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AN HPLC/PUSH-PULL PERFUSION TECHNIQUE FOR INVESTIGATING PEPTIDE METABOLISM

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We have combined the push-pull perfusion of radiolabelled peptide, with reverse phase - HPLC procedures as a valuable method for monitoring peptide metabolism in the CNS. This model not only permits studies on metabolism of neuropeptides at specific sites in the CNS, but more importantly, allows (unlike *in vitro* methods) the study to be made under the appropriate physiological conditions. We have demonstrated unambiguously for the first time, the metabolism of CCK-8 *in situ*.

Tissue levels of a peptide may be altered by changes in *de novo* biosynthesis, release, degradation or a combination of these; however very little is known about the mechanism(s) of inactivation of neuropeptides. This is in direct contrast to the classical transmitters, for which it is well established that as well as passive diffusion there are two active mechanisms whereby the action of a classical transmitter can be terminated after synaptic release. These are enzymatic degradation (e.g. inactivation of acetylcholine by acetylcholinesterase) and high affinity uptake systems (e.g. inactivation of biogenic amines, γ-aminobutyric acid and amino acid transmitters).

We report here the use of push-pull perfusion of radiolabelled peptide in combination with high performance liquid chromatography (HPLC)[1] of the perfusate as a valuable tool for monitoring peptide metabolism. This model not only permits studies on metabolism of neuropeptides at specific sites in the central nervous system (CNS), but more

[1] Abbreviations used: HPLC, High Pressure Liquid Chromatography; CNS, central nervous system; CCK-8, cholecystokinin octapeptide; CSF, cerebrospinal fluid.

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importantly, allows (unlike in vitro methods) the study to be made under the appropriate physiological conditions.

For this investigation, we have used the peptide cholecystokinin octapeptide (CCK-8). The molecular form originally isolated from porcine intestine was a 33 residue, single chain polypeptide (1). Other porcine and ovine forms have been sequenced subsequently (2). Moreover, using antisera raised against the porcine peptides, multiple forms of immunoreactive CCK have been identified in other species, including man (3). It is interesting to note that only very recently has the predominant form of CCK in human brain been characterised (4).

MATERIALS AND METHODS

Since ^{3}H-CCK-8 itself was not available, the labelled CCK-8 used for this experiment was (propionyl - ^{3}H) propionylated cholecystokinin which was obtained from Amersham Radiochemicals. Despite the manufacturer's specification of purity of the labelled peptide, the purity of the compound was rechecked using reverse phase HPLC just prior to the experiment. The fractions containing the labelled peptide were pooled, dried and subsequently used for the experiment.

Rats were stereotaxically implanted with push-pull cannulae into the third ventricle according to the atlas of Pellegrino and Cushman (5). After a one week recovery period rats were perfused with artificial cerebrospinal fluid (CSF),(made up of NaCl, 127mM; KCl, 3.73mM; $CaCl_2$, 1.8mM; KH_2PO_4, 1.18mM; $MgSO_4$, 1.18mM; $NaHCO_3$, 20mM) and containing bovine serum albumin (0.1% w/v), bacitracin (30 µg/ml), bestatin (20µM) and thiorphan (0.1µM). All chemicals used were obtained from Sigma (U.K. Ltd.) unless otherwise stated. Thiorphan was generously donated by Professor B. Roques (Faculte des Sciences, Paris, France).Each rat was perfused for a total of four and a half hours at a rate of 10µL/minute and consecutive samples were collected on ice. The first sample was a thirty minute collection and the others were all one hour collections. At the end of the perfusion, the samples were freeze dried in silanised flasks. An aliquot was removed from the remaining unused perfusion medium as a blank at the end of the experiment, and was treated identically to the perfusate samples. This sample gave an indication of any baseline degradative effects due simply to standing at room temperature for the duration of the experiment. The freeze dried samples were analysed by HPLC (6) to determine the ratio of intact ^{3}H-CCK-8 to total metabolites per sample. At the end of the perfusion experiment the cannula tip was histologically verified.

RESULTS AND DISCUSSION

The push-pull perfusion technique has been incorporated into a strategy for investigating the metabolism of neuro-

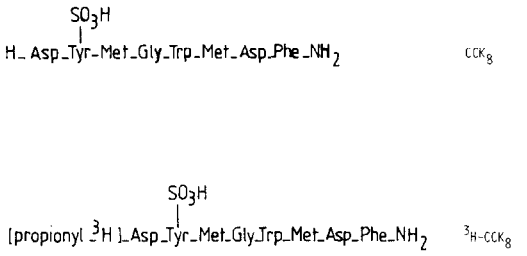

Fig. 1: Structure of cholecystokinin octapeptide and the labelled peptide used for the perfusion experiment.

peptides. Samples were perfused into the third ventricle of the brain of a rat and the perfusate was analysed by HPLC and liquid scintillation spectroscopy to identify whether radioactive metabolites had been formed. The method was tested using ^{3}H- propionylated CCK-8. Prior to perfusing this peptide, the commercial sample was purified by HPLC (see figure 2). Analysis of the HPLC profile obtained from purifying the labelled peptide revealed, rather surprisingly, 3 peaks (labelled A,B and C in figure 2). Inspection of the structure of both labelled and unlabelled octapeptide, figure 1, suggests that peak A is the

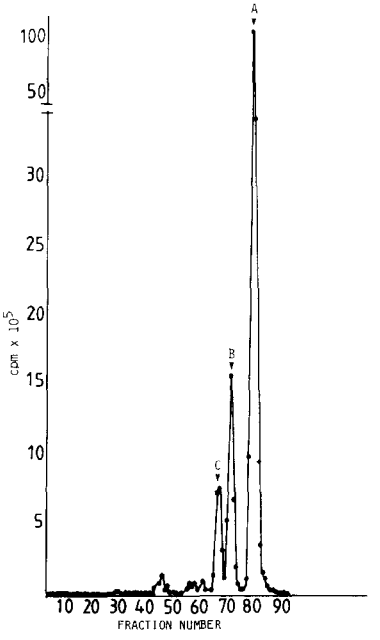

Fig. 2: Purification of ^{3}H-CCK-8 using reverse phase HPLC. Peak A represents the ^{3}H-peptide and peaks B and C are the oxidised forms.

unmodified ^{3}H- CCK-8 (^{3}H-propionylated cholecystokinin octapeptide) with peaks B and C being the oxidised forms of ^{3}H-CCK-8. The presence of two methionine residues could theoretically generate a minimum of two oxidised products. It is interesting to note here, that when the perfusion experiment was conducted with only peak A in the perfusion medium, peaks B and C were formed spontaneously, providing evidence for peaks B and C being oxidation products of CCK-8. All subsequent perfusion experiment were carried out using a pooled sample of A,B and C.

Typical levels of ^{3}H-CCK-8 and ^{3}H- metabolites obtained during the continuous <u>in situ</u> ventricular perfusion are shown in figure 5. For comparison, figure 4 shows the HPLC profile of the unused perfusion medium sampled at the end of the perfusion experiment. In addition to peaks A,B and C two additional radioactive peaks (D and E) were observed in the HPLC profile after perfusion had been carried out (see fig. 3) after 0.5hour. Interestingly, the percentage

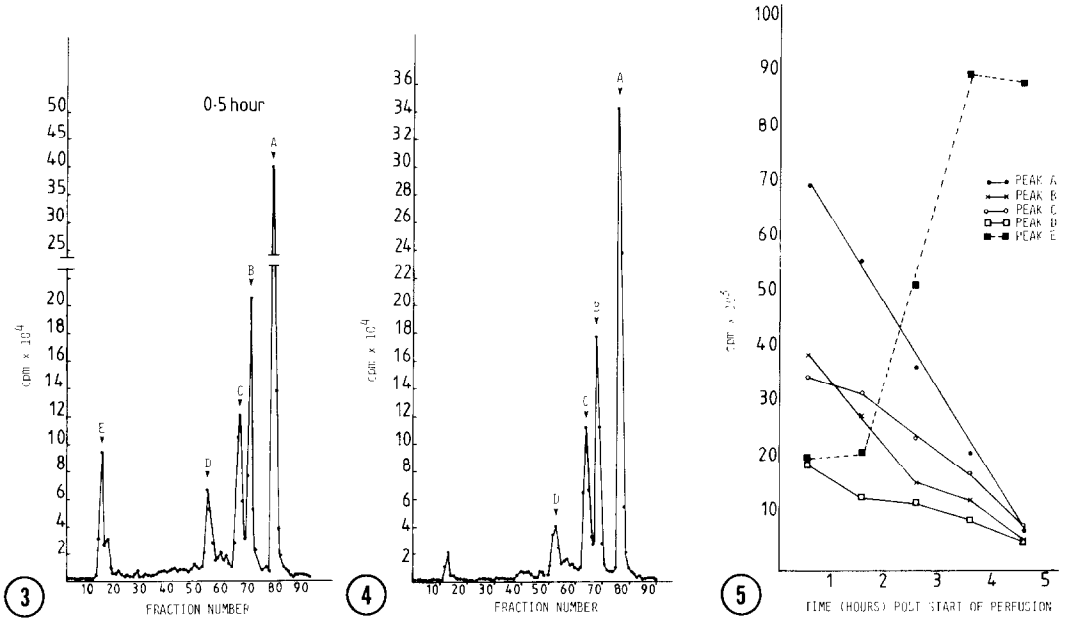

Fig. 3: HPLC profile after perfusion had been carried out for 0.5hour.

Fig. 4: HPLC profile of an aliquot of the unused perfusion medium containing the labelled peptide taken at the end of the perfusion experiment and treated identically to the perfusate samples.

Fig. 5: The time course of formation of metabolites of ^{3}H-CCK-8 <u>in vivo</u> . The loss of peaks A,B,C and D concomitant with the formation of peak E is clearly shown.

of peak D present in the perfusate samples at 0.5hour, (fig. 5)was observed to decrease with time, while conversely, the percentage of peak E was shown to have markedly increased over the same period. A concomitant reduction in A,B and C was observed and this is shown in figure 5 which clearly illustrates the loss of peaks A,B,C and D and the formation of peak E with time. From figure 5, it is clear that a metabolite of ^{3}H-CCK-8 has formed (peak E) which after a lag period (approximately one hour)increased linearly and reached a maximum at 3.5hours. Additionally, the figure shows that, in contrast to peaks B,C and D, the percentage of peak A declined linearly and rapidly with time.

In this research we have shown for the first time, the in situ metabolism of the small peptide, ^{3}H- propionyl CCK-8. The slow rate of degradation shown here should not be too surprising since the labelled CCK-8 used for this experiment was blocked at the N-terminus (see figure 1). Despite the fact that the free N-terminal amino acid group was blocked, conversion of the ^{3}H-CCK-8 to a more polar compound occurred. Figure 5 depicts the loss of ^{3}H-CCK-8 and the formation of its metabolites. It is not certain from this study whether peak E represents one product or whether more than one cleavage product has eluted at the same position.

Bacitracin, bestatin and thiorphan were included in the perfusion medium in an effort to reduce enzymatic degradation. Although these inhibitors (bestatin and thiorphan) have in the past been used specifically to reduce enzymatic degradation of enkephalins (aminopeptidase and enkephalinase activity)(7,8,9), there is no confirmatory evidence to indicate that the peptidases that are inhibited by these inhibitors are specific for the enkephalins. On the contrary, thiorphan has been reported to nonspecifically inhibit the degradation of other peptides, e.g. neurotensin (10), thus acting as a general dipeptidyl carboxypeptidase inhibitor.

Since there is no evidence to indicate the inactivation of CCK-8 by uptake mechanisms, it is reasonable to assume that enzymatic degradation plays a key role in the termination of its action. This rationale is further corroborated by

our findings in this study which unambiguously demonstrate the loss of the intact ^{3}H-CCK-8 peptide peak, concomitant with the formation of a metabolite.This metabolite elutes earlier than the intact CCK-8 peptide on reverse phase HPLC and is therefore considerably more polar. Further work is being carried out to identify this metabolite.

The experimental strategy used in this work for investigating the metabolism of CCK-8 in situ can be adapted for investigations on the metabolism of any neuropeptide at a particular site in the CNS.

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