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Direct demonstration of unique mode of natural peptide binding to the type 2 cholecystokinin receptor using photoaffinity labeling

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Abstract

Direct analysis of mode of peptide docking using intrinsic photoaffinity labeling has provided detailed insights for the molecular basis of cholecystokinin (CCK) interaction with the type 1 CCK receptor. In the current work, this technique has been applied to the closely related type 2 CCK receptor that also binds the natural full agonist peptide, CCK, with high affinity. A series of photolabile CCK analogue probes with sites of covalent attachment extending from position 26 through 32 were characterized, with the highest affinity analogues that possessed full biological activity utilized in photoaffinity labeling. The position 29 probe, incorporating a photolabile benzoyl-phenylalanine in that position, was shown to bind with high affinity and to be a full agonist, with potency not different from that of natural CCK, and to covalently label the type 2 CCK receptor in a saturable, specific and efficient manner. Using proteolytic peptide mapping, mutagenesis, and radiochemical Edman degradation sequencing, this probe was shown to establish a covalent bond with type 2 CCK receptor residue Phe¹²⁰ in the first extracellular loop. This was in contrast to its covalent attachment to Glu³⁴⁵ in the third extracellular loop of the type 1 CCK receptor, directly documenting differences in mode of docking this peptide to these receptors.

1. Introduction

As experience with the structural characterization of class A guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs) grows, with more than twenty such crystal structures now solved, the high level of conservation of the helical bundle domain structure has become clear [25]. This provides insights into the mode of docking of small molecule ligands targeting this region, and has facilitated the *in silico* prediction of the docking of similar ligands to analogous sites in other class A GPCRs [25]. It has also become clear that the extracellular loop and amino-terminal tail regions of these receptors are structurally quite diverse. These regions have traditionally been considered to be important for the docking of peptide ligands for these receptors [20].

The current work focuses on the mode of docking the peptide hormone, cholecystokinin (CCK), to the type 2 CCK receptor (CCK2R) using the direct method of intrinsic

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photoaffinity labeling. We have previously utilized this approach to map spatial approximations of six positions within the pharmacophore of this peptide with the type 1 CCK receptor (CCK1R) [1,6,7,12,16]. Previous photoaffinity labeling of both of these structurally related, yet functionally distinct, receptors through CCK ligand position 33 (based on the numbering of the 33-residue peptide first isolated from porcine intestine) [8,16] and outside the pharmacophore at the amino terminus of the peptide [4,8] have suggested that this ligand docks differently to the two subtypes of CCK receptors. This was also suggested based on peptide ligand structure-activity series [18], receptor mutagenesis studies [18], and fluorescent ligand probe analysis [13].

In the current work, we have examined photolabile probes with sites of covalent attachment in each position of CCK from 26 through 32, filling in every other position within the CCK pharmacophore. This has provided both additional structure-activity insights and new specific spatial approximation data for position 29 that continue to refine our understanding of the docking of the natural peptide ligand to the type 2 CCK receptor.

2. Materials and methods

2.1. Materials

Synthetic CCK-26-33 (CCK-8) was purchased from Bachem (Torrance, CA), while all other CCK peptides were custom synthesized. Cyanogen bromide (CNBr), solid-phase oxidant, N-chlorobenzenesulfonamide (Iodo-beads), and m-maleimidobenzoyl-N-hydroxysuccinimide were from Thermo Scientific Pierce (Rockford, IL). Concanavalin A (Con A)-immobilized lectin beads were from EY Laboratories (San Mateo, CA), soybean trypsin inhibitor was from Invitrogen (Carlsbad, CA), and Fura-2-acetoxymethyl ester (Fura-2AM) was from Molecular Probes (Eugene, OR). Fetal clone II culture medium supplement was from Hyclone laboratories (Logan, UT). All other reagents were of analytical grade.

2.2. Peptides

The photolabile CCK analogue used as the predominant probe in this study was (des-amino-Tyr)-Gly-[(Nle²⁸,Bpa²⁹,Nle³¹)CCK-26-33] (Bpa²⁹ probe) (Fig. 1). It contained a photolabile *p*-benzoyl-L-phenylalanine (Bpa) in position 29 at the amino-terminal end of the type 2 CCK receptor-binding pharmacophore as the site for covalent attachment. It incorporated a des-amino-tyrosyl residue at the amino-terminal end of the peptide as a site for radioiodination that was deficient in primary amino group to prevent peptide derivatization and cleavage during Edman degradation sequencing. It also included two norleucines in positions 28 and 31 to replace naturally-occurring methionines in these positions to prevent oxidative damage during radioiodination. Other photolabile analogues of CCK also utilized in this study included (des-amino-Tyr)-Gly-[(Bpa²⁶,Nle²⁸,Nle³¹)CCK-26-33] (Bpa²⁶ probe), (des-amino-Tyr)-Gly-[(NO₂-Phe²⁶,Nle²⁸,Nle³¹)CCK-26-33] (NO₂-Phe²⁶ probe), (*D*-Tyr)-Gly-[(Bpa²⁷,Nle²⁸,Nle³¹)CCK-26-33] (Bpa²⁷ probe), (*D*-Tyr)-Gly-[(NO₂-Phe²⁷,Nle²⁸,Nle³¹)CCK-26-33] (NO₂-Phe²⁷ probe) [1], (des-amino-Tyr)-Gly-[(BzBz)Lys²⁸,Nle³¹)CCK-26-33] ((BzBz)Lys²⁸ probe) [7], (des-amino-Tyr)-Gly-[(Nle²⁸,Bpa³⁰,Nle³¹)CCK-26-33] (Bpa³⁰ probe) [6], (des-amino-Tyr)-Gly-[(Nle²⁸,NO₂-Phe³⁰,Nle³¹)CCK-26-33] (NO₂-Phe³⁰ probe) [6], (des-amino-Tyr)-Gly-[(Nle²⁸,BzBz)Lys³¹)CCK-26-33] ((BzBz)Lys³¹ probe) [7], and (des-amino-Tyr)-Gly-[(Nle²⁸,Nle³¹,Bpa³²)CCK-26-33] (Bpa³² probe).

The photolabile probes and the CCK-like peptide that was radiolabeled and used in competitive ligand binding assays (*D*-Tyr-Gly-[(Nle²⁸,Nle³¹)CCK-26-33]) were synthesized by manual solid phase techniques. This was performed with Pal resin and Fmoc-blocked amino acids, with products purified by reversed-phase HPLC on octadecylsilane, as

described previously [22]. The expected molecular masses of the synthetic peptides were verified by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. Both the Bpa²⁹ probe and the CCK radioligand were radioiodinated using oxidation with Iodo-beads and Na¹²⁵I, as we have described [22]. Radiolabeled peptides were purified to yield approximate specific radioactivities of 2,000 Ci/mmol after purification by reversed-phase HPLC, as we have described [22].

2.3. Receptor Source

Chinese hamster ovary (CHO) cell lines stably expressing the hemagglutinin-tagged wild type human type 2 CCK receptors (CHO-CCK2R) [3] and M134L mutant (CHO-M134L-CCK2R) [8] that were prepared previously, were used for the current study. Cells were cultured at 37 °C in an environment containing 5% CO₂ on tissue culture plasticware in Ham's F12 medium with 5% fetal clone II supplement before being used for assays and for membrane preparations. Cells were passaged approximately twice a week and lifted mechanically before use. Membranes were prepared using a sucrose gradient as we have described [11], and were stored at -80 °C in Krebs-Ringers/HEPES (KRH) medium (25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM KH₂PO₄, 1.2 mM MgSO₄) containing 0.01% soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride, until ready for use in ligand binding and photoaffinity labeling studies.

2.4. Intracellular calcium assays

Biological activity was determined by examining the ability for a given ligand to stimulate intracellular calcium responses in CHO-CCK2R cells by measuring fluorescence of a calcium-sensitive dye in these cells after stimulation with CCK or each of the CCK photolabile analogues. This was performed using a FlexStation 3.0 (Molecular Devices, Sunnyvale, CA) and Softmax Pro 5.4 software, following a procedure similar to that we previously described [21]. The only modification was the use of 1.5 μM Fluo-8 (AAT Bioquest, Sunnyvale, CA) instead of Fura-2AM as the fluorescence indicator. Stimulated calcium responses were calculated as percentages of maximal levels stimulated by 1 μM CCK. All assays were performed in duplicate and repeated at least three times in independent experiments. The calcium concentration-response curves were analyzed and plotted using non-linear regression analysis in the Prism software suite v3.0 (GraphPad Software, San Diego, CA).

2.5. Ligand binding assays

The binding characteristics of the photolabile probes were determined in standard radioligand competition-binding assays [11]. In brief, 7.5 μg of CHO-CCK2R membranes were incubated at room temperature for 1 h with a constant amount of the CCK-like radioligand (~5 pM, ~20,000 cpm) in KRH medium containing 0.2% bovine serum albumin and 0.01% soybean trypsin inhibitor in the absence or presence of increasing concentrations of CCK or the relevant probe. Membrane-bound and free radioligand were separated by filtration through a Unifilter-96 GF/B filter plate in a FilterMate harvester (PerkinElmer Life Sciences), as we have described [2]. Radioactivity in the plate was quantified using the TopCount NXTTM instrument (Packard) with approximate 23 percent efficiency for this radioisotope.

2.6. Photoaffinity labeling of the type 2 CCK receptor

Approximately 50 μg of plasma membranes from cells expressing wild-type (CHO-CCK2R) or mutant (CHO-M134L-CCK2R) receptors, or from control non-receptor-bearing parental CHO cells were incubated with ~0.1 nM radioiodinated Bpa²⁹ probe in the absence or

presence of increasing concentrations of nonradioactive CCK ranging from 0 to 1 μM for 1 h at room temperature in the dark.

The reactions were then exposed to UV irradiation with 3500-Å lamps for 30 min at 4°C in a Rayonet photochemical reactor (Southern New England Ultraviolet, Hamden, CT). Membranes were solubilized with 1% Nonidet P-40 in KRH medium and receptors were purified using adsorption to Con A lectin beads prior to separation on 10% SDS-polyacrylamide (PAGE) gels [17]. Radiolabeled bands were visualized by autoradiography. The apparent molecular masses of radiolabeled receptor fragments were determined by interpolation on a plot of the mobility of ProSieve protein standards (Cambrex, Rockland, ME) (Invitrogen) versus the log values of their apparent masses.

2.7. Identification of receptor regions and sites of covalent labeling

For peptide mapping, photoaffinity labeling was performed in larger scale using approximately 200 μg membranes and 0.5 nM ^{125}I -Bpa²⁹ probe. After photolysis, Nonidet P-40 solubilization and Con A lectin-adsorption purification were performed as described above, and component proteins were separated on 10% SDS-PAGE gels. Labeled receptor bands were cut from gels, eluted, lyophilized, and precipitated with ethanol prior to peptide mapping.

Purified labeled receptor was deglycosylated by treatment with *N*-glycosidase F (PNGase F) following the manufacturer's protocol (Proenzyme, San Leandro, CA). CNBr cleavage of the purified labeled wild type and mutant receptor constructs was performed under conditions described previously [9], and the cleavage products were separated on 10% NuPAGE gels (Invitrogen, Carlsbad, CA) using MES running buffer. The apparent molecular masses of radiolabeled receptor fragments were determined by interpolation on a plot of the mobility of Multimark protein standards (Invitrogen) versus the log values of their masses.

After achieving definitive identification of the receptor fragment labeled with the Bpa²⁹ probe, the specific site of attachment was identified by radiochemical Edman degradation sequencing. For this, labeled wild type receptor was purified and cleaved using CNBr, and the resultant fragment (Gly¹¹⁸-Met¹³⁴) was purified on a gel to achieve radioactive homogeneity before being covalently coupled through the thiol group of Cys¹²⁷ with maleimidobenzoyl succinimide-derivatized *N*-(2-aminoethyl -1)-3-aminopropyl glass beads (Sigma). After this, repetitive cycles of manual Edman degradation were performed with quantitation of radioactivity released in each cycle as we have described [16]. This procedure was repeated three times in independent experiments.

2.8. Statistical analysis

All observations have been repeated in duplicate at least three times and are expressed as means $\pm$ S.E.M. Data were analyzed for differences using two-tailed *P*-value tests using InStat3 (GraphPad Software, San Diego, CA) with *P* < 0.05 considered to be significant.

3. Results

3.1. Screening of a series of photolabile CCK analogue probes for the type 2 CCK receptor

Shown in Figure 2 are curves reflecting the abilities of a series of photolabile probes to compete for binding of the CCK-like radioligand to membranes from the type 2 CCK receptor. Previous photoaffinity labeling of this receptor was performed with probes with sites of covalent attachment in position 33, at the carboxyl terminus, and at the amino terminus, outside the pharmacophore [8]. Screening of probes in all other positions between residue 26 and 32 showed that the order of affinity was CCK > NO₂-Phe²⁶ probe >

(BzBz)Lys²⁸ probe > Bpa²⁹ probe > NO₂-Phe²⁷ probe > NO₂-Phe³⁰ probe > Bpa²⁷ probe > Bpa²⁶ probe > (BzBz)Lys³¹ probe > Bpa³⁰ probe > Bpa³² probe (Fig. 2 and Table 1). While probes with photolabile nitro-phenylalanine (NO₂-Phe) in position 26 and benzoyl-benzoyl-lysine ((BzBz)Lys) in position 28 bound with high affinities (Table 1), the efficiencies of covalent labeling of the receptor using these probes were very low (data not shown). In contrast, the Bpa²⁹ probe exhibited adequate affinity (only 39-fold lower than that of natural CCK) and efficiency of covalent labeling to identify its site of covalent attachment (see below). All other probes had affinities that were too low to pursue for photoaffinity labeling.

Each of the probes was also studied for its ability to stimulate intracellular calcium responses in type 2 CCK receptor-bearing CHO-CCK2R cells. All of the probes, except for the NO₂-Phe²⁶ and Bpa³² probes, were full agonists, stimulating maximal responses that were no different from those stimulated by natural CCK (Fig. 2). The NO₂-Phe²⁶ probe, despite having relatively high binding affinity, had only minimal biological activity (Fig. 2 and Table 1) and was, therefore, not a candidate for photoaffinity labeling of a biologically relevant site. In contrast, the Bpa²⁹ probe was a full agonist, stimulating maximal levels of intracellular calcium in the CHO-CCK2R cells that were similar to that stimulated by CCK, with potency not significantly different from that of CCK (Fig. 2 and Table 1).

3.2. Photoaffinity labeling of the type 2 CCK receptor

The Bpa²⁹ probe was used in photoaffinity labeling studies of the type 2 CCK receptor, complementing similar studies previously done with the type 1 CCK receptor [12]. As shown in Figure 3, this probe labeled the receptor specifically and saturably, with labeling being inhibited by CCK in a concentration-dependent manner ($IC_{50} = 1.7 \pm 0.4$ nM). There was a single predominant band that was labeled with the Bpa²⁹ probe. This band migrated at molecular mass of approximate $M_r = 85,000$ -95,000 and shifted to approximate $M_r = 42,000$ after deglycosylation with PNGase F, as expected for this receptor. No radioactive band was observed in affinity labeled membranes prepared from non-receptor-bearing CHO cells.

3.3. Identification of the site of labeling

We previously demonstrated quantitative cleavage of the type 2 CCK receptor using CNBr [8], providing a useful tool for preliminary identification of labeled fragments, since this receptor contains ten methionine residues and CNBr cleavage would theoretically result in 11 receptor fragments ranging in mass from 0.1 to 11 kDa, with only one of these containing sites of glycosylation (Fig. 4). Here, we again utilized CNBr cleavage of this receptor labeled with the Bpa²⁹ probe. As shown in Figure 4, CNBr cleavage of the labeled type 2 CCK receptor yielded a labeled band that migrated at approximate $M_r = 4,000$ that did not shift after deglycosylation. Considering the mass of the attached Bpa²⁹ probe (1,508 Da) and the non-glycosylated nature of the labeled band, the labeled receptor fragment was tentatively identified as the fragment spanning the first extracellular loop and top of the third transmembrane segment (Gly¹¹⁸-Met¹³⁴, highlighted in grey circles in Fig. 4). To confirm this, we utilized a receptor mutant that eliminated one of the sites of CNBr cleavage adjacent to this fragment (M134L). This mutant was also labeled by the Bpa²⁹ probe specifically and saturably (data not shown), and its cleavage with CNBr yielded a single predominant labeled fragment migrating at approximate $M_r = 8,500$. This was clearly larger than the fragment of the wild type receptor treated similarly, confirming the identity of this band.

The identification of the specific site of covalent labeling of the type 2 CCK receptor with the Bpa²⁹ probe was achieved by Edman degradation radiochemical sequencing. This was applied to the purified, labeled Gly¹¹⁸-Met¹³⁴ fragment immobilized on maleimidobenzoyl succinimide-derivatized *N*-(2-aminoethyl -1)-3-aminopropyl glass beads. Figure 5 shows

that a major radioactive fraction eluted in the third cycle, representing the labeling of Phe¹²⁰ within the first extracellular loop of the receptor with this probe.

4. Discussion

It has been extremely challenging to determine the molecular basis of natural peptide docking to and activation of receptors. This relates to the flexibility of peptides and to the absence of consistent structural motifs within the loop and tail regions of GPCRs known to be important for peptide and protein ligand docking. These receptor regions are quite varied in their length and amino acid composition and are limited by few structural constraints. A disulfide bond is often present in this superfamily of receptors linking cysteine residues at the carboxyl-terminal end of the first extracellular loop (ECL1) above transmembrane segment three (TM3) and within ECL2. Nevertheless, there are examples of GPCRs in which the ECLs tend to converge and close off the center of the helical bundle [23] and those in which they splay out and expose the top of the bundle [26].

It might be predicted that two GPCRs that are as closely related in sequence as the type 1 and type 2 CCK receptors and that even are able to bind and to be activated by the same peptide ligand, CCK, would be structurally highly similar and would dock this peptide in similar manner. However, indirect studies suggest the presence of quite distinct modes of peptide binding. This includes peptide structure-activity series in which the CCK pharmacophore most critical for binding and activation of the type 1 CCK receptor includes the carboxyl-terminal heptapeptide amide with the sulfate moiety on the tyrosine residue seven positions from the carboxyl terminus (position 27) very important [5]. In contrast, the CCK pharmacophore most critical for binding and activation of the type 2 CCK receptor includes only the carboxyl-terminal tetrapeptide amide [18]. Tyrosine sulfation is not important for the type 2 CCK receptor. This is further emphasized by this region of CCK being shared by another natural hormone, gastrin, which binds to and activates the type 2 CCK receptor (also known as the gastrin receptor). Both hormones share their carboxyl-terminal pentapeptide-amide sequence.

Other data indirectly supporting differences in the mode of docking of natural ligands at the two types of CCK receptors include distinct fluorescence properties, such as lifetimes, anisotropy, and ease of quenching, of peptide probes having indicator fluorophores distributed throughout the peptides [13,14]. An alexa-indicator-containing probe with the fluorophore at the amino-terminal end of CCK-8 showed that lifetime and anisotropy were lower when bound to the type 2 CCK receptor than when bound to the type 1 CCK receptor, suggesting that the indicator in this position was more exposed to the aqueous milieu when docked at the type 2 CCK receptor [13]. These observations were confirmed and extended using probes with aladan situated at the amino terminus, mid-region, and carboxyl terminus of CCK-8 [14]. In that work, the carboxyl-terminal indicator was more easily quenched with water-soluble sodium iodide when docked at the type 1 CCK receptor than at the type 2 CCK receptor, suggesting that it might be inserted into the helical bundle in the type 2 CCK receptor. Analogous mutations in the two CCK receptors have also had different effects on CCK binding and activation [24]. While five amino acid residues in ECL1 and ECL2 of the type 1 CCK receptor were shown to affect CCK binding affinity, only three of the analogous mutations in the type 2 CCK receptor affected CCK binding affinity to that receptor.

There have also been limited observations that have more directly established differences in CCK docking to the two subtypes of CCK receptors [8,16]. The most striking difference is the different sites of covalent labeling of spatially approximated residues with a photolabile Bpa positioned at the carboxyl-terminal end of CCK analogues in position 33 [8,16]. This probe labeled Trp³³ in the type 1 CCK receptor, just above TM1, while it labeled Thr¹¹⁹ in

the type 2 CCK receptor in ECL1 [8,16]. In contrast to the differential docking of the carboxyl terminus of CCK in these two receptors that has been predicted based on these direct observations, predictions have been made that the carboxyl-terminal end of CCK dips into the helical bundle of both the type 1 and type 2 CCK receptors, based on more indirect interpretations of mutagenesis and peptide modification studies [15,19]. These types of structure-activity studies are often quite difficult to interpret, since changes in natural residues can alter receptor functions such as ligand binding and/or biological activity, by either direct or indirect means due to modification of folding and conformation.

Meaningful covalent labeling of a spatially approximated residue in a receptor through a photolabile moiety incorporated into a ligand of that receptor requires adequate affinity of the ligand to dock. When the probe is also an agonist analogue of the natural ligand, it provides another level of confidence that the docking is likely normal and that the determinants for inducing a productive conformational change in the receptor are intact. There is typically a balance that must be met between modifying the ligand in sites critical for interaction that can lower its binding affinity and biological potency or efficacy and placing the photolabile moiety so far away from important regions that the spatial approximation becomes less useful. In the current studies, we, therefore, attempted to insert a photolabile moiety into every position between 26 and 32 of CCK. Within the actual type 2 CCK receptor pharmacophore, representing the carboxyl-terminal tetrapeptide (positions 30-33), only the previous insertion of Bpa in position 33 [8] was tolerated. Benzophenone moieties like Bpa and (BzBz)Lys have optimal photochemical properties for photoaffinity labeling, not being quenched by water and able to regenerate the ground state until they react productively with an adjacent carbonyl group [10]. Unfortunately, benzophenones were not tolerated in positions 30, 31, or 32. In contrast, nitro-aryl groups have less ideal photochemical properties, but have been successfully utilized in photoaffinity labeling studies [1,6]. Indeed, the NO₂-Phe³⁰ probe bound with adequate affinity, but, unfortunately, the efficiency of forming a covalent bond with the receptor through this group was too low to be useful. This was also true of the NO₂-Phe²⁶ probe, although that probe also had no biological activity. It is not clear whether the low efficiency of covalent labeling with these probes reflects their less efficient photochemistry or absence of spatial approximation with the receptor as they dock.

The Bpa²⁹ probe was the most useful probe to covalently label the type 2 CCK receptor in this series. It bound in a specific and saturable manner with high affinity. It also was a full agonist, able to stimulate the same maximal biological response as natural CCK (or gastrin) at this receptor. This provided confidence that the spatial approximation constraint generated with this probe was likely meaningful. Of note, this probe labeled residue Phe¹²⁰ within ECL1 of the receptor, while it had labeled residue His³⁴⁷ within ECL3 in the type 1 CCK receptor [12]. This again provides direct evidence for distinct spatial approximation for residues along CCK close to and within its pharmacophore as docked productively at the type 2 and type 1 CCK receptor.

Phe¹²⁰ was also previously identified as an important residue in ECL1 of the type 2 CCK receptor in a mutagenesis study directed to examine possible interactions with ligand residue Trp³⁰ (referred to as Trp6 in a nine-amino acid analogue of CCK), immediately adjacent to residue 29 that is the focus of the current work [19]. Mutations of Phe¹²⁰ in which the natural residue was changed to Trp, Tyr, His, Met, Leu and Ala were prepared and studied. Of these, only the Trp replacement was tolerated without significant reductions in CCK binding affinity and biological potency. The F120W mutant was observed to yield five-fold gain in affinity and potency, explained in a working model by moving the aromatic side chains of the proposed interacting residues 1 Å closer to each other than for the natural residues. While this does not represent a proof of interaction, it is of interest, and is clearly

consistent with the current report of spatial approximation between receptor residue Phe¹²⁰ and the ligand residue immediately adjacent to Trp³⁰.

It is particularly interesting that three photoaffinity labeling spatial approximation constraints now exist for the type 2 CCK receptor (through peptide positions 24, 29, and 33), and all three residues that are covalently labeled are within ECL1 of this receptor. In Figure 6, we have highlighted the patterns of photoaffinity labeling of the type 2 and type 1 CCK receptors through positions along the pharmacophore of CCK as docked productively to both receptors. It is apparent that there are differences in the mode of docking this peptide for the two related receptors. While the position 24, 29, and 33 probes all label ECL2 of the type 2 CCK receptor, the position 24 and 29 probes label ECL3 of the type 1 CCK receptor and the position 33 probe labels a residue above TM1 in the amino-terminal tail of the type 1 CCK receptor. The other probes utilized for the type 1 CCK receptor photolabeling were not tolerated or efficient enough to label the type 2 CCK receptor. These observations also support differences in docking with those ligand residues likely not approximated with residues within the type 2 CCK receptor.

While we have collected an extensive set of additional constraints for the type 1 CCK receptor that has provided adequate insight to build a credible molecular model, analogous data do not currently exist for the type 2 CCK receptor. Because of flexibility in the loops and amino terminus of this receptor, we can only be confident in spatial approximation as docked, but we are not yet able to incorporate all of these into a meaningful three dimensional model. We can, however, be quite confident that this model will be distinct from that of the type 1 CCK receptor, with this peptide docked in different conformation and at a different site than that at the type 1 CCK receptor.

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References

1. Arlander SJ, Dong M, Ding XQ, Pinon DI, Miller LJ. Key differences in molecular complexes of the cholecystokinin receptor with structurally related peptide agonist, partial agonist, and antagonist. *Mol Pharmacol.* 2004; 66:545–52. [PubMed: 15322246]
2. Cawston EE, Lam PC, Harikumar KG, Dong M, Ball AM, Augustine ML, Akgun E, Portoghesi PS, Orry A, Abagyan R, Sexton PM, Miller LJ. Molecular basis for binding and subtype selectivity of 1,4-benzodiazepine antagonist ligands of the cholecystokinin receptor. *J Biol Chem.* 2012; 287:18618–35. [PubMed: 22467877]
3. Cheng ZJ, Harikumar KG, Holicky EL, Miller LJ. Heterodimerization of type A and B cholecystokinin receptors enhance signaling and promote cell growth. *J Biol Chem.* 2003; 278:52972–9. [PubMed: 14534299]
4. Ding XQ, Dolu V, Hadac EM, Holicky EL, Pinon DI, Lybrand TP, Miller LJ. Refinement of the structure of the ligand-occupied cholecystokinin receptor using a photolabile amino-terminal probe. *J Biol Chem.* 2001; 276:4236–44. [PubMed: 11050076]
5. Ding XQ, Pinon DI, Furse KE, Lybrand TP, Miller LJ. Refinement of the conformation of a critical region of charge-charge interaction between cholecystokinin and its receptor. *Mol Pharmacol.* 2002; 61:1041–52. [PubMed: 11961122]
6. Dong M, Hadac EM, Pinon DI, Miller LJ. Differential spatial approximation between cholecystokinin residue 30 and receptor residues in active and inactive conformations. *Mol Pharmacol.* 2005; 67:1892–900. [PubMed: 15774770]

7. Dong M, Lam PC, Pinon DI, Abagyan R, Miller LJ. Elucidation of the molecular basis of cholecystokinin Peptide docking to its receptor using site-specific intrinsic photoaffinity labeling and molecular modeling. *Biochemistry*. 2009; 48:5303–12. [PubMed: 19441839]
8. Dong M, Liu G, Pinon DI, Miller LJ. Differential docking of high-affinity peptide ligands to type A and B cholecystokinin receptors demonstrated by photoaffinity labeling. *Biochemistry*. 2005; 44:6693–700. [PubMed: 15850403]
9. Dong M, Wang Y, Pinon DI, Hadac EM, Miller LJ. Demonstration of a direct interaction between residue 22 in the carboxyl-terminal half of secretin and the amino-terminal tail of the secretin receptor using photoaffinity labeling. *J Biol Chem*. 1999; 274:903–9. [PubMed: 9873030]
10. Galardy RE, Craig LC, Printz MP. Benzophenone triplet: a new photochemical probe of biological ligand-receptor interactions. *Nat New Biol*. 1973; 242:127–8. [PubMed: 4513418]
11. Hadac EM, Ghanekar DV, Holicky EL, Pinon DI, Dougherty RW, Miller LJ. Relationship between native and recombinant cholecystokinin receptors: role of differential glycosylation. *Pancreas*. 1996; 13:130–9. [PubMed: 8829180]
12. Hadac EM, Pinon DI, Ji Z, Holicky EL, Henne RM, Lybrand TP, Miller LJ. Direct identification of a second distinct site of contact between cholecystokinin and its receptor. *J Biol Chem*. 1998; 273:12988–93. [PubMed: 9582333]
13. Harikumar KG, Clain J, Pinon DI, Dong M, Miller LJ. Distinct molecular mechanisms for agonist peptide binding to types A and B cholecystokinin receptors demonstrated using fluorescence spectroscopy. *J Biol Chem*. 2005; 280:1044–50. [PubMed: 15520004]
14. Harikumar KG, Pinon DI, Miller LJ. Fluorescent indicators distributed throughout the pharmacophore of cholecystokinin provide insights into distinct modes of binding and activation of type A and B cholecystokinin receptors. *J Biol Chem*. 2006; 281:27072–80. [PubMed: 16857665]
15. Henin J, Maigret B, Tarek M, Escricuit C, Fourmy D, Chipot C. Probing a model of a GPCR/ligand complex in an explicit membrane environment: the human cholecystokinin-1 receptor. *Biophys J*. 2006; 90:1232–40. [PubMed: 16326901]
16. Ji Z, Hadac EM, Henne RM, Patel SA, Lybrand TP, Miller LJ. Direct identification of a distinct site of interaction between the carboxyl-terminal residue of cholecystokinin and the type A cholecystokinin receptor using photoaffinity labeling. *J Biol Chem*. 1997; 272:24393–401. [PubMed: 9305898]
17. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970; 227:680–5. [PubMed: 5432063]
18. Miller LJ, Gao F. Structural basis of cholecystokinin receptor binding and regulation. *Pharmacol Ther*. 2008; 119:83–95. [PubMed: 18558433]
19. Paillasse MR, Deraeve C, de Medina P, Mhamdi L, Favre G, Poirot M, Silvente-Poirot S. Insights into the cholecystokinin 2 receptor binding site and processes of activation. *Mol Pharmacol*. 2006; 70:1935–45. [PubMed: 16998007]
20. Peeters MC, van Westen GJ, Li Q, AP II. Importance of the extracellular loops in G protein-coupled receptors for ligand recognition and receptor activation. *Trends Pharmacol Sci*. 2011; 32:35–42. [PubMed: 21075459]
21. Potter RM, Harikumar KG, Wu SV, Miller LJ. Differential sensitivity of types 1 and 2 cholecystokinin receptors to membrane cholesterol. *J Lipid Res*. 2012; 53:137–48. [PubMed: 22021636]
22. Powers SP, Pinon DI, Miller LJ. Use of N,O-bis-Fmoc-D-Tyr-ONSu for introduction of an oxidative iodination site into cholecystokinin family peptides. *Int J Pept Protein Res*. 1988; 31:429–34. [PubMed: 3410633]
23. Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, Thian FS, Chae PS, Pardon E, Calinski D, Mathiesen JM, Shah ST, Lyons JA, Caffrey M, Gellman SH, Steyaert J, Skinotitis G, Weis WI, Sunahara RK, Kobilka BK. Crystal structure of the beta2 adrenergic receptor-Gs protein complex. *Nature*. 2011; 477:549–55. [PubMed: 21772288]
24. Silvente-Poirot S, Escricuit C, Wank SA. Role of the extracellular domains of the cholecystokinin receptor in agonist binding. *Mol Pharmacol*. 1998; 54:364–71. [PubMed: 9687578]

25. Venkatakrisnan AJ, Deupi X, Lebon G, Tate CG, Schertler GF, Babu MM. Molecular signatures of G-protein-coupled receptors. *Nature*. 2013; 494:185–94. [PubMed: 23407534]
26. White JF, Noinaj N, Shibata Y, Love J, Kloss B, Xu F, Gvozdenovic-Jeremic J, Shah P, Shiloach J, Tate CG, Grisshammer R. Structure of the agonist-bound neurotensin receptor. *Nature*. 2012; 490:508–13. [PubMed: 23051748]

Highlights

We use photoaffinity labeling to explore mode of docking CCK to its type 2 receptor.

Ten photolabile CCK analogues have been characterized for type 2 CCK receptor.

Position 29 of CCK is spatially approximated to a residue in its receptor ECL1.

The modes of docking CCK to type 1 and 2 CCK receptors are different.

Fig.1.

Primary structure of the Bpa²⁹ probe. Shown are primary amino acid sequences of natural CCK-8 (CCK-26-33) and the Bpa²⁹ probe. Natural residues are illustrated in clear circles, whereas modified residues are filled with grey, or with black in the case of the photolabile Bpa site of covalent labeling.

Fig. 2.

Functional characterization of CCK probes incorporating photolabile residues in a variety of positions. Shown in the left panel are competition-binding curves using CCK and all listed photolabile CCK probes to compete for the binding of the CCK-like radioligand to CHO-CCK2R membranes. Values illustrated represent binding as percentages of saturable binding in the absence of the competing peptide, and are expressed as the means $\pm$ S.E.M. of data from three independent experiments performed in duplicate. The order of the peptides in the list corresponds to their order of highest to lowest affinity. Shown in the right panel are intracellular calcium responses to increasing concentrations of CCK and all the listed photolabile CCK probes in these cells. Data points represent the means $\pm$ S.E.M. of data from three independent experiments performed in duplicate, normalized relative to the maximal responses to CCK. The order of the peptides in the list corresponds to their order of highest to lowest potency.

Fig. 3. Photoaffinity labeling of the type 2 CCK receptor. Shown are representative autoradiographs of 10% SDS-PAGE gels used to separate the products of photoaffinity labeling of membranes from CHO-CCK2R cells with the Bpa²⁹ probe in the presence of increasing concentrations of competing CCK, as well as the deglycosylation product by PNGase F (left panel). As a control, labeling of the non-receptor-bearing CHO cell membranes in the absence of competitor is also shown. Also shown is the densitometric analysis of data from three similar experiments, with data points expressed as means $\pm$ S.E.M (right panel).

Fig. 4.

CNBr cleavage of wild type and mutant type 2 CCK receptor. Shown is a diagram of the predicted sites of CNBr cleavage of the human type 2 CCK receptor and the calculated masses of resultant fragments. Shown is a representative autoradiograph of a 10% NuPAGE gel used to separate the products of CNBr cleavage of the type 2 CCK receptor labeled with the Bpa²⁹ probe (middle panel). This cleavage resulted in a labeled band migrating at approximate $M_r = 4,000$ that did not shift after deglycosylation by PNGase F. CNBr cleavage of the labeled wild type and M134L mutant receptor membranes are compared in the panel on the right. The band from the M134L mutant receptor was larger than that from the wild type receptor, migrating at approximate $M_r = 8,500$.

Fig. 5. Identification of receptor residue labeled with the Bpa²⁹ probe. Shown is a representative profile of radioactivity eluted during Edman degradation of the labeled band. The peak in radioactivity was found in cycle 3, corresponding to the labeling of receptor residue Phe¹²⁰ within the first extracellular loop of the type 2 CCK receptor.

Fig. 6.

Graphic representation of sites of photoaffinity labeling of the type 1 and type 2 CCK receptors through photolabile moieties spread along the peptide pharmacophore. Shown is a two-dimensional representation of the topology of the CCK receptors with sites of covalent labeling highlighted. Colors are utilized to code for labeled residues within the amino terminus (green), ECL1 (yellow), ECL2 (blue), and ECL3 (red).

Table 1

Quantitative analysis of binding and biological activity data.

Peptides	CCK2R receptor binding affinities IC ₅₀ (nM)	Intracellular calcium responses EC ₅₀ (nM)
CCK	1.5 ± 0.3	0.2 ± 0.1
Bpa ²⁶ probe	205 ± 48 *	17.3 ± 5.9 *
NO ₂ -Phe ²⁶ Probe	24 ± 4 *	> 1000 *
Bpa ²⁷ probe	177 ± 42 *	6.0 ± 1.6 *
NO ₂ -Phe ²⁷ Probe	73 ± 27	1.1 ± 0.4
(BzBz)Lys ²⁸ Probe	57 ± 16 *	0.5 ± 0.2
Bpa²⁹ probe	58 ± 4 *	0.4 ± 0.1
Bpa ³⁰ probe	> 1000 *	83.3 ± 8.9 *
NO ₂ -Phe ³⁰ Probe	90 ± 10 *	1.3 ± 0.4
(BzBz)Lys ³¹ Probe	> 1000 *	36.0 ± 14
Bpa ³² probe	> 1000 *	> 1000 *

IC₅₀ values represent concentrations of each peptide ligand that were able to competitively inhibit the saturable binding of the CCK radioligand to the type 2 CCK receptor on CHO-CCK2R membranes (nM, means ± S.E.M.). EC₅₀ values represent concentrations of each peptide that were able to stimulate half maximal intracellular calcium responses from CHO-CCK2R cells (nM, means ± S.E.M.).

* indicates that the value is significantly different from the CCK control ($P < 0.05$).