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Benzodiazepine ligands can act as allosteric modulators of the Type 1 cholecystokinin receptor

Fan Gao^a, Patrick M. Sexton^b, Arthur Christopoulos^b, and Laurence J. Miller^{a*}

^aDepartment of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Scottsdale, AZ 85259 (F.G., L.J.M.)

^bDrug Discovery Biology Laboratory, Monash Institute of Pharmaceutical Sciences and Department of Pharmacology, Monash University, Clayton, Victoria 3800, Australia (P.M.S., A.C.).

Abstract

The cholecystokinin (CCK₁) receptor is a G protein-coupled receptor important for nutrient homeostasis. The molecular basis of CCK-receptor binding has been debated, with one prominent model suggesting occupation of the same region of the intramembranous helical bundle as benzodiazepines. Here, we used a specific assay of allosteric ligand interaction to probe the mode of binding of devazepide, a prototypic benzodiazepine ligand. Devazepide elicited marked slowing of dissociation of pre-bound CCK, only possible through binding to a topographically distinct allosteric site. This effect was disrupted by chemical modification of a cysteine in the benzodiazepine-binding pocket. Application of an allosteric model to the equilibrium interaction between a series of benzodiazepine ligands and CCK yielded quantitative estimates of each modulator's affinity for the allosteric site, as well as the degree of negative cooperativity for the interaction between occupied orthosteric and allosteric sites. The allosteric nature of benzodiazepine binding to the CCK₁ receptor provides new opportunities for small molecule drug development.

Keywords

Allosteric modulators; cholecystokinin receptor; cooperativity; G protein-coupled receptors

The Type 1 cholecystokinin (CCK₁) receptor is a Class I G protein-coupled receptor that is an important potential pharmacologic target, being activated by its natural peptide hormonal ligand to stimulate gallbladder contraction, pancreatic exocrine secretion, enteric motility, and satiety. Binding of CCK, the natural (orthosteric) ligand, to this receptor has been variably proposed to occur at the extracellular surface, where it interacts with receptor loop and tail domains,¹ or as dipping into the helical bundle, where benzodiazepine ligands have been proposed to act.² This latter model predicts a competitive interaction between CCK and small molecule benzodiazepine ligands, whereas the former model predicts distinct binding sites for the two ligands, allowing for allosteric interaction to occur.

A sensitive assay for identification of allosteric interactions is the analysis of ligand binding kinetics, in particular dissociation kinetics.³ Since orthosteric ligands exhibit mutually

*Please send all correspondence and reprint requests to: Laurence J. Miller, M.D., Mayo Clinic, 13400 East Shea Boulevard, Scottsdale, AZ 85259, Telephone: (480)-301-6650, Fax: (480)-301-6969, E-mail: miller@mayo.edu.

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exclusive binding, the rate of dissociation of a pre-bound orthosteric ligand cannot be altered by the presence of additional competitive ligands. In contrast, allosteric ligands occupy topographically distinct sites, allowing for simultaneous binding of both orthosteric and allosteric ligands with potential for cooperative interactions to occur that may manifest as increases or decreases in the rate of orthosteric ligand dissociation.⁴

The current project was designed to determine whether the binding sites for peptide and benzodiazepine ligands are indeed distinct or are overlapping, through analysis of the effect of the prototypic benzodiazepine compound, devazepide, on the rate of dissociation of radiolabeled CCK, and thus provide further evidence for the evaluation of existing models of CCK-receptor interaction. We demonstrate that the kinetics of dissociation of CCK were significantly altered by devazepide, indicative of an allosteric mode of binding. Furthermore, modification of Cys⁹⁴, postulated to reside within the intramembranous benzodiazepine-binding pocket, with a thiol-reactive methanethiosulfonate reagent, abolished the benzodiazepine effect on CCK dissociation. Moreover, we have also applied an allosteric model of ligand-receptor interaction to the analysis of the equilibrium binding properties of a series of benzodiazepine ligands to derive for the first time quantitative estimates of modulator affinity and cooperativity with CCK. A molecular model consistent with this distinct mode of peptide and small molecule compound binding is proposed.

CCK receptor binding was performed on a membrane preparation from a Chinese hamster ovary cell line engineered to stably express the rat CCK₁ receptor (CHO-CCKR).^{5,6} The CCK-like radioligand, [¹²⁵I]-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] ([¹²⁵I]CCK) (approximately 10 pM), was allowed to bind to membranes representing approximately 10 µg protein in Krebs-Ringers-HEPES (KRH) medium (25 mM HEPES at pH 7.4, 104 mM NaCl, 5 mM KCl, 1 mM KH₂PO₄, 2 mM CaCl₂, and 1.2 mM MgSO₄, supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.01 % soybean trypsin inhibitor (STI) and 0.2 % bovine serum albumin) for 60 min at room temperature. Bound and free radioligand were separated by centrifugation and washing with iced KRH medium, with the membrane-bound radioactivity quantified. Non-saturable binding represented less than 15 percent of total cpm bound, as determined in the presence of 1 µM non-radioactive CCK.

To explore whether a prototypic benzodiazepine ligand of the CCK receptor, devazepide (Merck & Co.), bound to an allosteric site or has overlapping determinants with the orthosteric site (as has been postulated),⁷ we studied CCK radioligand dissociation from the CCK receptor in the absence and presence of devazepide. After radioligand binding reached equilibrium, a saturating concentration of non-radioactive CCK (10 nM) was added to compete for the rebinding of dissociated radioligand. The amount of radioligand bound to the membrane receptors was determined over time, and the data were fitted to a single exponential decay equation using Prism 5.01 (GraphPad Software, San Diego, CA) to derive estimates of the radioligand dissociation rate constant, k_{off} . In some experiments, a second CCK receptor ligand (the benzodiazepine antagonist, devazepide, 10 nM) was introduced to determine its effect on the rate of CCK dissociation. Indeed, in the presence of devazepide, the dissociation of [¹²⁵I] CCK was significantly slowed from control (Fig. 1). The k_{off} of the radioligand decreased from $0.41 \pm 0.14 \text{ min}^{-1}$ to $0.10 \pm 0.02 \text{ min}^{-1}$ ($n = 3$, $p < 0.05$), supporting an allosteric mode of binding of the benzodiazepine.

To further probe the site of devazepide binding, we employed a strategy to modify a residue within the helical bundle that is believed to interact with the benzodiazepine⁷ and to thereby disrupt its binding to this allosteric site. For this, we utilized the thiol-reactive methanethiosulfonate reagent, Alexa⁵⁶⁸-MTSEA, to derivatize the free cysteine in position 94 in the second transmembrane segment facing the center of the helical bundle, as we have previously demonstrated.^{1, 8} This reagent was prepared by acylation of the free amino group

of 2-aminoethyl-methanethiosulfonate hydrochloride with Alexa⁵⁶⁸-*N*-hydroxysuccinimide ester, purifying the product to homogeneity using reversed-phase HPLC. The CHO-CCKR cell membranes were allowed to react with this reagent (final concentration 50 μ M) in KRH medium for 30 min. The unreacted Alexa⁵⁶⁸-MTSEA reagent was then removed by two cycles of centrifugation and washing. The CCK dissociation experiments were subsequently performed under identical conditions to those described above.

The kinetics of dissociation of the CCK radioligand from the receptor that had been derivatized with Alexa⁵⁶⁸-MTSEA were different from those observed with the non-derivatized receptor, perhaps reflecting a conformational effect on the receptor (Fig. 2). However, this was not further altered by addition of 10 nM devazepide (dissociation rate k_{off} of the radioligand was $0.08 \pm 0.03 \text{ min}^{-1}$ and $0.09 \pm 0.03 \text{ min}^{-1}$, respectively, $n = 3$, $p > 0.05$; Fig. 2). This was clearly quite distinct from the significant change observed after devazepide treatment of the non-derivatized, wild type receptor.

Equilibrium binding studies were also performed to quantify the parameters describing the allosteric interaction between devazepide and [¹²⁵I]CCK. In addition, we also applied this analysis to the interaction between [¹²⁵I]CCK and two other benzodiazepines developed by GlaxoSmithKline, compounds 6 and 8, which we showed in a prior study to interact competitively with devazepide⁹ and thus likely utilize a common binding site. These equilibrium binding data were fitted either to a simple one-site competitive-binding model ([¹²⁵I]CCK vs. CCK) or to an allosteric ternary complex model³ ([¹²⁵I]CCK vs. devazepide) to derive estimates of inhibitor K_B (equilibrium dissociation constant) and, for the allosteric model, the cooperativity factor, α , which is a measure of the magnitude and direction of the allosteric effect that one ligand exerts on the binding affinity of the other; values of α greater than 1 denote positive cooperativity, whereas values of α less than 1 (but greater than 0) denote negative cooperativity;³ values of α approaching 0 denote a high degree of negative cooperativity that is difficult to distinguish from simple competition in an equilibrium binding assay. In all instances, ligand affinity and cooperativity values were estimated as logarithms; the concentration of radioligand was 10 pM and the K_D was 0.22 nM. Statistical analyses were by Student's *t*-test, with $p < 0.05$ taken as the level of significance.

The results of this binding analysis are shown in Figure 3 and summarized in Table 1, where it can be seen that devazepide displayed a high degree of negative cooperativity with the orthosteric radioligand; the α value for the allosteric interaction approached zero, making it difficult to distinguish from a simple competitive interaction such as that observed with CCK. In contrast, both compounds 6 and 8 exhibited an inability to fully inhibit the specific binding of the orthosteric radioligand, clearly indicating that a ternary complex of radioligand, receptor and benzodiazepine persists at saturating concentrations of the modulator. Application of an allosteric ternary complex model to these data yielded the affinity and cooperativity estimates shown in Table 1; thus, compound 6 can only reduce orthosteric ligand affinity by a factor of approximately 4.5 ($\alpha = 0.22$), whereas compound 8 can only reduce orthosteric affinity by a factor of approximately 1.7 ($\alpha = 0.59$). To our knowledge, this is the first application of an allosteric model to quantify the binding properties of small molecule modulators at the CCK₁ receptor.

To illustrate the binding positions of the ligands utilized in the current study to the CCK₁ receptor, we utilized the Molsoft Internal Coordinate Mechanics (ICM) software.¹⁰ We previously developed a three-dimensional molecular model of the CCK₁ receptor that was based on homology with the structure of rhodopsin, in which we had attempted to independently dock a CCK peptide analogue¹ and a benzodiazepine ligand.⁹ It is noteworthy that another model of this receptor has also previously been proposed that has a very similar helical bundle structure and places the benzodiazepine-binding site in a similar position within

the helical bundle, but places the CCK-binding site quite differently.² In that model, the carboxyl-terminal end of CCK is clearly shown to occupy the same space as the benzodiazepine in the intramembranous helical bundle.²

Devazepide was docked to our CCK₁ receptor model using the small molecule docking module of ICM, directed by the mutagenesis data in the literature.⁹ In this process the ligand molecule was allowed to be fully flexible to search for the global minimum of the energy function that includes five grid potentials to calculate the interaction between the ligand and the receptor and the internal conformational energy of the ligand. The lowest energy conformation from the predicted conformational stack was selected as the docking orientation. Docking of CCK to the CCK receptor was also achieved by using the protein-protein docking module of ICM with rigid structure of CCK peptide in the docking, followed by 10,000 steps of random sampling and energy minimization of Monte Carlo simulation at 300 K to optimize the complex structure, with distance restraints from established spatial approximation constraints coming from published photoaffinity labeling studies.^{11–14} All the computational work was done on a Linux workstation with a Pentium IV Duo Core processor at 3.0 GHz frequency.

Highlighted in Figure 4A are the critical residues that have been reported to interact with the carboxyl terminus of CCK in the two distinct molecular models that have been proposed.^{1,2} In the model we have previously proposed, Trp³⁹ (Fig. 4A, highlighted in cyan) is situated adjacent to the CCK carboxyl terminus, based on photoaffinity labeling studies.¹¹ In the alternate model, the CCK carboxyl terminus is proposed to be positioned deep within the transmembrane helical bundle based on the effects of mutagenesis of Phe²¹⁸, Trp³²⁷, Ile³³⁰, and Phe³³¹ (Fig. 4A, highlighted in brown). This region of the receptor overlaps with the proposed benzodiazepine binding pocket.⁹ Figure 4B shows the simultaneous docking of CCK and devazepide that is now proposed based on our findings, reflecting the distinct sites of binding for these compounds. For CCK, the binding site resides at the extracellular face of the lipid bilayer, between the amino-terminal tail and the three extracellular loops. In contrast, devazepide binding occurs deep in the helical bundle, surrounded by several key interacting residues that have been identified by receptor mutagenesis, including Cys⁹⁴, Met¹²¹, Ser¹²⁴, Val¹²⁵, Ile³³⁰, Asn³³⁴, Leu³⁵⁷ and Ser³⁶⁴.⁹

To date, non-peptide ligands of the CCK₁ receptor have been predominantly viewed as classic antagonists or agonists, presumably acting at a site overlapping with the endogenous/orthosteric binding site of the natural ligand, CCK. Over the past 5 years, however, there has been an increasing recognition that G protein-coupled receptors may also possess allosteric sites that are topographically distinct from the orthosteric binding pocket. We now provide conclusive evidence that such a site exists on the CCK₁ receptor, and that this site is recognized by small molecule benzodiazepines.

A large body of data exists concerning structure-function analysis of the CCK₁ receptor and the potential mode of binding of both peptide ligands and non-peptide small molecule compounds. Although there is general agreement that the small molecule binding site resides deep within the transmembrane helical bundle, two distinct models of the mode of CCK binding to the CCK₁ receptor have been proposed; one is based predominantly on photoaffinity labeling and fluorescence resonance energy transfer measurements of modified CCK peptides,^{1,11–13} while the other is supported by extensive site-directed mutagenesis.² The former model, which has been proposed by our laboratory, places the CCK peptide on the extracellular face of the receptor, with the peptide in a configuration including a turn such that its carboxyl terminus is in close proximity to Trp³⁹ of the receptor amino terminus. In contrast, the latter model places the peptide in an extended conformation with its carboxyl terminus deep within the transmembrane bundle, such that it overlaps with the proposed small molecule binding pocket.

Standard equilibrium binding assays have suggested that many of the non-peptide compounds interact competitively with CCK, with full inhibition of binding of radiolabelled CCK peptides. This is true of the Sanofi series of compounds, such as SR146131,¹⁵ and also of the compound, devazepide, that is used in the current study. Superficially, these data are consistent with the model of CCK binding where the peptide carboxyl terminus and the non-peptide compounds overlap. However, allosteric compounds with high negative cooperativity can exhibit similar behavior to competitive inhibitors, such that these two classes of compounds are not readily distinguishable in equilibrium binding assays.³ This has also been demonstrated in our current study with respect to the equilibrium interaction between devazepide and [¹²⁵I]CCK. However, an examination of the interactive properties of compounds 6 and 8, two benzodiazepines developed by GlaxoSmithKline, clearly revealed binding behavior that was inconsistent with simple competition but could be reconciled with a simple allosteric ternary complex model. Given that we have previously shown devazepide to inhibit the ability of derivatives of compounds 6 and 8 to photoaffinity label the CCK₁ receptor,⁹ we hypothesize that all benzodiazepines that bind to this receptor utilize a common, allosteric site.

An allosteric mode of interaction would also favor the model of CCK-receptor interaction proposed from biochemical and biophysical interaction data. As such, the current study has exploited a specific assay for allosteric interaction, the ability to modify the dissociation rate of a prebound orthosteric ligand, and probed the mode of action of devazepide, which, as outlined above, is currently viewed as a competitive inhibitor of CCK binding on the basis of equilibrium binding data. Co-addition of devazepide caused a marked slowing of the dissociation of the radioiodinated CCK, which can only occur if it binds at a topographically distinct site to that of the peptide ligand. Thus, we conclude that the equilibrium binding properties of devazepide reflect high negative cooperativity rather than orthosteric competition.

We further probed the nature of the devazepide interaction through modification of Cys⁹⁴, a residue that plays a key role in non-peptide drug interaction.⁸ Derivatization of this residue with Alexa⁵⁶⁸-MTSEA, completely abolished the ability of devazepide to slow CCK dissociation, consistent with binding of the drug deep within the transmembrane helical bundle. However, the chemical modification also altered the control of CCK dissociation, potentially via conformational modification of the receptor, making clear interpretation of the effect on devazepide difficult. As outlined above, however, an allosteric mode of binding for devazepide is consistent with the ability of this compound to compete for binding of photoactive 1,5-benzodiazepine derivatives, whereas CCK was unable to modify compound binding.⁹

Our data thus demonstrate that the prototypic benzodiazepine antagonist, devazepide, is an allosteric inhibitor of CCK binding. Moreover, our application of a quantitative model of allosteric interaction to the binding properties of devazepide and other benzodiazepine modulators of the CCK₁ receptor allowed for the determination of modulator affinity estimates for the allosteric site, as well as measure of the cooperativity with the orthosteric ligand. These findings have significant implications for the development of small molecule non-peptide drugs acting at the CCK₁ receptor. For example, in the case of inhibitors, the activity of competitive antagonists is governed by affinity, whereas the behavior of allosteric inhibitors is a composite of both affinity and cooperativity.³ Structure-activity development that does not take the allosteric nature of drug interaction into account may lead to suboptimal compounds. The current study also provides additional evidence for evaluation of the potential mode of CCK peptide interaction with the receptor, and is consistent with the topographically distinct mode of peptide and small molecule binding that we have proposed.

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Figure 1. Dissociation of CCK_1 receptor-bound $[^{125}\text{I}]\text{CCK}$ in the absence (■) or presence (●) of devazepide (10 nM) from CHO-CCKR cell membranes.

Figure 2. Dissociation of CCK₁ receptor-bound [¹²⁵I]CCK in the absence (■) or presence (●) of devazepide (10 nM) from Alexa-MTSEA-derivatized CHO-CCKR cell membranes.

Figure 3. Effect of CCK (●), devazepide (○), compound 6 (□) or compound 8 (△) on the equilibrium binding of $[^{125}\text{I}]\text{CCK}$. Data for compounds 6 and 8 have been re-plotted from that presented in reference ⁹. Curves through the points represent the best fit of a competitive model (CCK) or an allosteric ternary complex model (devazepide, compounds 6 and 8).

Figure 4.

Panel A. CCK₁ receptor residues proposed to interact with the carboxyl terminus of CCK in two distinct models. Trp³⁹ (cyan) was identified by photoaffinity labeling and places the CCK carboxyl terminus at the extracellular face of the receptor; Phe²¹⁸, Trp³²⁷, Ile³³⁰ and Phe³³¹ (brown) are residues identified via mutagenesis data that have been used to alternatively place the CCK carboxyl terminus deep within the transmembrane helical bundle domain. *Panel B.* Computational docking of CCK (green) and devazepide (yellow) to the CCK₁ receptor.

Table 1
CCK₁ receptor ligand equilibrium binding parameters

Parameter	Inhibitor			
	CCK	Devazepide	Compound 6	Compound 8
LogK _B ^a	8.89 ± 0.04	8.35 ± 0.07	7.26 ± 0.11	6.39 ± 0.19
Logα ^b	n.a. ^c	< -100 (α ≈ 0)	-0.65 ± 0.08 (α = 0.22)	-0.23 ± 0.04 (α = 0.59)

^a negative logarithm of equilibrium dissociation constant for binding to the CCK₁ receptor.

^b negative logarithm of cooperativity factor, describing interactions between occupied orthosteric and allosteric sites; antilogarithm shown in parentheses.

^c not applicable.