

Small Synthetic Ligands of the Cholecystokinin-B/Gastrin Receptor Can Mimic the Function of Endogenous Peptide Hormones

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The gastric cholecystokinin-B/gastrin receptor (CCK-BR) is a key regulator of enterochromaffin-like cell function and proliferation. Over the last decade, a number of small non-peptide CCK-BR "antagonists" have been discovered. Here, we demonstrate that some of these non-peptide ligands in fact possess significant ability to activate the human CCK-BR, and are, therefore, more properly categorized as partial agonists. When tested in COS-7 cells transiently expressing the recombinant human CCK-BR, saturating concentrations of the small "peptoid" ligands PD 135,158 and PD 136,450 stimulated inositol phosphate formation to 23 and 43 percent, respectively, of the maximum response induced by a considerably larger endogenous peptide agonist, cholecystokinin octapeptide. In contrast, the benzodiazepine-derived CCK-BR ligand, YM022, acted as a "true" high-affinity antagonist of cholecystokinin-induced inositol phosphate formation ($pA_2 = 9.69$). Consistent with recent findings in animal experiments, our data reveal that small synthetic ligands have the potential to function as either CCK-BR agonists or antagonists. These dual properties of synthetic molecules must be considered when evaluating candidate drugs for human disease.

INTRODUCTION

The cholecystokinin-B/gastrin receptor (CCK-BR)^b is a seven transmembrane domain protein that modulates multiple functions in both the gastrointestinal tract and the brain [1, 2]. In the stomach, the CCK-BR has been well established to stimulate gastric acid secretion and mucosal cell growth [3, 4]. In particular, there is strong evidence suggesting that this receptor can induce the proliferation of enterochromaffin-like cells [5]. In the brain, the CCK-BR has been implicated in triggering anxiety attacks and may also modulate the perception of pain [6-9].

The CCK-BR is endogenously activated by structurally related endogenous peptide hormones and neurotransmitters, including the heptadecapeptide gastrin (G-17) and the octapeptide cholecystokinin (CCK-8). These peptides bind to the CCK-BR with affinities in the subnanomolar range and lead to the activation of phospholipase-C as a major second messenger pathway. Phospholipase-C, in turn, initiates the breakdown of membrane phospholipids to inositol phosphate metabolites and the subsequent release of intracellular Ca^{2+} as early signal transduction events [2, 4].

High affinity binding of endogenous peptide ligands to the CCK-BR is mimicked by considerably smaller synthetic molecules, which have been developed in an attempt to generate clinically useful CCK-BR antagonists. Such compounds include (i) "peptoid" derivatives that retain minimal structural features of the endogenous peptide agonists [10,

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^bAbbreviations: CCK-BR, cholecystokinin-B/gastrin receptor; G-17, gastrin heptadecapeptide; CCK-8, cholecystokinin octapeptide.

11], and (ii) a chemically different class of molecules with a common benzodiazepine-like backbone [12, 13]. Interestingly, we have recently discovered that these putative non-peptide antagonists occupy a ligand "pocket" within the transmembrane domains of the CCK-BR, similar to that described for biogenic amine receptors [14].

Given this considerable overlap of binding determinants, we speculated whether small synthetic CCK-BR ligands might have similar agonist potential as biogenic amines of similar size [15]. In fact, this hypothesis is supported by recent reports that "peptoid" compounds may act as partial agonists of the CCK-BR *in vivo*, with the ability to stimulate gastric acid secretion and ECL cell activation [16, 17]. In contrast, other investigators have been unable to detect any intrinsic activity of "peptoids" in the rat [18-21] and have, therefore, classified these compounds as antagonists both by *in vivo* and *in vitro* assays. Given these contradictory results, the postulated agonist potential of "peptoid" compounds remains to be further clarified, in particular when interacting with the human CCK-BR.

Using a simplified test system, we have, therefore, studied the abilities of several benzodiazepine-derived and "peptoid" ligands to stimulate the recombinant human CCK-BR when transiently expressed in COS-7 cells. These experiments clearly reveal considerable agonist properties of small synthetic CCK-BR ligands, reflected by their ability to trigger receptor-mediated inositol phosphate formation *in vitro*.

EXPERIMENTAL PROCEDURES

Materials

Cell culture media and fetal calf serum were obtained from GIBCO-BRL (Gaithersburg, MD) and from Intergen (Purchase, NY), respectively. Fura-2 (AM form) was obtained from Molecular Probes (Eugene, OR). ^{125}I -CCK-8 (2,200 Ci/mmol) and ^3H -myo-inositol (45-80 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Unlabelled CCK-8 (sulfated form) was obtained from Peninsula Laboratories (Belmont, CA). L-364,718, L-365,260 and YM022 were generously provided by Wyeth Research Ltd. (Taplow, UK). The "peptoid" compounds PD 135,158 and PD 136,450 were a gift from Parke-Davis Research Center (Cambridge, UK).

Binding experiments

COS-7 cells (1×10^6 /plate) were seeded onto 10 cm culture dishes (Costar, Cambridge, MA) and grown overnight in Dulbecco's modified Eagle medium with 10 percent fetal calf serum at 37°C. The cells were transfected with 5 μg of cDNA encoding the human CCK-BR, cloned into the eukariotic expression vector pcDNAI. The following day, cells were split into 24-well dishes (1×10^4 /well) (Costar). After an additional 24 hr, competition binding experiments were performed in Hank's balanced salt solution supplemented with 25 mM HEPES, pH 7.3, 0.2 percent bovine serum albumin and 0.15 mM phenylmethylsulfonyl fluoride, using 20 pM ^{125}I -CCK-8 as the radioligand. After an incubation for 80 min in the absence or presence of unlabelled competitor ligands, cell monolayers were washed three times in Hank's balanced salt solution and then hydrolyzed in 1N NaOH for γ -counting. All binding affinities (as well as signaling potencies, see below) were calculated by computerized non-linear curve fitting using GraphPad Prizm software (GraphPad, San Diego, CA).

Measurement of $[\text{Ca}^{2+}]_i$

Forty-eight hours after transfection with pcDNAI/CCK-BR, COS-7 cells were trypsinized, suspended in modified Krebs-Ringer bicarbonate buffer, and loaded with the Ca^{2+} fluorophore fura-2 (30 min at 37°C). Fluorescence changes after stimulation of cells with

increasing concentrations of CCK-8 were determined from fluorescence emission ratios at 340/380 nm, as previously described [22].

Measurement of inositol phosphate accumulation

Forty-eight hours after transfection, COS-7 cells were seeded into 6-well plates (3×10^5 /well) (Nunc). The cells were then pre-labelled overnight with 3 mCi/ml ^3H -myo-inositol in serum-free Dulbecco's modified Eagle medium. To assess total inositol phosphate formation, the media were replaced with phosphate-buffered saline containing 10 mM LiCl, and cells were incubated with the respective ligands for 30 min at 37°C. Inositol metabolites were extracted with methanol/chloroform; the upper phase was analyzed for inositol phosphates by strong anion exchange chromatography [23]. Inositol phosphate production was expressed as the percent of total cellular tritium content incorporated during an overnight exposure to ^3H -myo-inositol.

RESULTS AND DISCUSSION

We have previously demonstrated that the recombinant human CCK-BR, when transiently expressed in COS-7 cells, triggers CCK-8 induced inositol phosphate production and Ca^{2+} release from intracellular stores [1]. These *in vitro* findings are consistent with the well established coupling of the native CCK-BR to activation of phospholipase-C in gastric parietal cells [24-26], suggesting that the COS cell expression system reveals relevant functional properties of CCK-BR ligands. To further establish how receptor occupation by an agonist correlates with the induction of second messenger signaling, we extended our previous experiments by examining the concentration-effect relationship of CCK-8 induced second messenger signaling. As demonstrated as an example in Figure 1A, nanomolar concentrations of CCK-8 were sufficient to fully stimulate Ca^{2+} release in COS-7 cells transiently expressing the CCK-BR. Moreover, the concentration-response curve for ^{125}I -CCK-8 binding was virtually superimposable with those for CCK-8 induced inositol phosphate formation and intracellular Ca^{2+} release (Figure 1B), indicating that the recombinant CCK-BR is efficiently coupled to intracellular signaling in COS-7 cells. Therefore, this *in vitro* system provides high functional sensitivity and can be utilized to detect intrinsic agonist properties of CCK-BR ligands.

The small synthetic CCK-BR ligands analyzed in this study included the "peptoid" compounds, PD 135,158 and PD 136,450 [27, 28], as well as three benzodiazepine-based ligands, L-364,718, L-365,260, and YM022 [29-31]. All of these compounds were able to compete with the peptide radioligand, ^{125}I -CCK-8, for binding to the CCK-BR (Figure 2). Given the close correlation between receptor occupation and induction of intracellular signaling (see above), we used the data in Figure 2 to predict which ligand concentrations are likely to result in full receptor activation. In general, we tested the ability of individual compounds to trigger inositol phosphate production using a single ligand concentration which was at least 100-fold higher than required for 50 percent receptor occupation (indicated by the corresponding IC_{50} values in radioligand competition experiments, see legend of Figure 2). As an exception, the tested concentration of L-364,718 was limited to 5 μM . Although this concentration is only 20-fold higher than the corresponding IC_{50} of L-364,718 in binding experiments, it is still sufficient for approximately 95 percent receptor occupation.

At maximally effective concentrations, both PD 135,158 and PD 136,450 acted as partial agonists and stimulated inositol phosphate production to 23 ± 3 percent and 43 ± 2 percent, respectively, of the CCK-8 induced maximum (data not shown, means \pm SEM from three independent experiments). The activity of these two "peptoid" ligands was also confirmed by analysis of full concentration-response curves of CCK-BR mediated

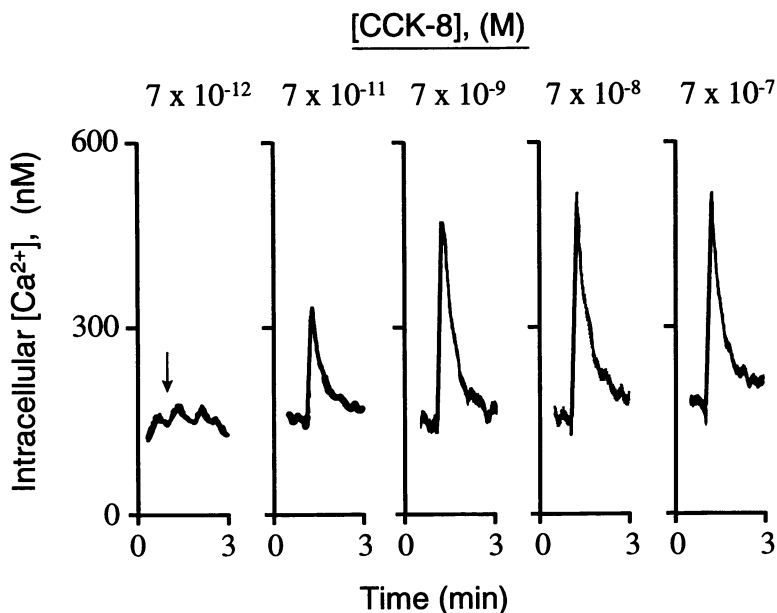


Figure 1A. CCK-8 triggers concentration-dependent increases of $[Ca^{2+}]_i$ in COS-7 cells expressing the human CCK-BR. An aliquot from a pool of simultaneously transfected COS-7 cells was tested at each CCK-8 concentration. No CCK-8-induced increase of $[Ca^{2+}]_i$ was detected in untransfected COS-7 cells (not shown).

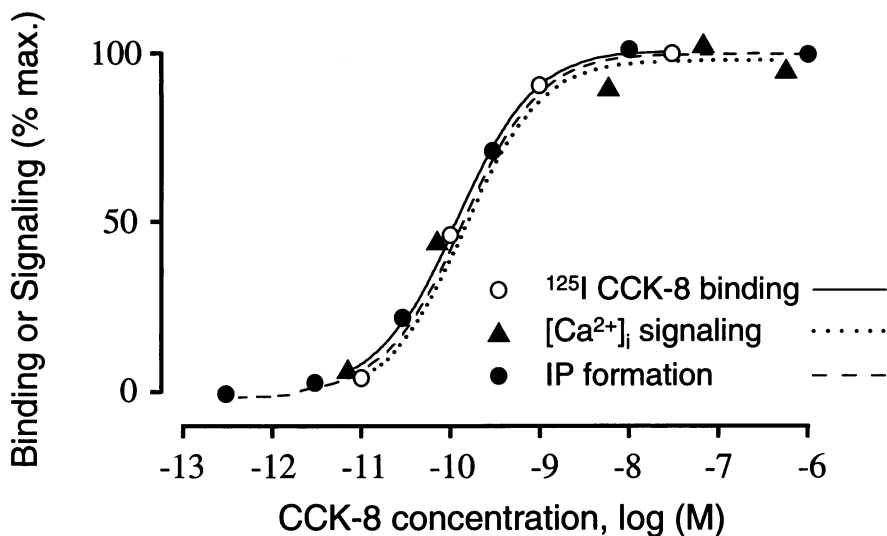


Figure 1B. Peak increases of $[Ca^{2+}]_i$ (see Figure 1A), when plotted as a function of CCK-8 concentration, follow a curve which is virtually superimposable with curves describing CCK-8 dependent inositol phosphate formation or receptor occupation. Receptor occupation was calculated from ^{125}I -CCK-8 competition binding studies with unlabelled CCK-8 (see Figure 2). All data in Figure 1 show the results of a single experiment with cells transfected on the same day, and are consistent with several replicate experiments where receptor binding and function were assessed on different days (e.g., data in Figures 2-4).

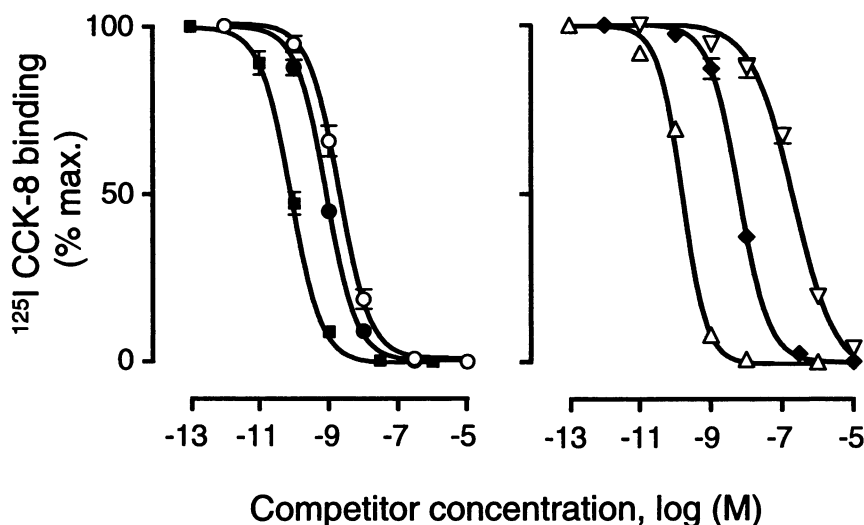


Figure 2. Concentration-dependent competition of unlabeled ligands for ^{125}I -CCK-8 binding to the recombinant human CCK-BR. Binding was tested with transiently transfected COS-7 cells (see methods). Calculated IC_{50} values (in nM, means \pm SEM of three independent experiments) for peptide and "peptoid" competitors (left panel) were 0.09 ± 0.01 for CCK-8 (■); 0.82 ± 0.14 for PD 136,450 (●); and 2.02 ± 0.39 for PD 135,158 (○), respectively. IC_{50} values for non-peptide ligands (right panel) were 0.18 ± 0.11 for YM022 (△); 6.04 ± 0.68 for L-365,260 (◆); and 226 ± 143 for L-364,718 (▽), respectively. Non-transfected cells showed no displaceable ^{125}I -CCK-8 binding (not shown).

inositol phosphate formation (Figure 3). The latter experiments revealed that the functional potencies of PD 135,158 and of PD 136,450 were fully consistent with their respective binding affinities (compare EC_{50} values in Figure 3 and IC_{50} values in Figure 2), again confirming the close correlation of receptor binding and functional parameters in our *in vitro* system. Interestingly, both tested "peptoid" ligands have been recently reported to act as partial agonists in the rat and have been demonstrated by *in vivo* experiments to stimulate gastric acid secretion [17] and the activity of histidine decarboxylase as a marker of mucosal ECL cell density [16]. In contrast, other experiments suggested that PD 136,450 has no appreciable intrinsic activity in the rat and acts as a true antagonist of gastric acid secretion [19, 21] and of gastrin-induced mucosal proliferation [20]. Furthermore, it has been reported that "peptoid" compounds including PD 136,450 and PD 135,158 antagonized CCK-BR mediated electrophysiological responses in the rat ventromedial hypothalamus [18].

Together, these apparently contradictory results illustrate the difficulty in classifying CCK-BR ligands using relatively complex native tissues or *in vivo* models, where drug-induced effects may depend on multiple interacting mechanisms and can be easily biased by the choice of experimental conditions. Moreover, it must be also considered that ligand function as an antagonist or as a partial agonist may heavily depend on the cellular microenvironment where native or recombinant receptors are expressed (e.g., in gastric parietal vs. nerve cells) and is also expected to be a function of receptor densities (which may differ even between rat strains). At the receptor level, we have recently shown that differences in drug efficacy can also result from minimal species differences in the amino acid sequence of their respective CCK-BRs [32]. Considering these limitations of characterizing drug pharmacology in animal experiments, it is important to verify the functional

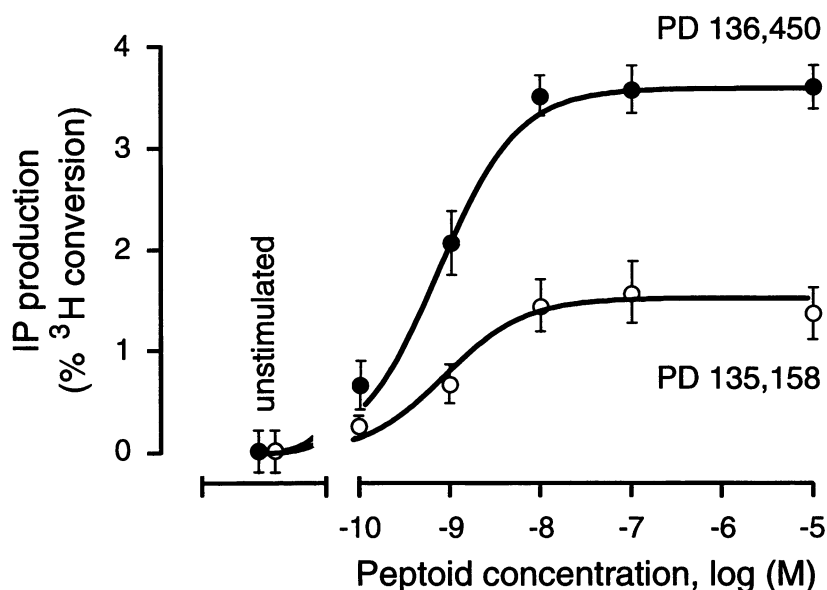


Figure 3. “Peptoid” compounds stimulate a concentration-dependent increase of inositol phosphate production in COS-7 cells expressing the recombinant human CCK-BR. Respective EC_{50} values (in nM, means \pm SEM) were 0.68 ± 0.31 for PD 136,450 (●, $n = 6$) and 1.04 ± 0.60 for PD 135,158 (○, $n = 3$). Data are corrected for inositol phosphate production in the absence of ligands (unstimulated cells), which approximated the value obtained in untransfected COS-7 cells not expressing the human CCK-BR (not shown).

properties of candidate drugs in a less complex model system that eliminates a large number of these confounding variables. In our test system, we have obtained unambiguous evidence that “peptoid” compounds can, in principle, activate the human CCK-BR isoform. Again, it is important to note that this finding, in itself, is not necessarily predictive of overall *in vivo* function but rather reflects properties of “peptoid” ligands, which may actually apply only to certain native cell types. The anticipated diversity in ligand efficacy is exemplified by a recent report where the recombinant human CCK-BR was expressed in CHO (instead of COS-7) cells, and the “peptoid” compound PD 135, 158 appeared to act as a “true” antagonist (rather than as a partial agonist) [33]. In any case, knowledge of the full spectrum of intrinsic activities to be anticipated will be a helpful guideline for evaluating the pharmacology of “peptoid” drugs in humans.

In contrast to “peptoid” molecules, all three benzodiazepine-derived compounds which were tested had very little, if any, ability to activate the human CCK-BR (Figure 4A). These compounds represent three generations of molecules in a promising series of putative CCK receptor antagonists which have been developed over the past decade.

L-364,718, the derivative with lowest affinity at the human CCK-BR (Figure 1), was one of the first synthetically generated high affinity ligands for any peptide hormone receptor (although it was targeted at the CCK-A receptor subtype, rather than the CCK-BR) [12, 29]. A derivative of this molecule, L-365,260, is considered the prototype CCK-BR non-peptide antagonist and was the first synthetic ligand for this receptor with affinity in the nanomolar range [30]. Recent efforts to further improve the affinity and receptor subtype selectivity of benzodiazepine-based ligands have resulted in the synthesis of derivatives like YM022 [31]. Consistent with its subnanomolar affinity in competition binding experiments with

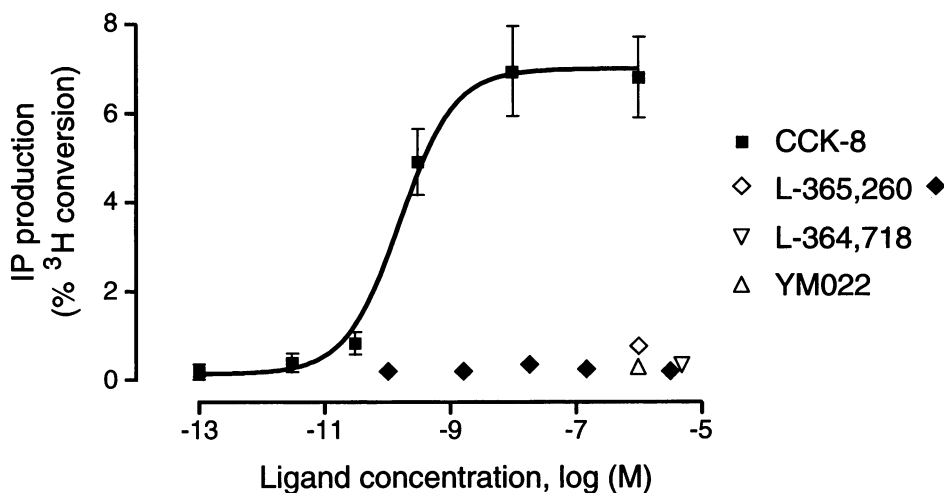


Figure 4A. CCK-8 stimulates a concentration-dependent increase of inositol phosphate production in COS-7 cells expressing the human CCK-BR ($EC_{50} = 0.15 \pm 0.8$ nM, mean \pm SEM of three independent experiments). In the same experiments, high concentrations of non-peptide ligands including L-365,260 (\diamond), L-364,718 (∇) and YM022 (\triangle) had little, if any, effect on inositol phosphate production. Data are corrected for inositol phosphate production in the absence of ligands (unstimulated cells). In addition to replicate testing at a single, high concentration (\diamond), the effect of L-365,260 was also assessed over a broader range of concentrations (\blacklozenge).

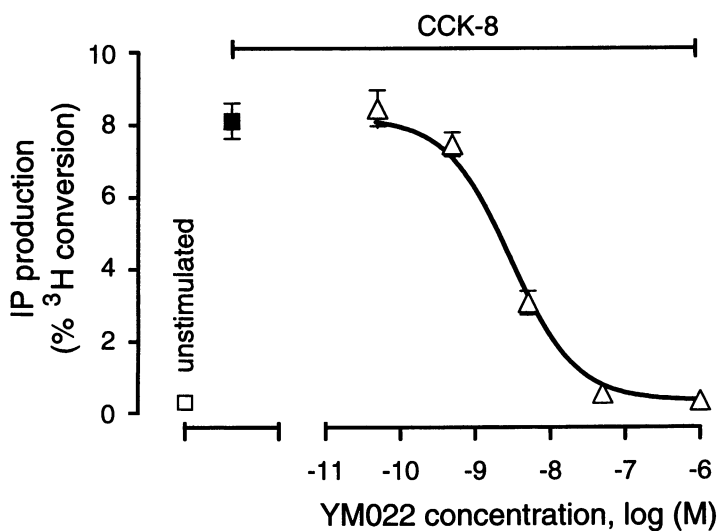


Figure 4B. Increasing concentrations of YM022 inhibit the effect of 2.5 nM CCK-8 ($IC_{50} = 3.2 \pm 1.1$ nM, $n = 3$). With an EC_{50} value of 0.15 nM for CCK-8 stimulated inositol phosphate production (see above), the antagonist pK_A value of YM022 can be estimated at 9.69 using the “null” method [36].

the human CCK-BR (Figure 2) and its apparent lack of intrinsic activity (Figure 4A), we were able to verify that YM022 acts as a high affinity antagonist and completely blocks CCK-8 induced inositol phosphate formation (Figure 4B). As expected for a competitive antagonist, the pA_2 value of YM022 (reflecting its functional affinity) was close to its IC_{50} value in competition binding experiments (compare legends of Figures 2 and 4B).

Although, by these experiments, benzodiazepine-based ligands appear to act as "true" antagonists and seem to have generally different functional properties than "peptoid" ligands, we have evidence that this distinction does not always apply. Preliminary data suggest that even benzodiazepine-based CCK-BR ligands have inherent agonist potential, which can be optimized by minor chemical modifications of these molecules [34]. Furthermore, ligands with similar benzodiazepine-like structure have recently been shown to act as partial or even full agonists of the CCK-A receptor subtype, both *in vivo* and *in vitro* [35]. With these precedents, it is likely that presently known non-peptide "antagonists" for peptide hormone receptors, due to their inherent potential, can be systematically modified to generate a broad range of clinically useful agonist drugs. Equally important, these intriguing findings should raise the awareness that apparent non-peptide "antagonists" for peptide hormone receptors could possess residual intrinsic activity which may result in unexpected, and possibly adverse, biological effects in the context of certain human diseases.

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