Peptides 46 (2013) 143–149

Contents lists available at SciVerse ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

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

Maoqing Dong, Laurence J. Miller*

Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic Scottsdale, Scottsdale, AZ 85259, United States

ARTICLE INFO

Article history:
Received 10 May 2013
Received in revised form 5 June 2013
Accepted 5 June 2013
Available online 14 June 2013

Keywords:
Cholecystokinin
Type 2 cholecystokinin receptor
Ligand binding
Photoaffinity labeling
G protein-coupled receptors

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 analog probes with sites of covalent attachment extending from position 26 through 32 were characterized, with the highest affinity analogs 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 Phe^{120} in the first extracellular loop. This was in contrast to its covalent attachment to Glu^{345} 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 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),

* Corresponding author at: Mayo Clinic, 13400 East Shea Boulevard, Scottsdale, AZ 85259, United States. Tel.: +1 480 301 4217; fax: +1 480 301 8387.
E-mail address: miller@mayo.edu (L.J. Miller).

0196-9781/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.peptides.2013.06.007

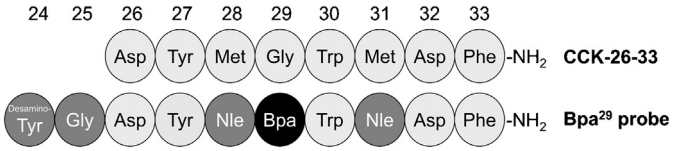

Fig. 1. Primary structure of the Bpa^{29} probe. Shown are primary amino acid sequences of natural CCK-8 (CCK-26-33) and the Bpa^{29} probe. Natural residues are illustrated in clear circles, whereas modified residues are filled with gray, or with black in the case of the photolabile Bpa site of covalent labeling.

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 analog used as the predominant probe in this study was (des-amino-Tyr)-Gly-[(Nle^{28},Bpa^{29},Nle^{31})CCK-26-33] (Bpa^{29} 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 analogs of CCK also utilized in this study included (des-amino-Tyr)-Gly-[(Bpa^{26},Nle^{28},Nle^{31})CCK-26-33] (Bpa^{26} probe), (des-amino-Tyr)-Gly-[(NO_2-Phe^{26},Nle^{28},Nle^{31})CCK-26-33] (NO_2-Phe^{26} probe), (*D*-Tyr)-Gly-[(Bpa^{27},Nle^{28},Nle^{31})CCK-26-33] (Bpa^{27} probe), (*D*-Tyr)-Gly-[(NO_2-Phe^{27},Nle^{28},Nle^{31})CCK-26-33] (NO_2-Phe^{27} probe) [1], (des-amino-Tyr)-Gly-[((BzBz)Lys^{28}, Nle^{31})CCK-26-33] ((BzBz)Lys^{28} probe) [7], (des-amino-Tyr)-Gly-[(Nle^{28},Bpa^{30},Nle^{31})CCK-26-33] (Bpa^{30} probe) [6], (des-amino-Tyr)-Gly-[(Nle^{28},NO_2-Phe^{30},Nle^{31})CCK-26-33] (NO_2-Phe^{30} probe) [6], (des-amino-Tyr)-Gly-[(Nle^{28}, (BzBz)Lys^{31})CCK-26-33] ((BzBz)Lys^{31} probe) [7], and (des-amino-Tyr)-Gly-[(Nle^{28},Nle^{31} Bpa^{32})CCK-26-33] (Bpa^{32} probe).

The photolabile probes and the CCK-like peptide that was radiolabeled and used in competitive ligand binding assays (*D*-Tyr-Gly-[(Nle^{28},Nle^{31})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 Bpa^{29} probe and the CCK radioligand were radioiodinated using oxidation with Iodo-beads and $Na^{125}I$, as we have described [22]. Radiolabeled peptides were purified to yield approximate specific radioactivities of 2000 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% CO_2 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 $CaCl_2$, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$) 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 analogs. 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 NXT™ instrument (Packard) with approximate 23% 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 Bpa^{29} 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 irritation 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.