Direct Identification of a Second Distinct Site of Contact between Cholecystokinin and Its Receptor*

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We have developed a biologically active analogue of cholecystokinin (CCK) that incorporates a photolabile benzylophenylalanine (Bpa) moiety in the middle of its pharmacophoric domain, which efficiently establishes a covalent bond with an interacting domain of the CCK receptor. This probe incorporated L-Bpa in the position of Gly29 of the well characterized, radioiodinatable CCK analogue, d-Tyr-Gly-[Nle28,31]-CCK-26–33. It was a potent pancreatic secretagogue (EC50 = 28 ± 6 nM) that was equally efficacious with natural CCK, and bound to the CCK receptor with moderate affinity (IC50 = 450 ± 126 nM). This was adequate to allow specific covalent labeling of the receptor. The labeled domain was within the cyanogen bromide fragment of the receptor including the top of TM6 (the sixth transmembrane domain), the third extracellular loop, and TM7 (the seventh transmembrane domain), as proven by direct Edman degradation sequencing. When this fragment was modified by the replacement of Val442 with Met to generate an additional site of cyanogen bromide cleavage, the labeled fragment was reduced in apparent size consistent with its representing the carboxyl-terminal portion of this fragment. Radiochemical sequencing of that fragment demonstrated covalent attachment of the probe to His347 and Leu438 in this domain. This represents the second experimentally demonstrated contact between a CCK analogue and this receptor, complementing the labeling of the domain just above TM1 (the first transmembrane domain) by a photolabile residue at the carboxyl terminus of CCK (Ji, Z. S., Hadac, E. M., Henne, R. M., Patel, S. A., Lybrand, T. P., and Miller, L. J. (1997) J. Biol. Chem. 272, 24393–24401). Both contacts are consistent with the conformational model of CCK binding proposed on the basis of the initial contact.

A molecular understanding of agonist-binding determinants provides important insights that may be useful in the development of drugs acting at the receptor of interest. While such insights are available for small, non-peptidyl agonists (2–4), our understanding of the molecular basis for peptide and protein hormone binding is much more primitive. Most insights have been derived from mutagenesis studies in which functional changes in ligand binding have been correlated with the modification of distinct receptor domains or residues (2–4). In favorable situations, a direct causal relationship can be established between a specific mutation and modified receptor function (5). However, the vast majority of mutation studies have only interfered with a receptor function. The ability to interpret such observations is, therefore, quite limited, because point mutations may alter receptor function via indirect mechanisms such as modification of receptor processing, conformation, or even regulation, rather than by eliminating a direct site of contact with an agonist.

Photoaffinity labeling has been a powerful approach to directly identify sites of interaction between a photolabile ligand and its receptor. However, it has been limited by the low efficiency of covalent labeling and selective nature of some labeling reagents (6). When these problems are added to their application toward site characterization within a sparse and physically chemically difficult target, the challenges of this approach become apparent. We have recently been successful in using this approach to identify the site of contact between a photolabile nitrophenylalanine positioned at the carboxyl terminus of cholecystokinin (CCK) and its receptor (1). While a single contact between ligand and receptor allows for an initial placement of the ligand into a potential binding site within the receptor, additional contacts would provide additional constraints that could be extremely useful for refining our understanding of this molecular interaction. In this work, we have developed an additional novel analogue of CCK that incorporates a photolabile benzophenylalanine (Bpa) into the mid-region of the peptide pharmacophore in the position of Gly29. Incorporation of such a benzophenone moiety has many theoretical and practical advantages (7). Such reagents are reactive enough to insert into alkyl carbon residues to establish a bond (6, 7). They are not quenched by water in the medium, making more reagent available to establish a covalent bond with adjacent proteins. When such a residue is optimally sited within a ligand, such that it is positioned adjacent to a receptor target upon binding, the efficiencies of covalent labeling can be very high (8). Indeed, there are examples reported of efficiencies of covalent labeling of 60–100% of bound receptor (9).

Characterization of this CCK analogue demonstrated that it is a full agonist and is an effective and efficient photoprobe. It specifically labeled His347 and Leu438 just outside of TM7 in the third extracellular loop of the CCK receptor. This has been a site previously targeted by mutation studies of other structur-
ally related peptide hormone receptors (4, 10). It is also the site predicted to be adjacent to Gly^{29} of CCK in a conformational model of the ligand-occupied receptor we recently reported (1). Additional well defined constraints should enhance our understanding of the molecular basis of binding and activation of this important receptor.

**MATERIALS AND METHODS**

**Reagents**

Wheat germ agglutinin-agarose was purchased from EY Laboratories, Inc., San Mateo CA, chromatographically purified collagenase and soybean trypsin inhibitor were from Worthington, and cholecystokinin-8 was from Peninsula Laboratories (Belmont, CA). The well characterized CCK analogues, d-Tyr-Gly-[Nle_{28,31},3-pNO_{2}-Phe^{33}]CCK-26–33 and d-Tyr-Gly-[Nle_{28,31},pNO_{2}-Phe^{33}]CCK-26–33 were synthesized as we have described previously (11, 12). Other reagents were analytical grade.

**Synthesis and Chemical Characterization of Receptor Probes**

T-Butoxycarbonyl-DL-Bpa was synthesized starting from p-chloromethylbenzophenone (Aldrich) as described by Kauer et al. (7). This was incorporated into a standard solid phase peptide synthetic protocol (13). Fmoc-Gly-Asp-Tyr-Nle-(DL-Bpa)-Trp-Nle-Asp-Phe-NH<sub>2</sub> was synthesized as we have reported, using standard solid phase methodology with t-butoxycarboxyl protection (except for the last residue) and using a p-methylbenzyldihyamine resin (13). This was cleaved from the resin by anisole/HF, lyophilized, and purified by semipreparative reversed-phase C-18 high performance liquid chromatography (HPLC), with a gradient of 10 to 60% acetonitrile in 0.1% trifluoroacetic acid. The peptide was then sulfated on the Tyr residue using sulfur trioxide pyridine complex, and purified by reversed-phase HPLC with a gradient of 10 to 60% acetonitrile in triethylamine-acetate, pH 5. The Fmoc group was removed with 20% piperidine/4-dimethylformamide, and the purified peptide was reacted with Bis-Fmoc-d-Tyr-ONSU or sulfosuccinimidyl-3-(4-hydroxyphenyl)propionate (to yield a probe with blocked amino terminus), as we have described (13). Fmoc protection was again removed, and the products were purified by HPLC. The probe with the blocked amino terminus was always used in experiments leading to Edman degradation sequencing.

Amino acid analysis and mass spectrometry confirmed the expected identity of the synthetic peptides. The D and L stereoisomers were separated by HPLC and identified by the method of Miller and Kaiser (14). The t-Bpa analogue was used for the present series of studies.

This receptor probe was iodinated with both ^{125}I and ^{127}I, using oxidative methods and HPLC purification, as described previously (15). The radioiodinated probe achieved a specific radioactivity of 2000 Ci/mmol, while the unlabeled iodinated probe was used to provide increased yields for ultimate efforts to purify and sequence the labeled receptor fragment.

**Cell and Tissue Preparations**

**Pancreatic Acini**—Dispersed rat pancreatic acini were prepared as we previously described (16). Briefly, pancreatic tissue was harvested from 80–120-g male Harlan Sprague-Dawley rats, and placed in iced, oxygenated Krebs-Ringers-Hepes (KRH) medium containing 25 mm HEPES, pH 7.4, 104 mm NaCl, 5 mm KCl, 1 mm KH_{2}PO_{4}, 1.2 mm MgSO_{4}.2 mm CaCl_{2}, 2.5 mm d-glucose, essential and nonessential amino acids, and 2 mm glutamine, with 0.2% bovine serum albumin and 0.01% soybean trypsin inhibitor. The pancreas was injected with 250 units of purified collagenase in 4 ml of KRH, followed by shaking in a 37 °C water bath at 120 strokes/min for 5 min, followed by mincing and manual shaking for 10 min. Dispersed acini were then filtered through 200-μm nylon, and washed twice with KRH.

**Chinese Hamster Ovary (CHO)-CCKR Cells**—CHO-CCKR cells, representing a rat CCK-A receptor-bearing CHO cell line which we previously established and characterized (15), were grown as a monolayer in flasks containing Ham’s F-12 medium supplemented with 5% Fetal Clone-2 (Hyclone Laboratories, Logan, UT). Cells were harvested me-
channically and used as a source of receptor-bearing plasma membranes. These were prepared by suspending the cells in 0.3 M sucrose containing 0.01% soybean trypsin inhibitor and 1 mM phenylmethylsulfonyl fluoride, and sonicating in a Sonifier cell disrupter (Heat Systems-Ultrasonics, Inc., Plainview, NY) at setting 7 for 10 s. The concentration of sucrose in the homogenate was adjusted to 1.3 M with 2.0 M sucrose, and the membrane band at the sucrose interface was then harvested and diluted with ice water. This was pelleted by centrifugation at 225,000 × g for 1 h. The membrane band of cellular content secreted.

**Biological Activity Assay**

Amylase release assay was utilized to determine the biological activity of the receptor probe (16). For this, dispersed rat pancreatic acini were incubated for 30 min at 37 °C in the absence or presence of secretagogue. At the end of the incubation, cells were separated from medium by centrifugation through NuSil oil (William P. Nye, Inc., New Bedford MA). The phadebas assay (18) for amylase (Pharmacia Diagnostics, Uppsala Sweden) was applied to aliquots of a total cellular lysate and to each supernatant, allowing the calculation of percentages of cellular content secreted.

**Receptor Binding and Affinity Labeling**

**Receptor Binding**—Receptor binding was characterized using 2–5 μg of CHO-CCKR membranes or intact cells grown in 24-well culture dishes (approximately 500,000 cells/well) incubated at room temperature for 1 h in KRH medium with radioligand (3–5 pM) and various concentrations of competing cold peptide. For membrane binding, bound and free radioligand were separated using a Cell Harvester (Skatron Instruments, Inc. Sterling, VA) with receptor-binding filter mats. Cells were lysed with 0.5 M NaOH to release cell-bound ligand. Bound radioligand was quantified in a gamma spectrometer.

**Affinity Labeling**—Affinity labeling was performed similarly, using a larger quantity of membrane (50–100 μg) and iodinated ligand (50–100 μl). When incubations were complete, samples were washed twice with 0.5 M NaCl containing 0.1% Nonidet P-40, and once with water. It was then resuspended in endoglycosidase F buffer (0.1 M NaH2PO4, pH 6.1, 50 mM EDTA, 0.1% Nonidet P-40, and 1% 2-mercaptoethanol) with 2 units of endoglycosidase F, and incubated for 20 °C overnight. The precipitate was pelleted by centrifugation at 14,000 × g for 10 min and rinsed with 500 μl of iced ethanol and then dried in a vacuum centrifuge. Samples were suspended in cyano gen bromide (2.5 mg/tube) in 70% trifluoroacetic acid or formic acid for 72 h at room temperature in the dark. Sample volumes were reduced under vacuum and washed twice with water in preparation for separation on a NuPAGE gel using MES running buffer. The affinity labeled receptor was visualized by autoradiography, cut out, eluted, and lyophilized. It was then suspended in cyano gen bromide cleavage (2.5 mg/tube) in 70% trifluoroacetic acid or formic acid for 72 h at room temperature in the dark. Sample volumes were reduced under vacuum and washed twice with water in preparation for separation on a NuPAGE gel using MES running buffer. The affinity labeled receptor was visualized by autoradiography, cut out, eluted, and lyophilized. This was then further purified by HPLC.

**HPLC**—Two sequential HPLC steps were utilized to further purify the receptor fragments to homogeneity for Edman degradation sequencing. The first HPLC step was performed with a Vydac analytical C-4 column (catalog no. 214TP5415), utilizing a 1 ml/min flow rate and a
membrane and subjected to Edman degradation sequencing using an Applied Biosystems automated instrument.

The radioiodinated cyanogen bromide fragment of the V342M mutant receptor was purified following analogous steps. Radiochemical sequencing of the peak labeled fraction eluting from HPLC was also performed, in manner analogous to that previously reported (1). For this, the relevant labeled receptor-containing fraction was coupled to N-(2-aminoethyl)-3-aminopropyl glass beads either through its α-carboxyl group with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide at pH 5.0 or through a cysteine sulphydryl side chain. The latter was accomplished by derivatizing amino group-expressing glass beads with m-maleimidobenzoyl-N-hydroxysuccinimide ester at pH 7.0, quenching remaining amino reactivity with Tris, and then adding the labeled receptor fragment. The peptide-bound beads were treated with triethylamine:methanol:phenylisothiocyanate (1:7:1, v:v) for 5 min at 50 °C. They were then washed three times with 400 µl of ethyl acetate and dried under vacuum. Beads were then incubated with 50 µl of trifluoroacetic acid for 5 min at 25 °C, and elution was achieved with three successive 200-µl aliquots of methanol. Cycles were repeated for up to 15 complete cycles. Radioactivity in each pooled eluate was quantified in a gamma spectrometer. Noncovalently bound radioiodated fragments were eluted from the beads with a minimum of three complete cycles excluding the phenylisothiocyanate, before initiating formal cycle number one.

RESULTS

Receptor Probe—The photolabile CCK receptor probe incorporating the l-Bpa residue was synthesized and purified to homogeneity on reversed phase HPLC. Its identity was confirmed by mass spectrometry and it was quantified by amino acid analysis. The determination of the identity of the l- and d-Bpa stereoisomers was based on the differential sensitivity to digestion by aminopeptidase M (14). Only digestion of the earlier eluting peak (identified as the l-Bpa analogue) yielded a product that comigrated with a parabenzylophenylalanine standard, while the specificity of this protease did not permit the release of this residue from the d-Bpa analogue.

The probe was radioiodinated using the solid phase oxidant, iodosobeads (Pierce), and its products were purified by reversed phase HPLC, using methods analogous to those we have reported (20). The radioiodinated product was fully resolved from unlabeled peptide to yield a specific radioactivity of 2000 Ci/mmol. This reagent was stored in the dark at ~70 °C until ready for use.

Biological Activity—The probe was an agonist, stimulating dispersed rat pancreatic acini to secrete amylase in a concentration-dependent manner (Fig. 1, left). These are cells that naturally express the wild type CCK receptor. The probe was also a potent secretagogue (EC50 = 28 ± 6 nM), and was as efficacious as the native hormonal agonist, CCK-8.

Receptor Binding and Affinity Labeling—Competition binding studies were performed with a well characterized native CCK-like radioligand (125I-D-Tyr-Gly-L(Nle28,31)CCK-26–33) (11), and enriched plasma membranes were prepared from...
CHO-CCKR cells (15). The L-Bpa analogue competed for binding of this radioligand in a concentration-dependent manner, with an IC₅₀ of 450 ± 126 nM (Fig. 1, right).

When used in affinity labeling experiments, the photolabile probe covalently labeled a protein that migrated on a SDS-polyacrylamide gel in the M₉ 85,000–95,000 position of the CCK receptor (11, 21). Its identity was further supported by its migration at M₉ 42,000 after deglycosylation with endoglycosidase F (Fig. 2), a position well established for the core protein of the CCK receptor (21). As can also be seen in Fig. 2, unlabeled CCK inhibited the affinity labeling of the receptor band in a concentration-dependent manner.

The efficiency of covalent labeling of the CCK receptor with the L-Bpa analogue was 14-fold greater than that using ¹²⁵I-D-Tyr-Gly-(Nle₂⁸,₃¹,Bpa₂⁹)CCK₂₆–₃₃ in the absence or presence of competing unlabeled CCK (left). Also shown is a representative autoradiograph of a 10% Tris-Tricine gel used to separate cyanogen bromide fragments of the wild type CCK receptor and the V342M mutant receptor affinity labeled with this reagent (right). The labeled cyanogen bromide fragment of the mutant receptor migrated as a fragment smaller than that of the wild type receptor. It had an approximate M₉ 5,500, corresponding to the fragment extending from residue 343 to 374. This was typical of 11 similar experiments. As definitive evidence for the identity of the labeled fragment, it was also exposed to Edman degradation sequencing after extensive purification, as described under “Materials and Methods” (Fig. 4). This resulted in the identification of Ile-Phe-Ser-X-Asn-Ala-Trp in cycles two through eight. The residues in cycles one and five were ambiguous. This sequence exists only in fragment 14, and residues are in the appropriate positions relative to the site of cyanogen bromide cleavage at Met₃₂⁸, thus confirming its identity.

The location of labeling within this receptor domain was further defined by mutagenesis to introduce an additional site for cyanogen bromide cleavage within this fragment (V342M). CCK bound to this mutant receptor similarly to the wild type CCK receptor (Fig. 5). The mutant receptor was affinity labeled efficiently (Fig. 6), and cleavage with cyanogen bromide demonstrated the labeling of a fragment of M₉ 5,500, consistent with its representing the carboxyl-terminal portion of the previously described fragment, extending from residue 329 to 374.

The site of covalent attachment of probe to receptor was further defined by radiochemical sequencing using Edman degradation with quantification of the radioactivity in the released cleavage products, analogous to that recently reported (1). This resulted in the identification of His₃₄⁷ and Leu₃₄⁶ as the site of covalent attachment of the L-Bpa analogue probe. This was chosen because of the nicely spaced distribution of methionine residues in the CCK receptor sequence (Fig. 3), and the efficient and specific cleavage of peptide chains at such residues with this reagent. After reduction, alkylation, and cyanogen bromide cleavage of the radiochemically pure affinity-labeled receptor, only a single labeled band was observed which migrated on urea-SDS-polyacrylamide, NuPAGE or Tris-Tricine gels at M₉ 6,700 ± 400 (Fig. 3). When corrected for the mass of the receptor probe (M₉ 1,647), this best corresponded with the calculated mass of the fragment extending from residue 329 to 374 (5,129 + 1,647 = 6,776). Consistent with this assignment, as expected, treatment with endoglycosidase F did not modify the electrophoretic migration of this peptide (data not shown).

Active Site Identification of Cholecystokinin Receptor

Affinity labeling approaches are ideal to define sites of contact between molecules that interact with high affinity and structural specificity, such as a ligand and its receptor. The
specificity of this interaction is provided by the reproducible spatial geometry of the interacting functionalities within those molecules. If a photolabile moiety can be introduced into an agonist ligand, without interfering with its ability to bind and activate its receptor, it can function as a probe of receptor domains adjacent to it. The most useful such probes incorporate a photolabile moiety into a domain that is intrinsic to the pharmacophore, thereby being in a position to potentially define critical sites of molecular contact.

In designing such “intrinsic” probes, there is often a trade-off between the use of a relatively small moiety that may be less likely to interfere with conformation and function, and the use of a bulkier substituent that may have more ideal characteristics of photolability or reactivity. An example of the former may involve replacing an existing aryl group with an aryl-azide (6). Given the advantages provided by a benzophenone which were discussed in the introduction (6, 7), we attempted to find a position for this residue within the receptor-binding domain of CCK. Extensive primary structure-activity data exist for the action of this hormone at the type A CCK receptor (23). The position of Gly29 was utilized, due to experimental data indicating that modifications of that residue are tolerated (24), and a recent molecular model of the ligand-bound receptor that suggests that a Bpa substitution would be tolerated at this position (1).

We have often utilized n-Tyr-Gly-[(Nle28,31)CCK-26–33] as a “parent” probe for the CCK receptor that is stable in oxidizing conditions and radiiodinatable (25). In this work, we further modified that CCK analogue to replace Gly29 with l-Bpa. This analogue was a potent pancreatic secretagogue that bound with adequate affinity to allow efficient covalent attachment to the receptor.

Like previous analogues of CCK, which incorporate photolabile residues within the pharmacophore of this hormone (12, 26), the Bpa analogue affinity labeled the same M₉, 85,000–95,000 molecule previously described to represent the CCK receptor on the pancreatic acinar cell. We have recently demonstrated the identity of that molecule with the recombinant receptor molecule encoded by the cDNA clone originally isolated (15). In this work, we took further advantage of the CHO-CCKR cell line that we established to express 25-fold more receptors on the surface than on the pancreatic acinar cell (15), to provide an enriched source of this receptor for biochemical characterization. The Bpa analogue covalently labeled that molecule with efficiency 14-fold greater than that of the established CCK receptor probe, 125I-n-Tyr-Gly-[(Nle28,31)pNO₂-Phe33]CCK-26–33 (12, 22).

The purification of the affinity labeled receptor could not utilize the most efficient purification modality now available, a specific ligand-affinity column (27), since the ligand-binding site was already occupied. Instead, we utilized more traditional physical methods for purification, following the receptor and ultimately the fragment of interest by monitoring the position of elution of the radioactivity. This approach requires efficient covalent labeling of the receptor, since a very small labeled receptor fragment might be expected to elute differently than the same fragment not covalently bound to the probe. For this, the benzophenone-containing probe offers substantial advantage over a variety of previously described reagents that have substantially lower efficiencies of covalent labeling of the receptor (22).

The active sites of G protein-coupled receptors have been of substantial interest (2–4). This is particularly true as we enter the era of rational drug design. Receptors have long been very effective drug targets. Anything that can be done to improve the ability to develop reagents with improved selectivity, potency, or bioavailability would be very useful. Until recently, most of our knowledge of G protein-coupled receptor agonist binding sites was restricted to those receptors that bind small ligands, such as the 11-cis-retinal chromophore and biogenic amines, in binding sites within the helical bundle transmembrane domain (2–4). With the cloning of cDNAs that encode peptide and protein ligand receptors in this superfamily, attention has focused much more on extracellular loop regions as the principle ligand-binding domain. The rules are not nearly as clear for such receptors as for the small ligand receptors.

It is of particular interest that the domain labeled in this study includes the carboxyl-terminal half of the third extracellular loop and TM7 of the CCK receptor. Indeed, this is a domain that includes residues that have substantial impact in mutagenesis studies of the substance P receptor (10, 28). It is reassuring that the same domain is identified with two quite distinct experimental approaches. The specific residues that were labeled with this probe included His347 and or Leu348 just outside of TM7 in the third extracellular loop. This contact is fully compatible with the model we recently proposed for the peptide agonist ligand-occupied receptor, which was based on an initial contact between the carboxyl terminus of the ligand and Trp29 in the domain just above TM1 (1). Indeed, these residues were even predicted to represent the best receptor contact for the mid-region of CCK in that work.

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