μM for G-17. Tritiated inositol phosphate production was expressed as a percentage of the total ^3H incorporated into the cells, means ± S.E. of three to five independent experiments. B, the endogenous peptide agonists CCK-8 and G-17 stimulate inositol phosphate production with similar potencies when compared at the wild type and the constitutively active CCK-BRs. Mean EC_{50} values in nanomolar (95% confidence intervals) at the wild type (closed symbols) versus the mutant receptor (open symbols), respectively, were 0.16 (0.12–0.20) versus 0.10 (0.07–0.15) for CCK-8 (left panel); five independent experiments, no significant difference, p > 0.05 and 0.24 (0.18–0.32) versus 0.23 (0.11–0.51) for G-17 (right panel), three independent experiments, no significant difference, p > 0.05. For comparison of potencies, inositol phosphate production was normalized using the respective values in unstimulated cells (no ligand) as 0% and in maximally stimulated cells expressing either the wild type or the constitutively active CCK-BR (10^-6 M CCK-8 or G-17) as 100%. In the figure, symbols represent means ± S.E.
TABLE I

| Peptide | Ki (nM) | Ki (nM) |
|---------|---------|---------|
| Peptides |         |         |
| Gastrin-17 | 1.35 ± 0.28 | 0.78 ± 0.16 |
| CCK-8 | 0.12 ± 0.01 | 0.07 ± 0.01 |
| CCK-8US | 0.20 ± 0.78 | 1.16 ± 0.51 |
| CCK-4 | 11.6 ± 4.0 | 7.97 ± 2.08 |
| Peptoids |         |         |
| PD-135,158 | 2.25 ± 0.61 | 1.01 ± 0.19 |
| PD-136,450 | 0.99 ± 0.10 | 0.59 ± 0.12 |
| Non-peptides |         |         |
| L-740,093 | 0.19 ± 0.02 | 0.18 ± 0.04 |
| YM022 | 0.07 ± 0.01 | 0.08 ± 0.01 |
| L-364,718 | 150 ± 42 | 170 ± 34 |
| L-365,260 | 7.2 ± 0.9 | 7.8 ± 1.5 |
| L-740,093 | 19.5 ± 1.5 | 16 ± 1.4 |

L-740,093 (20), the most efficacious agonist of the tested ligands. Reflecting its relatively strong signaling potential, (S)-L-740,093 also functions as a partial agonist at the wild type CCK-BR (Fig. 2A). Further confirming the agonist activity of (S)-L-740,093, stimulation of the wild type CCK-BR with this compound triggers a concentration-dependent increase in second messenger signaling (Fig. 3). Therefore, (S)-L-740,093 provides the first example of a “true” non-peptide agonist for the human CCK-BR.

It is noteworthy that the mirror image (R-enantiomer) of the non-peptide agonist (S)-L-740,093 has the opposite functional properties. (R)-L-740,093 reduces basal signaling of the constitutively active receptor close to that of the wild type isoform (Fig. 2A) and thereby satisfies the criterion of an inverse agonist. Non-peptide inverse agonists have recently been identified for two other types of peptide receptors, i.e. the thyrotropin-releasing hormone and the AT1A angiotensin II receptors (26, 22). Like (R)-L-740,093, each of these non-peptide inverse agonists appears to have no intrinsic activity at the respective wild type receptors and was therefore originally classified as an antagonist. Together, these examples illustrate that inverse agonism is likely a hidden property of many ligands which are currently considered “agonists.” To enable more definitive classification of these compounds, further functional characterization using constitutively active receptors will be required. The discovery of non-peptide inverse agonists provides a compelling rationale for developing a new class of drugs, targeted at constitutively active peptide hormone receptors which result in human disease (23, 24). For example, the pathogenesis of thyroid adenomas has been linked to constitutively active thyroid stimulating hormone receptors (25), drugs which “silence” these overactive proteins could potentially be utilized to inhibit tumor growth. Similarly, inverse agonists could delay the onset of precocious puberty in patients with constitutively active luteinizing hormone receptors (26).

The concentration-dependent activity of (S,R)-L-740,093 at the wild type and mutant CCK-BRs supports the pharmacological classification of these compounds as agonist and inverse agonist, respectively (Figs. 3 and 4A). It is intriguing to alter the steric conformation of these compounds in order to accommodate their function. An observation which parallels our findings with the L-740,093 enantiomers was recently reported for synthetic derivatives of the carboxyl-terminal cholecystokinin tetrapeptide fragment, CCK-4. Two of these peptoid ligands, PD-149,164 and PD-151,932, which are distinguished only by their steric conformation, were found to act as agonist and antagonist, respectively, at the CCK-A receptor subtype (27). It remains to be established whether stereoisomers of ligands for other peptide hormone receptors will also have opposite functional properties.

Unlike the L-740,093 enantiomers, YM022 has minimal effect on the basal activity of either the wild type or the mutant CCK-B/gastrin receptors (Fig. 2A). This lack of intrinsic activity, and the ability of YM022 to block CCK-8-induced inositol phosphate formation (17), are consistent with the expected properties of an antagonist. To further validate the functional classification of the CCK-BR non-peptide ligands, the interaction of YM022 with S- and R-740,093 was studied. (S)-L-740,093 induced inositol phosphate production was inhibited by YM022 in a concentration-dependent manner (Fig. 4B, top), further supporting that (S)-L-740,093 functions as a non-peptide agonist. In addition, YM022 was able to attenuate the inhibitory effect of (R)-L-740,093 on the constitutively active...
ternary complex model of receptor activation which has been posited as another general property of constitutively active (constitutive) CCK-B receptors. Consistent with the expectation of increased agonist potencies, our data reveal that (S)-L-740,093 is slightly more potent at the constitutively active CCK-BR (EC_{50} = 2.5 nM; see Fig. 3) than at the wild type receptor (EC_{50} = 2.5 nM; see Fig. 3). However, we were unable to detect any potency shifts for the peptide ligands CCK-8 and gastrin (see Fig. 1B), despite the fact that both of these full agonists have considerably higher efficacy than the partial agonist, (S)-L-740,093. The latter findings suggest limitations of the extended ternary model of receptor activation in predicting how constitutive receptor activity affects agonist potency for a given ligand. Consistent with our observations, it has been previously noted for other constitutively active mutants that the potencies of peptide agonists show little or no changes versus corresponding values at the respective wild type receptor isoforms (22, 39–41). It is possible that the extended ternary complex model of receptor activation is most applicable to small ligands (e.g., (R)-L-740,093 and biogenic amines), whereas larger ligands (e.g., peptides) may interact with the receptor in a less predictable fashion.

The apparent lack of generalizable rules regarding potency shifts at constitutively active receptors can be best explained by the “cubic ternary complex” model of receptor activation (42). This recently proposed model refines earlier theories by considering additional receptor- and ligand-specific variables and multiple receptor states which may be involved in agonist-mediated receptor activation. The model acknowledges that several of these factors may be altered by receptor mutations which confer constitutive activity, and implies that these changes will not necessarily result in potency increases.

In addition to revealing both consistencies with and limitations of existing models of receptor activation, our observations extend current knowledge regarding the versatility of benzodiazepine-based molecules as potent and selective ligands for a wide range of different receptors (43). Based on the precedent provided, it appears likely that such molecules are preferred structures not only for the development of specific antagonists, but may also provide promising templates for novel receptor agonists. Consistent with this generalization, it has been recently reported that certain benzodiazepine derivatives can act as mixed CCK-A receptor agonists/CCK-BR antagonists (44). However, although structural similarities exist between these 1,5-benzodiazepine-derived CCK-A receptor agonists and 1,4-
Figure 4. YM022 antagonizes both the agonist and inverse agonist activities of (S,R)-L-740,093 enantiomers, respectively, at the mutant CCK-BR (Leu325 \( \rightarrow \) Glu). Tritiated inositol phosphate production was expressed as a percentage of the total \(^3\)H-incorporated into COS-7 cells expressing the constitutively active Leu325 \( \rightarrow \) Glu CCK-BR mutant. In the figure, symbols represent mean \( \pm \) S.E. The number of independent experiments (n) is indicated for each panel. All values are corrected for inositol phosphate production in control cells (no receptor, see Fig. 1A). A, top panel: (S)-L-740,093 triggers a concentration-dependent increase in inositol phosphate production, with a mean EC\(_{50}\) (95% confidence interval) of 0.7 (0.6–0.8) nM (n = 3). Bottom panel, in contrast, (R)-L-740,093 causes a concentration-dependent inhibition of constitutive activity, with a mean IC\(_{50}\) (95% confidence interval) of 1.7 (0.5–6.3) nM (n = 3). B, top panel: YM022 inhibits inositol phosphate production triggered by 10 nM (S)-L-740,093, with a mean IC\(_{50}\) (95% confidence interval) of 4.0 (1.6–9.9) nM (n = 4). Bottom panel, in the presence of the inverse agonist (R)-L-740,093 (20 nM), YM022 partially restores basal inositol phosphate production of the constitutively active CCK-BR. The half-maximal effect of YM022, mean (95% confidence interval), was observed at 16.6 (7.0–39.3) nM (n = 3). Note that YM022, by itself, is a weak inverse agonist, and is therefore unable to completely restore the basal level of signaling (compare control values (no ligand) in A).

Figure 5. Enhanced ligand-induced signaling at the mutant CCK-BR (Leu325 \( \rightarrow \) Glu) correlates with ligand activity at the wild type receptor. Inositol phosphate formation was induced by peptide, peptoid, or non-peptide ligands in COS-7 cells transiently expressing either the wild type or the constitutively active receptor isoforms (see legend, Fig. 1). The concentrations at which ligands were tested are described in the text and in Figs. 1A and 2A, legends. In addition to compounds which have been discussed earlier, data are shown for L-364,718, a benzodiazepine derivative which preferentially binds to the CCK-A receptor (47) (tested at 5 \( \mu \)M) and for two additional peptides, cholecystokinin tetrapeptide (CCK-4; tested at 3 \( \mu \)M) and unsulfated CCK8 (CCK-8US; tested at 1 \( \mu \)M). In cells expressing either the wild type or the constitutively active receptor, G-17 induced signaling was assigned an efficacy value of 100%, whereas basal inositol phosphate production in the absence of ligand (open square) was defined as 0%. Ligand-induced signaling (closed squares) was normalized to these reference values. The correlation between wild type and mutant efficacy values for each of the tested ligands in the graph is approximated by a rectangular hyperbola, \( \frac{Y}{X} = \frac{a}{X} + b \), with a and b representing constants and X and Y representing ligand activity at the wild type and mutant receptors, respectively. A least square fit of this function to the data points is indicated by the dashed line. As implied by this rectangular hyperbolic correlation, we hypothesize that ligand efficacies may approach a “ceiling” that cannot be exceeded at either receptor isoform.

At present, there are only a few other examples of non-peptide agonists which can mimic the function of endogenous peptide hormones (45, 46). Constitutively active receptors, as exemplified by the Leu325 \( \rightarrow \) Glu mutant, hold promise as sensitive probes for the systematic screening of non-peptide ligands for intrinsic activity since these receptors lead to a systematic efficacy increase, regardless of ligand structure (see above). As an important advantage in the search for agonist potential, the proposed strategy may be applicable independent of the chemical structure of non-peptide ligands, and could therefore be utilized to re-assess a large variety of already known non-peptide ligands which have been classified as antagonists for different peptide hormone receptors. Once identified, lead agonist mimetics can then be structurally modified as necessary, to optimize receptor specificity, oral bioavailability, and pharmacokinetic properties. This approach should accelerate pharmacological discovery and design of novel ligands for therapeutic use.

Benzodiazepine-based CCK-BR ligands (tested in the present study), the two groups of compounds are clearly distinguished by the configuration of their respective benzodiazepine cores and by the different composition of attached substituents. As a common theme, the respective substituents appear to play a key role in determining the level of ligand intrinsic activity both at the CCK-A and the CCK-B/gastrin receptors.

For example, the two benzodiazepine-based CCK-BR ligands (tested in the present study), the two groups of compounds are clearly distinguished by the configuration of their respective benzodiazepine cores and by the different composition of attached substituents. As a common theme, the respective substituents appear to play a key role in determining the level of ligand intrinsic activity both at the CCK-A and the CCK-B/gastrin receptors.
ate the identification and development of new drugs, applicable for the treatment of a broad spectrum of diseases.

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