Vol. 147, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1987 Pages 346-353

ESTABLISHMENT OF A NEW SHORT, PROTEASE-RESISTANT, AFFINITY LABELING REAGENT FOR THE CHOLECYSTOKININ RECEPTOR

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Received June 16, 1987

Summary: Proteolytic degradation of radioligands is an important source of artifact in affinity labeling of receptor proteins. To complement our previous characterization of the pancreatic acinar cell cholecystokinin (CCK) receptor, we synthesized D-Tyr-Gly[(Nle^{28,31})CCK-26-33]. The amino terminal D-enantiomer of tyrosine provided a site for oxidative iodination, a free amino group for cross-linking, and rendered the peptide resistant to aminopeptidases. The decapeptide was oxidatively iodinated and purified by reverse-phase HPLC to 2,000 Ci/mmol, to yield a probe which was equal in potency and efficacy to CCK-8, and which bound to rat pancreatic membranes in a rapid, reversible, temperature-dependent, specific, saturable and high affinity manner. This probe was resistant to aminopeptidase degradation, and maintained its ability to bind to receptor after incubation with pancreatic membranes or dispersed cells. Affinity labeling of pancreatic membranes with this analogue identified an M_r=85,000-95,000 molecule. This analogue offers several advantages over existing probes and should be useful for future studies of this and other CCK receptors.

Introduction: Cholecystokinin (CCK) is a polypeptide hormone with multiple physiological functions, including stimulation of pancreatic enzyme secretion. Structure-activity studies at this target have shown that the carboxy-terminal heptapeptide is adequate and sufficient for full biological activity and potency.

We and others have used affinity labeling to biochemically characterize the subunit structure of the pancreatic acinar cell CCK receptor (1-7). This technique requires a radioligand with high specific radioactivity which retains high affinity receptor binding and possesses a reactive nucleophilic group, such as a free amino group, for chemical cross-linking. Studies using the long probe, ^{125}I-Bolton Hunter-CCK-33, consistently have identified a major M_r=80,000 plasmalemmal binding protein and a variety of less consistent minor components on this tissue (2-6). The amino groups on this probe, however, are far from the carboxy-terminal binding region, theoretically increasing its chance of labeling a non-binding, "near neighbor" protein. We, therefore, designed two decapeptide analogues of CCK which possess free amino groups near the binding region

0006-291X/87 $1.50

(^{125}I-Bolton Hunter-Lys-Gly-[CCK-26-33], ^{125}I-Tyr-Arg-[(Thr^{28}, Nle^{31})CCK-26-33]) (1). These probes labeled a M_r=85,000-95,000 protein in rat pancreatic membranes, which is distinct from that labeled with the longer CCK-33 based probe (1). However, those analogues were susceptible to tryptic and/or aminopeptidase proteolysis.

Since ligand degradation diminishes the efficiency of receptor labeling and can release iodinated peptide fragments capable of being cross-linked, further complicating interpretation of results, it was important to design a more stable affinity-labeling reagent. In this work, we report the synthesis, oxidative radioiodination, and validation of ^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33], an oxidation- and aminopeptidase-resistant decapeptide for affinity labeling of CCK receptors. When used with the amino-reactive cross-linking reagent, DSS, this probe labeled the M_r=85,000-95,000 protein previously identified on rat pancreatic plasma membranes (1,8). We also demonstrated the importance of aminopeptidase degradation of ligand in pancreatic acinar cells and plasma membranes by comparing this probe with its analogue possessing the L-enantiomer of tyrosine.

Materials and Methods

Synthesis and iodination of peptides. The peptides were synthesized by a combination of solid-phase and solution techniques. The product was purified on a Beckman gradient HPLC with a semi-preparative Vydac C-18 column (218TP1010), run at 1ml/min with a linear gradient from 10% to 60% acetonitrile with 0.05M triethylamine-acetate (pH 5.0). D-Tyr-Gly-[(Nle28,31)CCK-26-33] eluted as a symmetrical peak at 21.5 min while the L-tyrosine derivative eluted slightly later. The products were collected, divided into 5µg aliquots and stored at -79°C.

D- and L-Tyr-Gly-[(Nle^{28,31})CCK-26-33] were oxidatively iodinated with Na^{125}I and N-chlorobenzenesulfonamide as we have previously described (11). The reaction products were separated by reversed-phase HPLC using a Vydac C-18 column (218TP54) run isocratically at 1ml/min with 30% acetonitrile/70% 0.1M triethylamine-acetate (pH 5.0).

Biological activity. The biological activities of labeled and unlabeled D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] were determined by measuring their abilities to stimulate amylase secretion by dispersed pancreatic acini. The acini were prepared from 125-150g male rats (Harlan Sprague-Dawley) by sequential enzymatic and mechanical dissociation according to the method of Schultz, et al (9). All incubations were carried out in a modified Krebs-Ringer-Hepes (KRH) buffer, pH 7.4, containing 25mM Hepes, NaCl (104mM), KCl (5mM), $MgSO_4$ (1.2mM), $CaCl_2$ (2mM), KH_2PO_4 (1mM), D-glucose (2.5mM), 0.2% BSA, 0.01% soybean trypsin inhibitor (STI), and essential and non-essential amino acids. Acini containing one to five million cells were incubated with various concentrations of labeled or unlabeled ligand or CCK-8 in a 1ml volume for 30 minutes at 37°C. Total amylase content and amylase release were assayed by the method of Bernfeld (10).

Receptor binding studies. Pancreatic plasma membranes were prepared from 125-150g male rats (Harlan Sprague-Dawley) using a method previously reported (2). In standard assays, 15-20µg of pancreatic membrane protein and 9pM (15,000 cpm) radioligand were incubated in 0.5ml KRH containing 1mM phenylmethylsulfonyl-fluoride. Bound and free ligand were separated by centrifugation (10,000xg) at 4°C with subsequent washing. Non-specific binding was

measured in the presence of 0.1μM unlabeled CCK-8. Association and dissociation experiments were performed as we have previously described (11).

Binding to dispersed acinar cells was done similarly, separating by centrifugation and washing (800xg).

Degradation of Radioligands. Aminopeptidase M: Equal concentrations ($2X10^5$cpm, 450pM) of ^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] and ^{125}I-L-Tyr-Gly-[(Nle^{28,31})CCK-26-33] were incubated with 40mU of aminopeptidase M in 200μl of phosphate buffered saline (PBS) at 37°C. The reaction products were analyzed by thin layer chromatography (TLC) using silica gel plates and chloroform: methanol:32% acetic acid (5:3:1, vol:vol) solvent. Dried plates were exposed to x-ray film (Kodak XAR-5) for 2 hrs. at -70°C.

Pancreatic membranes and acinar cells:Radioligand was incubated with 100μg of pancreatic membranes or ten million acinar cells for 30 min at 37°C. Label in the supernatant was then analyzed by TLC using the solvents described above. Further, dissociated label was tested for its ability to rebind to membranes or cells in a standard binding assay. Results are expressed as a percentage of the specific binding obtained with the same concentration of fresh radioligand.

Affinity labeling of the CCK receptor. Binding was carried out with approximately 500pM ^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] as described above. Membrane pellets were resuspended in 100μl KRH buffer without BSA, and DSS dissolved in dimethylsulfoxide was added to a final concentration of 250μM. After a 5 minute reaction at 4°C, cross-linking was quenched with 50 μl of 0.2M Tris (pH 7.4) and the membranes pelleted by centrifugation (10,000 x g) and analyzed by SDS slab gel electrophoresis.

Membrane pellets were solubilized in sample buffer containing 0.125M Tris, pH 6.8 with 4% SDS, 10mM EDTA, 15% sucrose, and 0.01% bromphenol blue with or without 0.1M dithiothreitol (DTT). Samples were run on 10% polyacrylamide slab gels (11cm x 1.5mm) containing 2mM EDTA according to the method described by Laemmli (12). The gels were stained in Coomassie Blue (13), then dried and exposed to x-ray film (Kodak XAR-5) at -70°C for 1-10 days using a DuPont Quanta III® intensifying screen. M_r values for affinity labeled proteins were calculated from a plot of log M_r vs. mobility of standard proteins: myosin (200,000), β-galactosidase (116,500), phosphorylase β (92,500), BSA (66,200), ovalbumin (45,000) and carbonic anhydrase (31,000)).

Results

Biological activity. D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] and its iodinated product were as potent and efficacious in stimulating amylase secretion from dispersed rat pancreatic acini as CCK-8 (Fig 1). All peptides demonstrated maximal stimulation at 0.3nM, and supramaximal inhibition of amylase secretion typical for CCK peptides.

Binding studies. Binding of ^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] to pancreatic membranes was a linear function of membrane protein concentration from 2.5μg/ml- 25μg/ml. Greater than 95% of total binding was abolished by 0.1μM unlabeled CCK-8. Binding association was rapid, with greater than 50% of binding occurring within five minutes at 25°C, and temperature-dependent, (Fig 2). Binding was reversible, with rapid dissociation at 37°C, and little or none at 4°C.

CCK-8 competed for binding of the radioloigand in a concentration-dependent manner, with 50% of binding inhibited by 0.5nM CCK-8 (Fig 3). Desulfated CCK-8 had a lower affinity, with 50% of binding inhibited by 1μM peptide. This

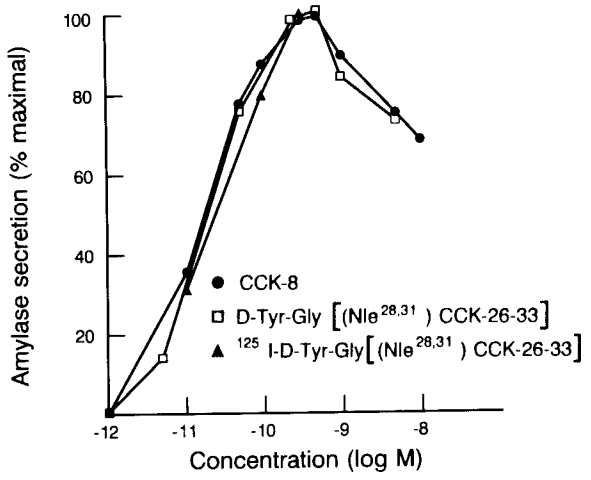

Figure 1. Ability of CCK-8 (●-●), D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] (□-□) and ^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] (▲-▲) to stimulate amylase secretion from dispersed rat pancreatic acini. Maximal amylase secretion was the same for all three peptides. Each value was the same for all three peptides. Each value represents the mean of two separate experiments done in triplicate.

reflects the abilities of these peptides to stimulate pancreatic enzyme secretion, and is consistent with interaction at the physiologically relevant pancreatic CCK receptor. In addition, these results closely approximate those obtained for ^{125}I-Bolton Hunter labeled CCK-33 and CCK-8 (14,15).

Analysis of Probe Stability to Enzymatic Degradation. Following incubation with pancreatic membranes, cells, or aminopeptidase M, ^{125}I-D-Tyr-Gly-[(Nle^{28,31})

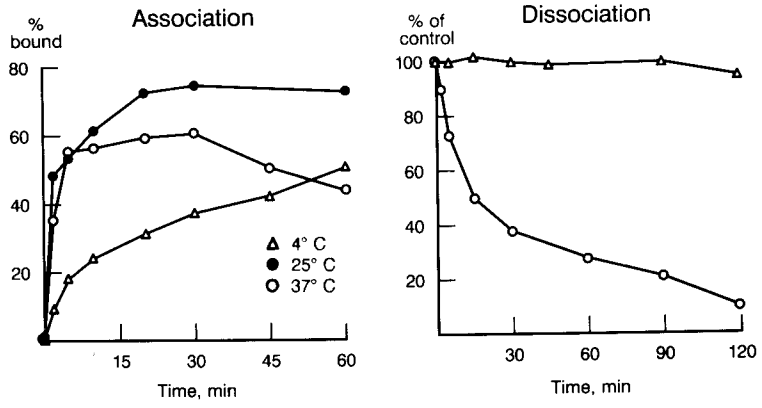

Figure 2. Time and temperature dependence of association and dissociation of binding of ^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] to pancreatic membranes. Association data are expressed as a percentage of total radioactivity specifically bound to the membrane pellet. In dissociation studies, the membrane pellet and its bound radioactivity were resuspended in fresh buffer and incubated at 4°C or 37°C. Values are expressed as a percentage of bound radioactivity at the beginning of the second incubation. In these studies, values represent the mean of two experiments performed in duplicate.

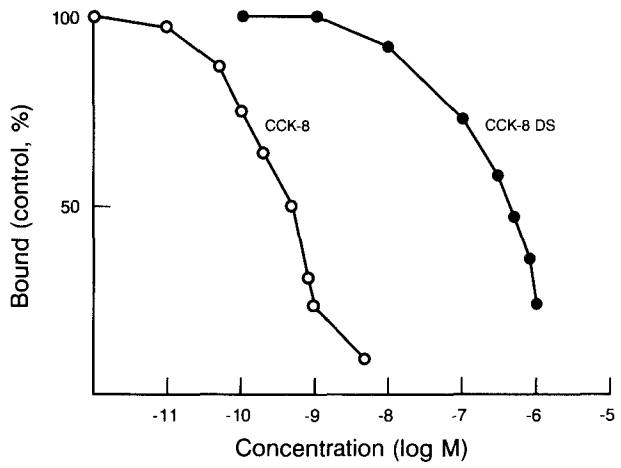

Figure 3. Ability of CCK-8 (o-o) and desulfated CCK-8 (●-●) to compete for binding of ^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] to pancreatic membranes. Values are the mean of three experiments performed in triplicate and represent a percentage of the radioactivity bound in the absence of competing peptide.

CCK-26-33] was not degraded, co-migrating with control radioligand on TLC (Fig 4). ^{125}I-L-Tyr-Gly-[(Nle^{28,31})CCK-26-33], under the same conditions, was degraded to iodotyrosine, which migrated with a slower R_f than the control radioligand. Sensitivity to aminopeptidase M degradation was demonstrated by complete degradation at 2 min (Fig 4).

Furthermore, after dissociation, ^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] fully retained its ability to specifically rebind to pancreatic membranes (91 ± 14%,

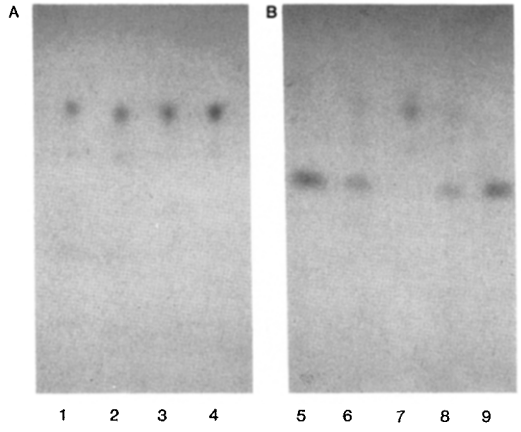

Figure 4. TLC analysis of probe stability to enzymatic degradation. Panel A is an autoradiogram of studies using ^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] and Panel B using ^{125}I-L-Tyr-Gly-[(Nle^{28,31})CCK-26-33]. Lanes 3 and 7 are untreated radioligands with an identical Rf=0.70. Experimental conditions include: Radioligand after a 30 min incubation at 37°C with pancreatic membranes (1,5) or cells (2,6), radioligand after incubation with 40mU of aminopeptidase M at 37°C for 30 seconds (8) or 2 min (4,9).

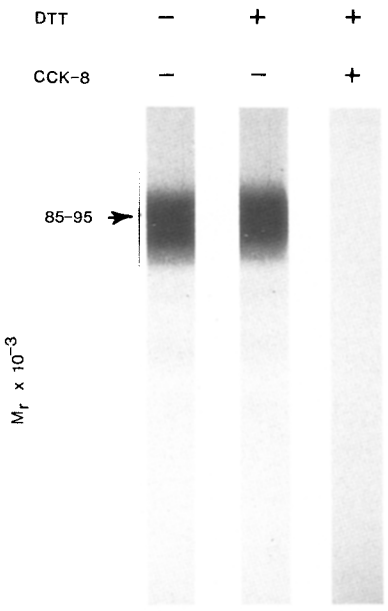

Figure 5. Affinity labeling of pancreatic membranes with ^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] and DSS. Membranes were incubated with 500pM radioligand with or without 0.1μM CCK-8. Cross-linking was done with 250μM DSS. Samples were prepared for SDS-PAGE with or without dithiothreitol (DTT).

X ± SEM) or acinar cells (95 ± 13%) when compared with fresh radioligand. In contrast, dissociated ^{125}I-L-Tyr-Gly[(Nle^{28,31})CCK-26-33] bound poorly to pancreatic membranes (13 ± 10%) and cells (17 ± 27%) when compared with the binding of fresh peptide.

Affinity labeling. An M_r=85,000-95,000 protein was labeled in pancreatic membranes with ^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] and DSS (Fig 5). The labeling was abolished by competing CCK-8 (0.1μM), and no change in labeling pattern was observed when membranes were solubilized in the presence or absence of the reductant dithiothreitol. This band co-migrated with the component previously reported by us with other short probes and a variety of affinity and photoaffinity labeling techniques (1,8).

Discussion

The probe described in this report for affinity labeling of CCK receptors, D-Tyr-Gly-[(Nle^{28,31})CCK-26-33], has many desirable features. Because of the norleucine substitutions for methionine residues, it is resistant to oxidative conditions known to abolish the biological activity of native CCK peptides. The amino terminal tyrosine of this analogue provides a site for an oxidative iodination, a simpler and more efficient method of labeling than the acylation reactions required for native CCK derivatives (e.g. ^{125}I-Bolton Hunter reagent).

Finally, the D-enantiomer of tyrosine confers relative resistance to aminopeptidases while retaining a free amino group for cross-linking.

We validated this probe in the rat pancreas, a classical target for CCK. D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] retained full biological activity despite the substitutions for the methionine residues, manipulations of the amino terminus and oxidative iodination. This is consistent with previous studies with other CCK and gastrin analogues (11,16). The binding characteristics of this probe were consistent with a ligand-receptor interaction, being rapid, reversible, temperature-dependent, saturable and specific. The affinity and structural specificity of the binding of ^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] was appropriate for an interaction with the physiologically relevant CCK receptor.

To be useful in affinity labeling, a probe needs a reactive chemical group. The amino terminus of ^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] was shown to be accessible for interaction with amino reactive cross-linkers. The same M_r=85,000-95,000 pancreatic membrane protein we identified in previous studies with other short probes (1,8) was labeled, confirming the importance of the position of the cross-linkable amino group relative to the binding region of CCK in affinity labeling of the pancreatic receptor.

Proteolysis of peptide ligands is another confounding variable in the interpretation of both pharmacologic and direct binding studies. In affinity labeling, proteolysis is particularly important because ligand degradation reduces the strength of the "signal" and contributes to the "noise" by releasing peptide fragments capable of non-specifically labeling non-receptor proteins.

D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] was designed to be resistant to two important types of protease degradation. First, it contains no basic amino acid residues for a tryptic-like cleavage site. The amino terminal D-tyrosine was used to prevent aminopeptidase degradation. As expected, ^{125}I-D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] was resistant to aminopeptidase degradation. The importance of this modification was confirmed in side-by-side recovery experiments comparing analogues terminating in the D- or L-enantiomers of tyrosine. As assessed by either TLC or receptor rebinding studies, the majority of ^{125}I-L-Tyr-Gly-[(Nle^{28,31})CCK-26-33] was degraded while the D-tyrosine analogue remained intact after incubation with pancreatic membranes or dispersed acinar cells. Thus, there was significant aminopeptidase activity in this tissue directed against an iodinated amino terminal tyrosine which was blocked by the use of the D-enantiomer of this residue.

Similar protease activities are found at many sites, and the approach used in this study may be useful for affinity labeling of other peptide hormone receptors. Specifically, D-Tyr-Gly-[(Nle^{28,31})CCK-26-33] should be useful for the characterization of CCK binding proteins at other targets and serve as an oxidation and protease-resistant "backbone" for the development of other affinity and photoaffinity probes.

Acknowledgements: The authors wish to thank Ms. Delia Pinon for her technical assistance and Ms. Marilyn Breyer for her help in preparing the manuscript.

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