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METABOLIC STABILITY OF THE LHRH ANTAGONIST ANTIDE TO CELL-SURFACE PEPTIDASES

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Summary: The susceptibility to hydrolysis of LHRH and the decapeptide analogue Antide has been compared. The hydrolysis of LHRH by pig kidney brush border membranes is inhibited by phosphoramidon (I_{50} = 5.6 nM) implicating endopeptidase-24.11 in the initiation of hydrolysis. Under conditions in which LHRH is fully degraded by brush border membranes, Antide was completely resistant to hydrolysis. Similar results were obtained with purified preparations of both endopeptidase-24.11 and angiotensin converting enzyme. These data confirm that the remarkable duration of action of Antide is due principally to its stability to hydrolysis by cell-surface peptidases.

A major site for the clearance and degradation of circulating luteinizing hormone-releasing hormone (LHRH; luliberin) is the proximal tubule of the kidney (1,2). Since LHRH is blocked at both N- and C-termini it is not susceptible to hydrolysis by exopeptidases. Two renal brush border enzymes, however, can initiate hydrolysis of LHRH *in vitro* (3). Endopeptidase-24.11 (EC 3.4.24.11) cleaves LHRH at the Ser^4-Trp^5 and Gly^6-Leu^7 bonds and, to a lesser extent, at His^2-Trp^3 (2,3). Angiotensin converting enzyme (EC 3.4.15.1) has been shown to release both the N-terminal and C-terminal tripeptides of LHRH through endopeptidase action (4,5). Endopeptidase-24.11 appears to function in the inactivation of LHRH *in vivo* in the rat (2). In addition to its location in the brush border, endopeptidase-24.11 co-localises with gonadotrophic cells in the adenohypophysis, suggesting that it may also play a role in LHRH metabolism at that site (6). Another metalloendopeptidase, designated endopeptidase-24.15, has also been implicated in LHRH metabolism at some sites but this is predominantly a cytosolic enzyme rather than an ectoenzyme and is not located in the renal brush border (7,8).

During the last decade, progress has been made in the design of potent antagonists of LHRH (9). However, such antagonists also

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released histamine from mast cells and produced cutaneous wheal reactions (10). Ljungqvist et al. (11) designed, synthesized and bioassayed 52 new peptides and found that one, named Antide, showed potent anti-ovulatory activity yet a negligible release of histamine. Antide was also found to have a duration of action in the monkey and rat which was not anticipated (11,12), as well as oral activity. This antagonist was only sparingly soluble in a formulation and it was speculated that the exceptional duration of action might be associated with its lipophilicity producing a deposition of the peptide on injection. Alternatively this may be attributable to the presence of several D-amino acids, producing resistance to enzymic cleavage, although some cell-surface peptidases (e.g. renal dipeptidase, EC 3.4.13.11) can handle D-amino acid-containing peptides efficiently (13). Although D-amino acid substituents may diminish cleavage at a particular scissile bond, the resultant change in conformation may inhibit or even enhance cleavage at more distant sites (2). It is important, therefore, to examine directly the metabolic stability of peptide hormone analogues.

Here we therefore compare the metabolism of LHRH and Antide by brush border membranes as well as purified preparations of endopeptidase-24.11 and angiotensin converting enzyme. The peptide analogue is shown to possess remarkable metabolic stability mirroring its prolonged action *in vivo*.

MATERIALS AND METHODS

Materials- Antide was synthesized by the solid-phase method as described previously (11). LHRH was purchased from Cambridge Research Biochemicals, Harston, Cambridge, UK and phosphoramidon was from Peninsula Laboratories Europe, St Helens, UK. The angiotensin converting enzyme inhibitor enalaprilat (MK422) was a gift from Merck & Co. (Rahway, NJ, USA). Other materials were from sources previously described.

Enzymes- Endopeptidase-24.11 and angiotensin converting enzyme were affinity purified as described previously (14,15). They were both apparently homogeneous as assessed by SDS-polyacrylamide gel electrophoresis.

Brush border membranes- These were prepared from pig kidney as described by Booth & Kenny (16). They were stored in batches at -70°C.

Hydrolysis of peptides- The incubation mixture (vol 100µl) contained 100 mM-Tris/HCl, 150 mM-NaCl, pH 7.4, 50 µM-peptide and 5 µg of brush border membrane protein or 875 ng purified peptidases. Samples were incubated at 37^0 C for 2 h. The incubation was terminated by heating to 100^0C for 4 min and the samples were centrifuged before analysis.

Analysis of peptide products by h.p.l.c.- This was performed as described previously (5) except that the acetonitrile gradient was 5 - 45% (v/v).

RESULTS

The hydrolysis of LHRH was compared by kidney brush border membranes, endopeptidase-24.11 and angiotensin converting enzyme. Under the conditions used the membrane preparation and endopeptidase-24.11 completely degraded the peptide. The h.p.l.c. profiles of the incubation mixtures are shown in figs 1a & 1b where it can be seen that the patterns of product formation are similar for membranes and endopeptidase-24.11 although their relative proportions differ. This

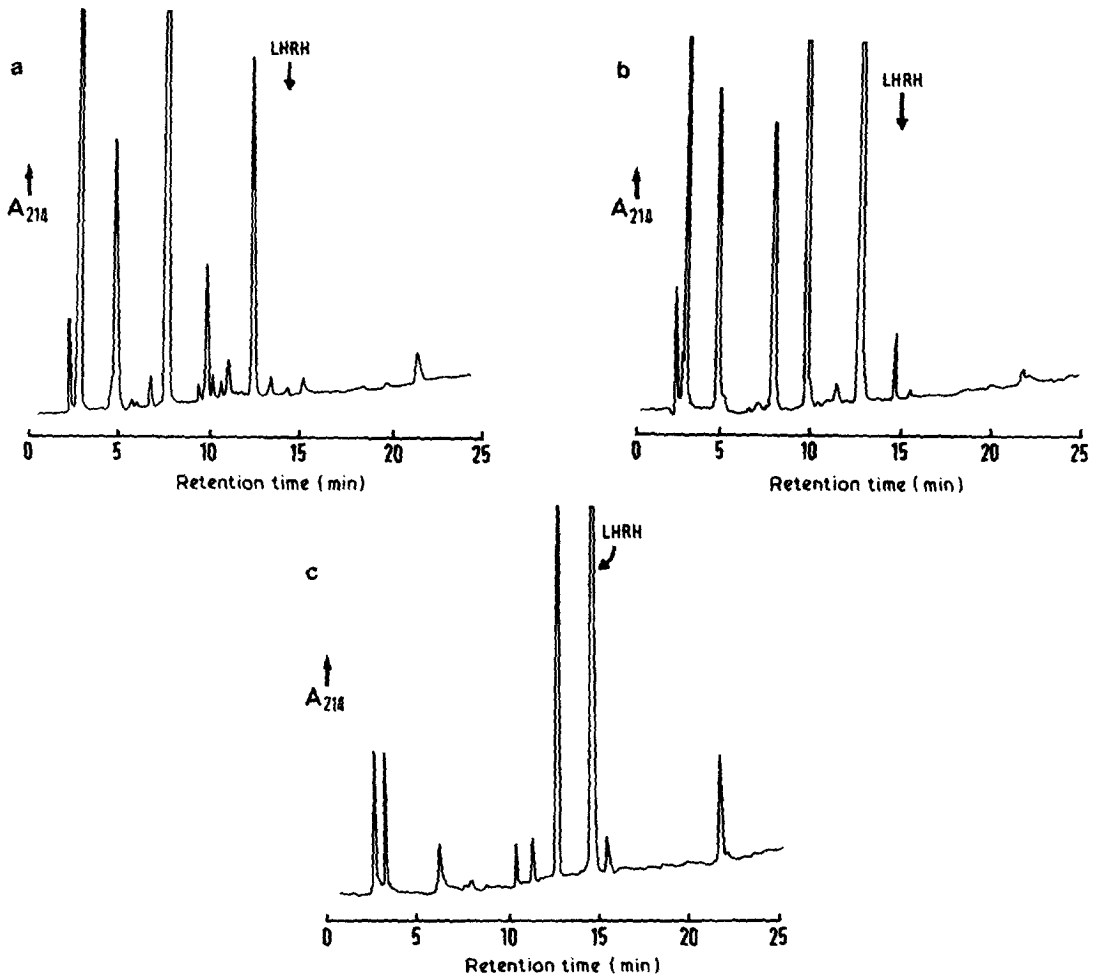

Fig.1. H.p.l.c. analysis of peptide products of LHRH hydrolysis by a brush border membrane preparation, endopeptidase-24.11 and angiotensin converting enzyme.

The metabolism of LHRH (50 μM) was examined (a) with brush border membranes (5 μg protein; 2 h), (b) endopeptidase-24.11 (875 ng; 2 h) and (c) angiotensin coverting enzyme (875 ng; 2h). See the Methods section for details. Products were then resolved by H.P.L.C.

reflects the presence of other activities in the membrane preparation that can further degrade primary products of metabolism. Angiotensin converting enzyme degrades LHRH much more slowly than endopeptidase-24.11 with only 20% degradation after 2 h incubation (fig 1c). Further evidence that endopeptidase-24.11 is the primary enzyme initiating hydrolysis in the brush border preparation is shown in fig. 2 where the specific inhibitor phosphoramidon completely blocks LHRH metabolism by brush border membranes with an I_{50} value of 5.6 nM. This is comparable to the value obtained with purified enzyme. Under the same conditions, no degradation of Antide could be detected with brush border membranes, endopeptidase-24.11 or angiotensin converting enzyme. Even with prolonged incubation with brush border membranes (5 µg protein, 8 hours) no significant metabolism was observed (fig. 3).

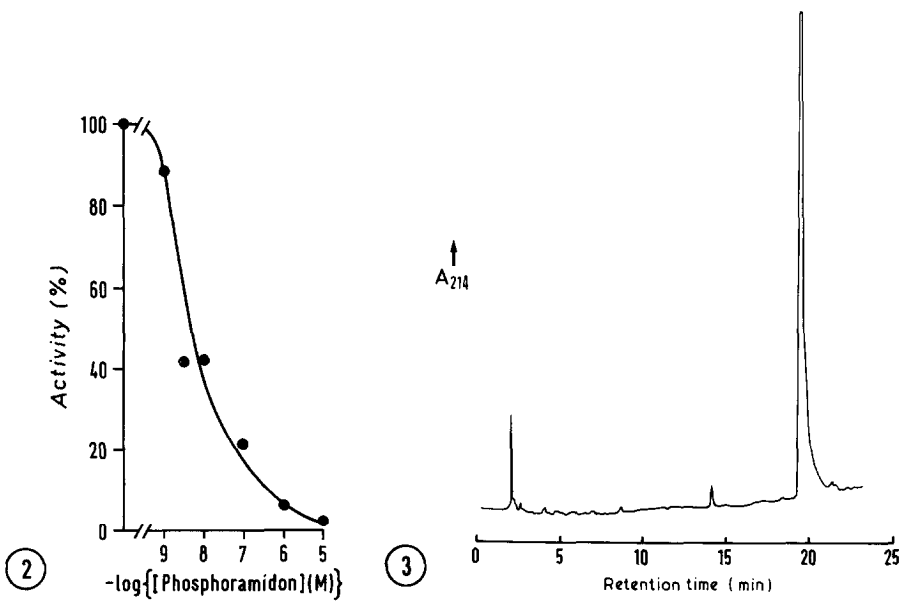

Fig.2. Phosphoramidon inhibition of hydrolysis of LHRH by brush border membranes.

Brush border membranes (1 µg protein; 2 h) were incubated with LHRH in the presence of a range of concentrations of phosphoramidon. Hydrolysis was assessed by h.p.l.c. separation and quantification.

Fig.3. H.p.l.c. analysis of antide hydrolysis by brush border membranes.

Antide was incubated with brush border membrane protein (5 µg) for 8 h. The reaction mixture was then resolved by h.p.l.c. An identical h.p.l.c. profile was observed in the presence of phosphoramidon (1 µM), enalaprilat (1 µM) or membranes that had been incubated at 100°C for 5 min.

DISCUSSION

The LHRH antagonist Antide possesses remarkable duration of action *in vivo* (11) which is presumed to relate to a greater metabolic stability, although this has not been directly tested previously. Structural modifications to a peptide may render some peptide bonds resistant to hydrolysis but may make other bonds more susceptible. Other properties of the peptide analogue, such as its lipophilicity, may also contribute to its prolonged action. A major site for the clearance and inactivation of LHRH is the kidney (1,2). The renal brush border membrane is extraordinarily rich in peptidases, with one or two endopeptidases complemented by a battery of exopeptidases ensuring hydrolysis of most peptides that reach the proximal tubule. It is therefore a valuable model system for studies of peptide hormone degradation. At least twelve ecto-peptidases have been characterized to date in the brush border membrane (see e.g. 8 for review). However, endopeptidase-24.11 appears to be the primary enzyme initiating the hydrolysis and inactivation of many peptides including enkephalins, tachykinins, atrial natriuretic peptides, endothelins as well as LHRH (17-19). Although angiotensin converting enzyme can also hydrolyse *in vitro* LHRH and is present in the renal brush border, its contribution to LHRH degradation appears to be minimal since phosphoramidon inhibits LHRH breakdown virtually completely (fig. 2). Enalaprilat (10 μM) had no significant inhibitory effect on LHRH metabolism by brush border membranes. When Antide is exposed to brush border membranes or purified peptidases even for extended periods of time up to 8 hours, no degradation is seen, establishing its metabolic stability. This emphasizes the potential clinical role of Antide.

It is clear from the present work, and other studies, that the design of stable peptide analogues, particularly those with blocked N- and C-termini, requires inbuilt resistance to endopeptidase-24.11 as a primary consideration. Endopeptidase-24.11 is identical to the acute lymphoblastic leukaemia antigen (CALLA) (20) which is expressed in abundance on the surface of lymphocytes of ALL patients. The raised circulating concentration of endopeptidase-24.11 in such patients may well alter the clearance of a variety of peptide hormones including LHRH. Inhibitors of the endopeptidase may therefore be beneficial to maintain hormone levels in the normal range.

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