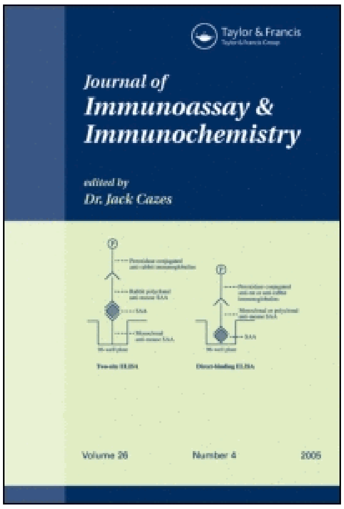

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^{125}I-(Thr$_{34}$, Nle$_{37}$)- CCK$_{31-39}$ A Non Oxidizable Tracer for the Characterization of CCK Receptor on Pancreatic Acini and Radio-Immunoassay of C-Terminal CCK Peptides

Daniel Fourmy[a]; Lucien Pradayrol[a]; Nicole Vaysse[a]; Christiane Susini[a]; Andre Ribet[a]
[a] INSERM U 151, TOULOUSE, FRANCE

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^{125}I-(Thr_{34}, Nle_{37})- CCK_{31-39} A NON OXIDIZABLE TRACER FOR THE CHARACTERIZATION OF CCK RECEPTOR ON PANCREATIC ACINI AND RADIO-IMMUNOASSAY OF C-TERMINAL CCK PEPTIDES

Daniel FOURMY, Lucien PRADAYROL, Nicole VAYSSE, Christiane SUSINI and André RIBET

INSERM U 151, CHU Rangueil, Chemin du Vallon, 31054 TOULOUSE
FRANCE

Short title : ^{125}I-(Thr, Nle)-CCK-9 a non oxidizable CCK tracer

ABSTRACT

A derivative of the C-terminal nonapeptide of CCK, namely (Thr_{34}, Nle_{37}) - CCK_{31-39} was radio-iodinated by conjugation with ^{125}I-Bolton-Hunter reagent. The labelled peptide was purified by RP-HPLC on a C-18 column. Validation of the iodinated peptide was performed by measuring its biological integrity and by studying its binding characteristics on pancreatic acini. ^{125}I-(Thr, Nle)-CCK-9 present the same ability to stimulates amylase release than (Thr, Nle)- CCK-9 and CCK-8. Binding of the radio-ligand to CCK receptors is specific, reversible, saturable. Inhibition of the binding by CCK-related peptides correlates well their biological potencies. ^{125}I-(Thr, Nle)-CCK-9 is able to interact with high affinity CCK receptors. Furthermore, ^{125}I-(Thr, Nle)-CCK-9 is recognized by C-terminal CCK directed antibodies. This reliable tracer could be used as a replacement of CCK-8 since it is protected from risks of oxidation.

INTRODUCTION

Cholecystokinin was initially isolated from porcine duodenum as a 33-amino-acid peptide hormone (1, 2) that stimulates both

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pancreatic enzyme secretion and gallbladder contraction (3). It is now well established that cholecystokinin exists in multiple molecular forms in the intestine and in the nervous system (4). Several peptides ranging in length from 39 to 4 amino-acids (CCK-39, CCK-33, CCK-12, CCK-8, CCK-4) have been identified (1, 5, 6, 7). The largest molecular forms (CCK-39, CCK-33) might correspond to biosynthetic precursors of the octa- and tetra-peptides, which might be the physiologically active forms. Several studies support this view (8, 9, 10). Rehfeld et al. have demonstrated that CCK-8 and CCK-4 are the predominant molecular forms synthetized in brain neurons and that there exists a precursor-product relationship between the large forms and CCK-8 (9). Application of low amounts of CCK-8 and CCK-4 to postsynaptic membranes strongly excite hyppocampal neurons (11). Thus, CCK-peptides are now recognized as neural peptides with a possible role in the control of satiety (12). "In vitro", CCK-8 is severalfold more potent than CCK-39 and CCK-33 as stimulant of enzyme secretion (13, 14), but in man "in vivo", CCK-8 and CCK-33 were found to be equipotent (15). Several studies have demonstrated the predominant release of CCK-8 in blood (8, 10). These results are much debated (16) because of difficulties in measuring CCK peptides in biological fluids. Additionnal insight into the physiological roles and relative importance of the different molecular forms of CCK can be obtained by receptor binding studies. Until now, interactions of CCK with its receptors have been essentially studied with the radio-iodinated derivative of CCK-33 prepared by conjugation of the peptide to the labelled Bolton-Hunter reagent (13, 14). Such a radio-ligand has permitted characterization of high affinity receptors in the brain (17) and pancreas (13, 14). In light of the relatively low activity of CCK-33 compared to that of CCK-8, and the possibility of enzymatic processing of the iodinated portion from the C-terminal active sequence (13), ^{125}I-CCK-33 might not be the best tracer for the study of CCK receptors. Tritiated derivatives of CCK-8 (18) or caerulein (19, 20) have also been used in such studies, but the low specific activity of the ligands did not permit a good characterization of high affinity binding sites. Recently, synthesis of iodinated CCK-8 derivatives with high specific activity using the Bolton-Hunter reagent (21) and an iodinated imido-ester (22) have been published. Such radio-ligands are expected to be more resistant to enzymatic cleavages than ^{125}I-CCK-33 ; however they possess two methionines which are sensitive to oxidation which can lead to the loss of bioactivity of the tracer during storage, incubation and/or steps of studies of CCK receptors using chromatographic purification.

In the present communication we relate the synthesis and purification of a non-oxidizable ^{125}I-CCK tracer obtained by conjugation of a derivative of the nona-peptide C-terminal of the CCK, $[(Thr_{34}, Nle_{37})\text{-}CCK_{31\text{-}39}]$ to ^{125}I-Bolton-Hunter reagent. The validation of the labelled CCK-peptide is done using binding studies with pancreatic acini and tests of immunoreactivity using antibodies directed to the C-terminal sequence of CCK.

MATERIALS AND METHODS

Chemicals

^{125}I-Bolton-Hunter reagent (N-Succinimidyl 3 - 4-hydroxy, 5-^{125}I-iodophenyl propionate) with a specific activity of 2000 Ci/mmol was obtained form Amersham France. Acetonitril was purchased from Fluka lab. The C-terminal nonapeptide (Thr$_{34}$, Nle$_{37}$) CCK$_{31-39}$ was synthetized by Prof. E. Wünsch from Max Plank Institut für Biochemie, Munchen, West, Germ. Peptides were purchased from the following : caerulein and bombesin : Farmitalia, Milan, Italia ; CCK-4 : Interchim, Montluçon, France ; CCK-8 and VIP : C.R.B. laboratories, Cambridge, England ; CCK-39 : GIH Research Unit, Karolinska Institute, Stockholm, Sweden ; Secretin: Hoffman La Roche laboratories, Bâle, Switzerland ; Glucagon and Insulin : Novo Industries pharmaceutiques, Paris, France.

Synthesis and purification of ^{125}I-(Thr, Nle)-CCK-9

(Thr, Nle)-CCK-9 was radio-iodinated by conjugation of the peptide to mono-iodinated ^{125}I-Bolton-Hunter reagent. An aliquot of the ^{125}I-Bolton-Hunter reagent corresponding to 0.5 mCi was transferred to a reaction minivial and dried under a gentle stream of argon. Five micrograms of peptide dissolved in 20 µl of 0.1 M sodium borate buffer, pH = 8,5 were added to the iodation vial. The mixture was strongly stirred and incubated at 0°C for 1 hour. Excess of free ^{125}I-Bolton-Hunter reagent was reacted with 250 µl of 0.4 M glycine in 0.1 M sodium Borate pH = 8,5.

Purification of the iodinated CCK peptide was performed by reverse-phase HPLC on a C-18 µBondapak column as previously described (23). Radioactive fractions were diluted with 1 ml of storage solution containing 12 % (w/v) of bovine serum albumin and freezed at - 30° C.

Biological validation of ^{125}I-(Thr, Nle)-CCK-9

Standard medium used for preparation of acini and for incubations was a Krebs-Hepes solution pH = 7.4 gassed with 95 % O_2. This was composed of 25 mM Hepes, 103 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM Mg SO_4, 0.5 mM $CaCl_2$, 5 mM glucose, 2 mM glutamine 0.2 % Bovine serum albumin, 1 % essential amino-acid solution 1 % non essential amino-acid solution. Pancreatic acini from Guinea pig were prepared as previously described (24). The procedure involved an enzymatic digestion by collagenase (50 U/ml, 40 min at 37° C) followed by mechanical dissociation of the tissue through glass pipettes.

*Bioassay of ^{125}I-(Thr, Nle)-CCK-9 and CCK related peptides

Biological activity of the tracer was controlled by measuring its ability to stimulate amylase release on pancreatic acini, and was compared to that of (Thr, Nle)-CCK-9. Various CCK-peptides were also tested. An aliquot of the acini suspension (0.5 ml) was incubated in duplicate at 37°C for 15 min with appropriate reagents (0.5 ml). At the begining and at the end of the incubation period, 0.8 ml of medium was centrifuged at 10 000 xg for 15 sec. The supernatant (0.5 ml) was assayed for amylase activity by the method of Bernfeld (25). Total amylase activity was measured on four separated aliquots of cell suspension treated with Triton X-100 2 % in 20 mM phosphate buffer. Results were expressed as the percent of maximum amylase release elicited with each stimulant.

*Binding characteristics of ^{125}I-(Thr, Nle)-CCK-9

Binding studies were performed at 37°C in standard incubation medium supplemented with 1.3 % (w/v) bovine serum albumin, 0.1 % (w/v) bacitracin, 0.03 % soybean trypsin inhibitor (Sigma). Samples of the cell suspension (0.2 ml) were incubated in duplicate with the radio-ligand alone or with the various molecules tested (CCK, dbcGMP, VIP,...)(50 µl). At the end of the incubation period 180 µl of the suspension was transferred to plastic microtubes containing 180 µl medium plus 12 % albumin at 0°C and were centrifuged (10 000 xg). The supernatant was aspirated, and the bottom of tube that contains radioactivity associated with cells was then measured. Specific binding was defined as the excess binding over that in blanks containing 10^{-6} M (Thr, Nle)-CCK-9 and IC_{50} as the concentration of peptide that inhibited 50 % of specific binding. For association, dissociation and competition studies the concentration of radio-ligand used was of about 6.10^{-11} M. Dissociation was studied by incubating acini with the radio-ligand for 90 min at 37° ; then 10^{-6} M (Thr, Nle)-CCK-9 was added, and residual binding was measured at various times. Scatchard plot analysis of the binding was done by two methods : incubation of pancreatic acini with a constant concentration (6.10^{-11}) of ^{125}I-(Thr, Nle)-CCK-9 diluted with increasing concentrations of (Thr, Nle)-CCK-9 and by incubating pancreatic acini with increasing concentrations of ^{125}I-(Thr, Nle)-CCK-9 ranging from 10^{-12} M to 10^{-9} M.

Immunoreactivity of ^{125}I-(Thr, Nle)-CCK-9

A commercial antiserum (Merseyside laboratories, Warrington, England) raised against the C-terminal tetrapeptide common to gastrin and CCK (CCK-4) was used to test immuno-reactivity of the tracer. The assay was performed by incubating at + 4° C for 16 hours in a 20 mM sodium barbital buffer pH = 8.0 containing 0.1 % (w/v) bovine serum albumine 100 µl of tracer dilution (5000 cpm) with 100 µl of antiserum. Displacement of bound peptide was achieved by adding doses of CCK peptides ranging from 1 to 10^4 fmol/ assay. Total volume of incubation medium was 500 µl. Antibody-bound label was separated from free by adding 250 µl (10 mg) of amberlite resin CG400 in a 20 mM Tris buffer pH = 8.0. The supernatant which contained ^{125}I-(Thr, Nle)-CCK-9 bound to antibody was transferred to a plastic tube and counted.

RESULTS

Preparation and purification of ^{125}I-(Thr, Nle)-CCK-9

Figure 1 illustrates chromatographic profiles obtained from the HPLC of an aliquot of the ^{125}I-(Thr, Nle)-CCK-9 solution. Radioactivity was eluted in the three main peaks : Peak 1 and peak 2 were identified to correspond to the iodinated glycine and the hydrolysis product of ^{125}I-Bolton-Hunter reagent (23).

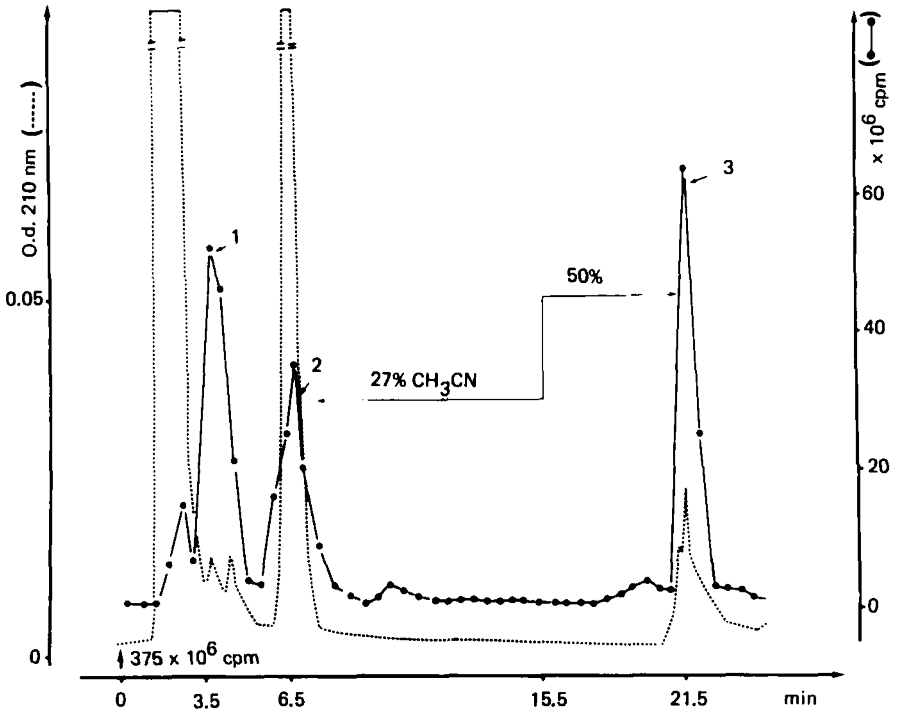

FIGURE 1 : RP-HPLC profile of an aliquot of labelling medium of (Thr,Nle)-CCK-9. Column : µBondapak C-18 (0.39 x 30 cm) ; mobile phase : triethyl ammonium phosphate buffer 0.25 N, pH : 3.5 + Acetonitrile. HPLC was run in a 27 % acetonitrile buffer for 15 mn, then this ratio was increased to 50 %. Flow rate : 2 ml/min. Back pressure : 1500 PSI. Molecules were monitored by absorption at 210 nm (------) and measurement of radioactivity (●——●) in the 1 ml fractions collected.

The last peak eluted in a 50 % acetonitril buffer was the only one that contained molecules exclusively with characteristics of the CCK tracer, since these molecules bind specifically to the CCK receptors on the pancreatic acini and are recognised by antibodies reacting with the C-terminal portion of CCK. The average recovery of radioactivity in the biologically active fraction was 25 % of total radio-activity, used in the labelling procedure. In addition, a chromatographic recovery calculated on the basis of the percent radioactivity eluted within 30 minutes greater than 85 % was obtained. The UV profile at 210 nm showed one larg peak at the void volume of the column which contains polar components such as borate, glycine. The UV peak at 6.5 min corresponds to the un-reacted (Thr, Nle)-CCK-9. Finally a small UV peak is observed at the same retention time as that of the CCK tracer. This last peak might correspond, at least in part, to the labelled peptide.

Ability of ^{125}I-(Thr, Nle)-CCK-9 to stimulate amylase release

The dose-response curve (fig. 2) revealed that ^{125}I-(Thr, Nle) CCK-9 has an ability to stimulate amylase release identical to that of (Thr, Nle)-CCK-9. Concentrations of the labelled peptide were calculated by using the specific activity of 2000 Ci/mmol (that of the Bolton-Hunter reagent). Concentrations of (Thr, Nle)-CCK-9 and its iodinated derivative which elicited half-maximal and maximal responses were 10^{-10} M and 10^{-9} M respectively.

Binding studies of ^{125}I-(Thr, Nle)-CCK-9 to pancreatic acini

*Time course of association (fig. 3) and dissociation (fig. 4)

When incubating labelled peptide 6.10^{-11} M with acini at 37°C, a specific binding to acini was obtained. Total binding (and specific binding) increased, reached equilibrium at 60 min and remained constant to at least 120 min. Non specific binding remained constant for 120 min and did not exceed 10 %. At the equili-

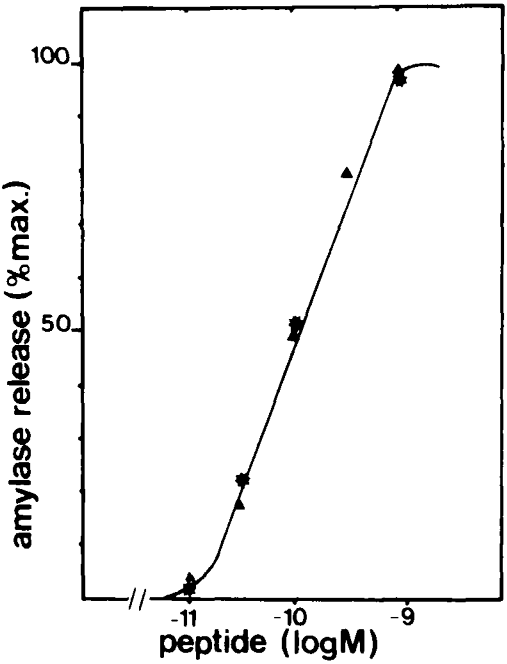

FIGURE 2 : Ability of ^{125}I-(Thr,Nle)-CCK-9 to stimulate amylase release on pancreatic acini. Acini were incubated at 37° C for 15 min with ^{125}I-(Thr,Nle)-CCK-9 (✱——✱) and (Thr,Nle)-CCK-9 (▲——▲). At the end of incubation period 10 % of total amylase was released in medium.

brium, a B/F ratio of 7.2 ± 0.9 % corresponding to 3.8 ± 0.6 fmol of ^{125}I-(Thr, Nle)-CCK-9 bound by mg of acinar proteins was found. The amount of specific binding was plotted following the equation $\ln \frac{Beq}{Beq-B} = K_{obs} \times t$. Beq is the specific binding at equilibrium ; B is the specific binding at the time considered, S the concentration of the radio-ligand, K_{obs} the rate constant of association observed. The plot which is linear indicates that the binding reaction followed pseudo-first order-kinetics.

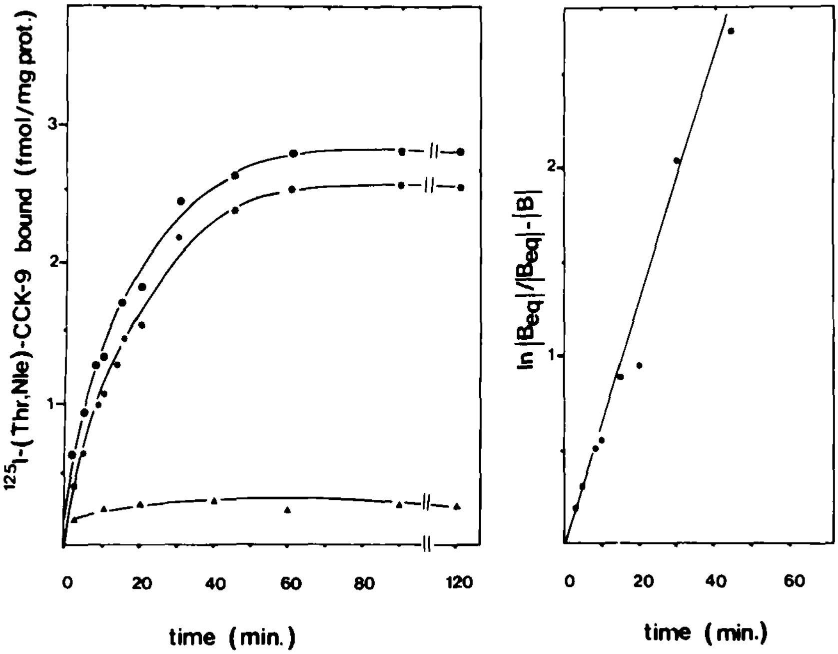

FIGURE 3 : Time course of binding of ^{125}I-(Thr,Nle)-CCK-9 to pancreatic acini. (a typical experiment). Left panel ; acini were incubated at 37° C with the radio-ligand alone (● —— ●) or in presence of (Thr,Nle)-CCK-9 10^{-6} M (non-specific binding) (▲—▲). Right panel : the data are linearized.

Dissociation of ^{125}I-(Thr, Nle)- CCK-9 in presence of 10^{-6} M of (Thr, Nle)-CCK-9 added to the incubation medium is shown in fig. 4. When the data was expressed according to the equation $\ln \frac{B}{Beq} = k_{off} x t$, a biphasic relationship was found. Because of the rapide dissociation rate of the first component, its dissociation rate constant could not be accurately calculated. However, the dissociation rate constant corresponding to the second step which has slower kinetic was 13,5 10^{-3} min^{-1}.

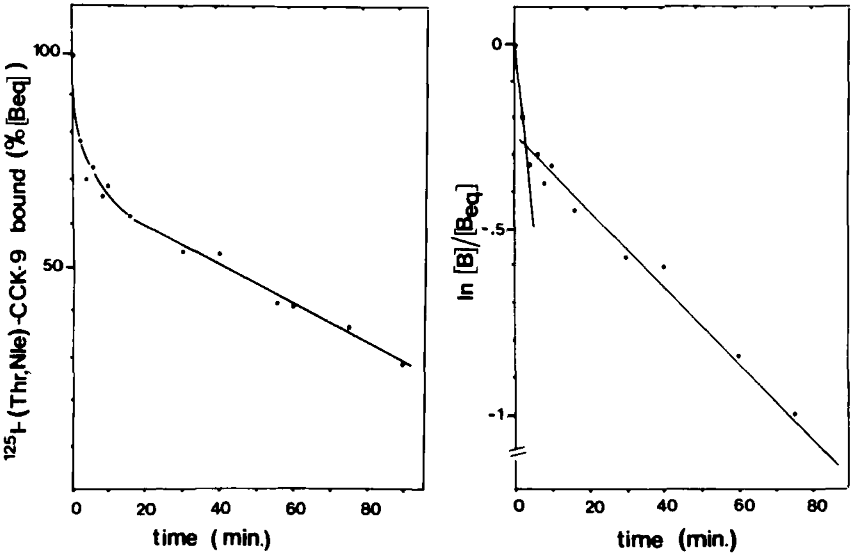

FIGURE 4 : Time course of dissociation of ^{125}I-(Thr,Nle)-CCK-9 after binding to acini for 90 min. (a typical experiment). Left panel : radio-ligand that remained specifically bound after adding 10^{-6} M (Thr,Nle)-CCK-9 was measured at the times indicated. Right panel ; data are linearized.

*Saturation analysis of ^{125}I-(Thr, Nle)-CCK-9 binding (fig. 5)

When the radio-ligand was progressively diluted by increasing concentrations of non-labeled peptide, binding was saturable showing an upward-curved Scatchard plot. These results are compatible with two orders of binding sites, a high affinity site with a Kd : 1-2.10^{-10} M having a binding capacity of 20-30 fmol/mg of proteins and a lower affinity site with a Kd : 3-4.10^{-8} M having a binding capacity of ~ 500 fmol/mg of proteins.

When acini were incubated with increasing concentrations of radio-ligand, Scatchard plot was some-what different with that observed in presence of increasing concentrations of non-labelled

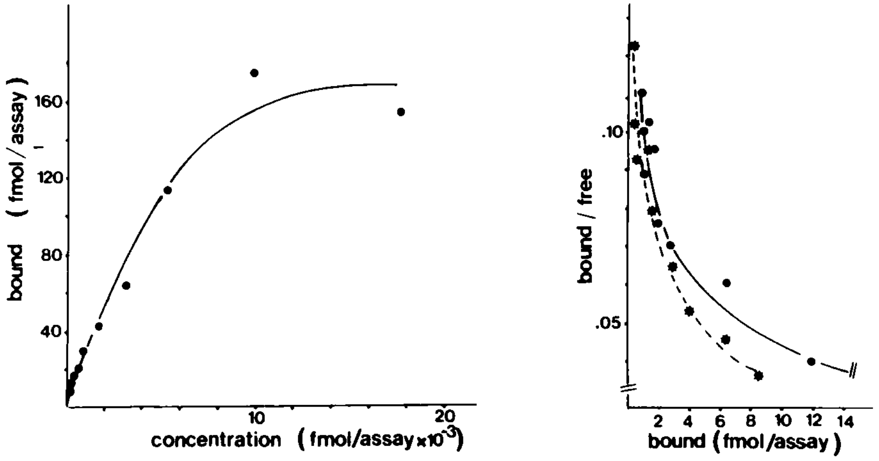

FIGURE 5 : Scatchard plot of ^{125}I-(Thr,Nle)-CCK-9 binding (a typical experiment). Left panel : the radio-ligand 6.10^{-11} M was incubated with acini for 90 min in presence of increasing concentrations of (Thr,Nle)-CCK-9 for testing saturation of binding sites. Bound peptide is plotted as a function of concentration. Right panel : binding of ^{125}I-(Thr,Nle)-CCK-9 obtained by incubating the radio-ligand alone (✱——✱) with acini is compared to that of ^{125}I-(Thr,Nle)-CCK-9 in presence of (Thr,Nle)-CCK-9 (o—o).

peptide but high affinity sites (Kd : 1.10^{-10} M) with characteristics similar to that previously found, could be identified.

*Relationship of biological activity to receptor binding and specificity

Ability of related CCK peptides to competitively inhibit ^{125}I-(Thr, Nle)-CCK-9 binding versus their ability to stimulate amylase release on pancreatic acini was studied (fig. 6). Inhibition curves were parallel and all the peptides tested progressively and completly inhibited the binding of the radio-ligand.

Half-maximal inhibition concentration (IC$_{50}$) gave the following relatives potencies : caerulein : IC$_{50}$: 2.10^{-10} M,

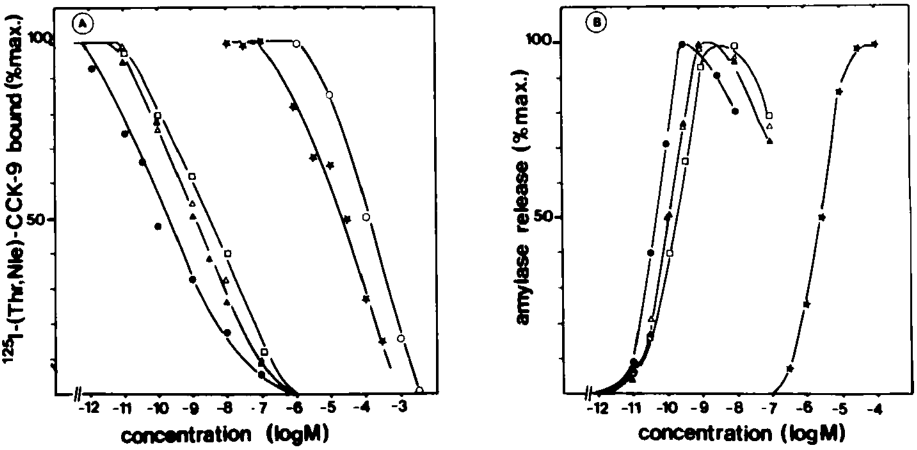

FIGURE 6 : Relationship of biological activity to receptor binding on pancreatic acini. A. Ability of CCK related peptides to stimulate amylase release. Acini were incubated at 37° C for 15 min. B. Ability of CCK related peptides to inhibit binding of ^{125}I-(Thr,Nle)-CCK-9 to acini. Acini were incubated with the radioligand alone 6.10^{-11} M or in presence of various concentrations of reagents: Caerulein (●——●) ; CCK-8 (△——△) ; (Thr,Nle)-CCK-9 (▲——▲) ; CCK-39 : (□——□) ; CCK-4 (✱——✱) and dibutyryl cyclic GMP (○——○).(Results are the mean of at least 3 separated experiments).

CCK-8 and (Thr, Nle)-CCK-9 : IC_{50} : 10^{-9} M, CCK-39 : IC_{50} : 4.10^{-9} M ; CCK-4 : 2,10^{-5} M.

Dibutyryl cyclic GMP, an antagonist of CCK receptors (14) inhibited the binding with an IC_{50} : 10^{-4} M. Various agents were tested for their abilities to inhibit the binding of ^{125}I-(Thr, Nle)-CCK-9. Secretin, vasoactive intestinal peptide, glucagon, insuline, bovine pancreatic polypeptide, bombesin, dopamine and carbamylcholine did not inhibit binding of ^{125}I-(Thr, Nle)-CCK-9.

The dose-response curves for CCK-related-stimulated amylase release (fig. 6) are also parallel. The peptides used were able to elicite a maximal amylase release of 10 % after a 15 min

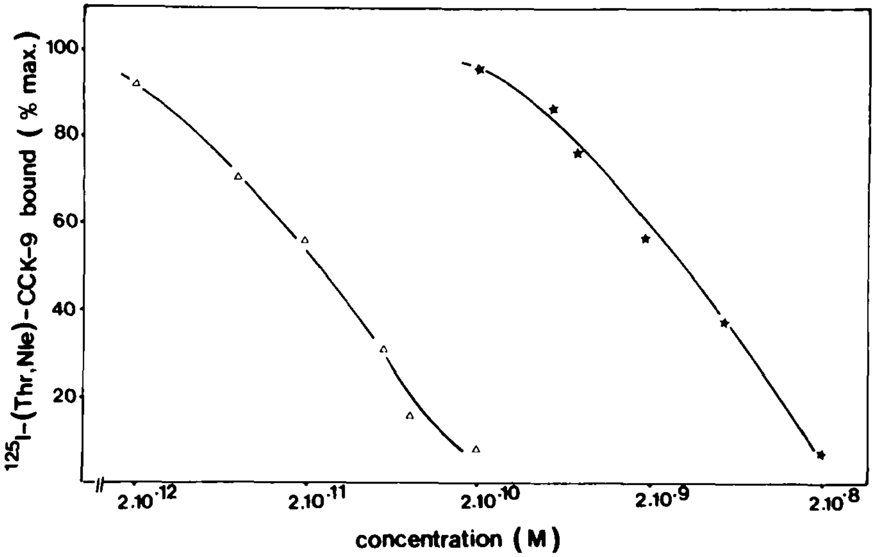

FIGURE 7 : Competitive inhibition of ^{125}I-(Thr,Nle)-CCK-9 to antibody by CCK-8 (△—△) ; CCK-4 (★—★). Tracer was incubated with antibody alone or in presence of increasing concentrations of peptides (shown in abscissa) at 4° C for 16 h in a 20 mM barbital buffer pH = 8.0.

incubation period. Doses which produced half-maximal stimulations were : caerulein : 5.10^{-11}M, CCK-8 and (Thr, Nle)-CCK-9 : 10^{-10} M, CCK-39 : 2.10^{-10} M ; CCK-4 : 3.10^{-6} M. These results are in good agreement with the ability of these peptides to interact with ^{125}I-(Thr, Nle) CCK-9 receptors.

Degradation of ^{125}I-(Thr, Nle)-CCK-9 during incubation with acini

In a first attempt radio-ligand containing medium incubated 120 min with acini was compared with fresh labelled peptide, for its ability to bind to acini. Associations kinetics were identical and indicated that the binding activity of the radioligand was retained during incubation with acini. In a second type of experiment were compared immuno-reactivity of the radio-ligand previously exposed to acini with that of fresh tracer using five dilutions of the C-terminal CCK antiserum. Only 70 % of CCK-like immunoreactivity was retained after a 120 min incubation period with pancreatic acini.

^{125}I-(Thr, Nle)-CCK-9 as tracer in radio-immunoassay

When 2 fmol of radio-ligand were incubated in presence of the C-terminal CCK directed antibody, 40 % (B/F) of radioactivity was bound to antibody. Increasing concentrations of CCK-8 and CCK-4 progressively inhibited the binding. Concentrations of CCK-8 and CCK-4 which inhibited 50 % of initial binding were 1.2 10^{-11} M and 1.2 10^{-9} M respectively.

DISCUSSION

Although the structure of CCK was determined ten years ago, only recently have biologically active iodinated CCK-33 been reported (26, 27). Two iodinated derivatives of CCK-8 have been reported (21, 22). But the main CCK radio-ligand used in CCK-receptors studies is still radio-iodinated CCK-33. The preparation method which involves conjugation of the peptide to iodinated Bolton-Hunter reagent, avoids oxidation during the reaction and preserved biological activity of the hormone contrary to the chloramine T method initially used (26, 27). But the other risks of oxidation can occur during purification, storage, and incubation with target structures owing to the fact that the labelled molecules possesse one methionine that is necessary to the biological activity on pancreas (28).

Recently, a new nonoxidable CCK nonapeptide analogue has been synthetised (29). In this molecule, the methionine-34 was substituted with threonine, as for caerulein and the methionine-37 with Norleucine. (Thr, Nle)-CCK-9 was found to be equipotent with CCK-8 (28). The present studies on pancreatic acini confirm these last results and favour the hypothesis of an hydrophobic role of the methionine-37 residue in the biologically active portion of CCK. Similar results were obtained with little gastrin (30), and conformational calculation on gastrin C-terminal tetrapeptide supports these views (31).

With the non-oxidizable CCK nonapeptide analogue, we prepared an iodinated tracer used in interaction hormone-receptor studies and in RIA determinations. The purification method involved reverse-phase HPLC which has the distinct advantage of producing a good separation of the labelled peptide from the unsubstituted peptide, in contrast to conventional methods (22, 26, 27). A high specific activity could be anticipated. To compared biological potency of ^{125}I-(Thr, Nle)-CCK-9 with the native peptide, we compared their ability to stimulate amylase release in acini, using the concentration of the labelled component calculated on the basis of a 2000 Ci/mmol specific activity. The molecules were found to be equipotent which indicates that ^{125}I-(Thr, Nle)-CCK-9 possessed an intrinsic activity identical with that of the native peptide and presented probably a specific activity near 2000 Ci/mmol. Thus, one molecule of ^{125}I-Bolton-Hunter reagent was incorpored in the peptide probably via the NH_2 terminal α amino group. Identity of the iodinated (Thr, Nle)-CCK-9 with the nonapeptide and CCK-8 was completed by assessing its affinity for CCK receptors on pancreatic acini. Binding of the radio-ligand was specific, saturable and reversible. Relative potencies of the CCK related peptide to inhibit ^{125}I-(Thr, Nle)-CCK-9 binding correlated well with their relative ability to stimulate amylase release in pancreatic acini. (Thr, Nle)-CCK-9 and CCK-8 in particular, have the same potencies to inhibit binding of ^{125}I-(Thr, Nle)-CCK-9. Scatchard plot analysis of the binding indicated that the radio-ligand used identified the high affinity CCK receptors presents on pancreatic acini (13, 14). Caracteristics of the sites were assessed by two means : one using increasing concentration of radio-ligand, a second using a progressive dilution of the radio-ligand by increasing concentration of native peptide. K_d values found in these two set of experiments were identical which confirms intrinsic affinity of two molecules are similar. We found a dissociation rate constant which is in good agreement with values found by others (13,14,21, 32). however, using CCK-8 or CCK-33 iodinated derivatives, the same authors obtained more rapid association kinetics (13, which lead to an equilibrium state at 15-20 min, whereas 60 min are necessary in our study.

Enzymatic cleavage of ^{125}I-BH-CCK-33 in medium after binding to acini was reported using trichloroacetic acid precipitability (13). ^{125}I-BH-CCK-8 was found to be resistant to enzymatic degradations since its binding activity was retained during incubation with acini (21). Using this last property we found no evidence for the degradation of ^{125}I-(Thr, Nle)-CCK-9 in the medium. However, using CCK antibodies, we found a drop of immuno-reactivity of the radio-ligand after a 2 hours incubation period. Amino-peptidases might be responsible of the radioligand immunoreactivity decrease. In fact, aminopeptide conversion of CCK-9 to CCK-8 have been described (33).

Immunoreactivity tests indicated that ^{125}I-(Thr, Nle)-CCK-9 is recognized by CCK C-terminal directed antibodies. As shown from the first results obtained, a sensitive radioimmunoassay of C-terminal molecular forms of CCK could be developped with ^{125}I-(Thr, Nle)-CCK-9 as tracer.

In conclusion, a new iodinated probe for CCK receptors has been validate by assessing its biological and binding properties on pancreatic acini. It could be used as tracer in RIA determinations and in binding studies as a replacement of CCK-8 since it is protected from all risks of oxidation.

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Address requests for reprints to Daniel FOURMY INSERM U 151.

Bat L3, CHU Rangueil 31054 TOULOUSE CEDEX FRANCE

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