J. Pharm. Pharmacol. 2000, 52: 785–795
Received September 22, 1999
Accepted March 9, 2000

Investigations of the In-vitro Metabolism of Three Opioid Tetrapeptides by Pancreatic and Intestinal Enzymes

EVA KRONDAHL, HANS VON EULER-CHELPIN*, ACHIM ORZECHOWSKI*, GUNILLA EKSTRÖM* AND HANS LENNERNÄS

*Department of Pharmacy, Division of Pharmaceutics, Uppsala University, Box 580, BMC, SE-751 23 Uppsala, Sweden and *Preclinical Development, AstraZeneca R&D Södertälje, SE-151 85 Södertälje, Sweden*

Abstract

The metabolism of three opioid tetrapeptides, Tyr-D-Arg-Phe-Nva-NH_2, Tyr-D-Arg-Phe-Phe-NH_2 and Tyr-D-Ala-Phe-Phe-NH_2, was investigated in the presence of pure pancreatic enzymes (trypsin, chymotrypsin, elastase, carboxypeptidase A and carboxypeptidase B), as well as in the presence of pure carboxylesterase and aminopeptidase N. The cleavage patterns of the pure pancreatic enzymes were then compared with those found in rat and human jejunal fluid. Metabolism was also studied in homogenates from different intestinal regions (duodenum, jejunum, ileum and colon) and in enterocyte cytosol from rats. The effect of various protease inhibitors was investigated in the jejunal homogenate. The parent peptides were assayed by high-performance liquid chromatography and metabolites were identified by means of liquid chromatography–mass spectrometry.

Of the pure enzymes, the quickest hydrolysis of the peptides was observed for the pancreatic enzymes chymotrypsin, trypsin and carboxypeptidase A. In most cases they formed the corresponding deamidated tetrapeptides (chymotrypsin and trypsin) or tripeptides with a missing C-terminal amino acid (carboxypeptidase A). Regional differences in intestinal metabolism rates were found for all three peptides ($P < 0{\cdot}001$), with the highest rates observed in jejunal and/or colonic homogenates. The deamidated tetrapeptides were formed both in rat intestinal homogenates and in enterocyte cytosol. Metabolism in the jejunal homogenate was markedly inhibited by some serine and combined serine and cysteine protease inhibitors.

In conclusion, the C-terminal amide of these tetrapeptides did not fully stabilise them against intestinal deamidase and carboxypeptidase activities. The significant hydrolysis of the peptides by pure chymotrypsin, trypsin and carboxypeptidase A showed that lumenal pancreatic proteases might be a clear metabolic obstacle in oral delivery even for small peptides such as these tetrapeptides.

Endogenous opioid peptides have long acted as model peptides for the development of new analgesic drugs. Most of the endogenous opioid peptides known today, for example enkephalins, dynorphins, and β-endorphin, are produced from three precursor proteins, namely proopiomelanocortin, proenkephalin and prodynorphin (Akil et al 1984; Höllt 1986). These opioid peptides exert their effects by interacting with μ, δ and κ subtypes of the opioid receptors (Lord et al 1977). Enkephalins and dynorphins are considered to be endogenous ligands for δ- and κ-opioid receptors, respectively, while β-endorphin has about the same affinity for μ- and δ-opioid receptors (Akil et al 1984). Recently, two tetrapeptides, both with high affinity and selectivity for the μ-opioid receptor, were isolated from mammalian brain (Zadina et al 1997). It was suggested that these peptides, endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2), are natural ligands for the μ-opioid receptor.

Although it is recognised that opioid peptides exert their analgesic effects in the central nervous system (CNS), there is also evidence for a peripheral mechanism of action, especially under

Correspondence: H. Lennernäs, Department of Pharmacy, Division of Pharmaceutics, Uppsala University, Box 580, BMC, SE-751 23 Uppsala, Sweden.

inflammatory conditions (Barber & Gottschlich 1992). Synthetic opiate analgesics clinically used today are often associated with serious central side effects, such as respiratory depression, physical dependency and tolerance (Duthie & Nimmo 1987), which may limit their general use. Hence, peripherally acting opioid analgesics could be one way of treating inflammatory pain while avoiding these side effects. Several synthetic, peripherally acting opioid peptides have been synthesised and evaluated for their antinociceptive action (Hardy et al 1988, 1989).

A major problem with peptides as drugs is their susceptibility to enzymatic hydrolysis when administered in-vivo. Enkephalins are very sensitive to hydrolysis by, for example, aminopeptidases (Dodda Kashi & Lee 1986; Shibanoki et al 1992; Langguth et al 1997). Different chemical approaches, such as the incorporation of D-amino acids, amidation of the C-terminal carboxylic acid, cyclisation, phosphorylation and sugar coupling, have resulted in more stable enkephalin analogues (Dodda Kashi & Lee 1986; Weber et al 1991; Dass & Mahalakshmi 1996; Mizuma et al 1996; Langguth et al 1997). For instance, [D-Ala^2]-metenkephalinamide has a half-life of hydrolysis in ileal homogenate from the albino rabbit that is 15 times longer than that of metenkephalin (Dodda Kashi & Lee 1986). Metkephamid, another synthetic metenkephalin analogue, is stable in the presence of various pancreatic enzymes but is metabolised by aminopeptidases in brush-border membrane vesicles of the rat intestine (Langguth et al 1994a, 1997).

Tyr-D-Arg-Phe-Nva-NH_2 (TArPN; Nva = norvaline), Tyr-D-Arg-Phe-Phe-NH_2 (TArPP) and Tyr-D-Ala-Phe-Phe-NH_2 (TAPP) are potent synthetic μ-opioid selective tetrapeptides, which are designed to have restricted access to the CNS and their action limited to peripheral opioid receptors. TArPP has been reported to be 150 times more potent in the peripheral inflammatory formalin model compared with the centrally mediated tail-flick test (Alari & Martel 1996). However, these three opioid tetrapeptides are sensitive to hydrolysis by enzymes in jejunal fluid and mucosal homogenate from rat jejunum, despite the fact that they have an unnatural D-amino acid in the second position (D-Arg or D-Ala) and a protected C-terminus (an amide) (Krondahl et al 1997).

Although it is well established that peptides usually are easily hydrolysed in the gut, oral dosing still represents the preferred route of administration. More research is therefore required to better understand the mechanisms behind the metabolism and the relation between peptide structure and metabolic activity. Consequently, a strategy for chemical modifications in order to synthesise more stable peptides could be established. This is of special interest since proteomics is one of the most important post-genomic approaches, in which the cellular protein expression can be investigated (Abbott 1999). Its future as a drug discovery tool might renew interest in peptide metabolism in order to be able to develop drugs with peptide structures that can be given orally.

The purpose of this study was to perform in-depth investigations of the enzymes involved, together with the pathways and regional localisation of the intestinal metabolism of three opioid tetrapeptides. Thus, incubations of the peptides were performed in the presence of pure enzymes and intestinal fluid, in homogenates from different intestinal regions and in enterocyte cytosol from rats. The effects of various protease inhibitors were investigated in the jejunal homogenate. Finally, metabolites were identified by means of liquid chromatography–mass spectrometry (LC–MS).

Materials and Methods

Chemicals and buffer solutions

TArPN·2HCl, TArPP·2HCl and TAPP·HCl were synthesised at BioChem Therapeutics (Montreal, Canada). The pancreatic enzymes, namely trypsin (bovine), chymotrypsin (bovine), elastase (porcine), carboxypeptidase A (CP-A, bovine) and carboxypeptidase B (CP-B, porcine), together with liver carboxylesterase and kidney aminopeptidase M (referred to below as aminopeptidase N, AP-N), came from Boeringer Mannheim GmbH, Germany. The inhibitors (Table 1) were all purchased from Sigma Chemical Co. (St Louis, USA), except for the Complete Mini EDTA-free cocktail tablets and Pefabloc SC, which were obtained from Boeringer Mannheim GmbH, Germany. Acetonitrile (LiChrosolv) and acetic acid (AG) came from Merck KGaA, Germany. Other chemicals used were of analytical grade.

A phosphate buffer (pH 6·5 and 290 mmol kg^{-1}) consisting of NaCl (48 mM), KCl (5·4 mM), Na_2HPO_4 (28 mM), NaH_2PO_4 (43 mM), mannitol (35 mM) and D-glucose (10 mM) was used for the incubations with intestinal homogenates and AP-N. For the other incubations, the buffer was modified to pH 7·4 (pancreatic enzymes and carboxylesterase) or pH 7·0 (cytosol). There was no adsorption of any of the peptides on the plastic or glass materials used.

Table 1. The enzyme inhibitors and their concentrations. BNPP, bis(*p*-nitrophenyl)phosphate; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; EDTA, ethylenediaminetetraacetic acid; TLCK, *N*α-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; ZPCK, *N*-CBZ-L-phenylalanine chloromethyl ketone; endo-24.11, endopeptidase 24.11; ACE, angiotensin converting enzyme; SSAO, semicarbazid-sensitive amino oxidase.

Inhibitor	Inhibitor of	Concentration (mM)
Aprotinin	Serine proteases	0·01
Bestatin	Aminopeptidases	0·1
BNPP	Carboxylesterases	1
Cephalothin	Chymotrypsin	1
Cocktail[a]	Serine proteases, aminopeptidases, endo-24.11	
Complete Mini[b]	Serine and cysteine proteases	[c]
E-64	Cysteine proteases	0·1
EDTA	Metalloproteases	1
Lisinopril	ACE	0·01
Paraoxon	Carboxyl- and cholinesterases	1
Pefabloc SC	Serine proteases	1·2
Pepstatin	Aspartic proteases	0·05
Puromycin	Aminopeptidases	0·25
Semicarbazid	SSAO	1
Thiorphan	Endo-24.11	0·01
TLCK	Trypsin, other serine and cysteine proteases	1
TPCK	Chymotrypsin, other serine and cysteine proteases	1
ZPCK	Serine and cysteine proteases	1·2

[a]1·2 mM Pefabloc SC, 0·1 mM bestatin and 0·01 mM thiorphan (Krondahl et al 1997).
[b]Complete Mini EDTA-free cocktail tablets.
[c]1 tablet to 10 mL incubation mixture.

Animals

Male Sprague-Dawley rats (Crl : CD(SD)BR, Charles River, Uppsala, Sweden) were housed together in controlled conditions (21 ± 1°C, 40–60% air humidity, 12-h light–dark cycle) at the Biomedical Center, Uppsala. The rats were acclimatised for at least 1 week before the experiments. During that time they had free access to tap water and regular pellet food (R36, Lactamin AB, Vadstena, Sweden) until 14–20 h prior to being killed, when food was withdrawn. The study was approved by the Animal Ethics Committee in Uppsala (C352/95).

Preparation of jejunal fluid, intestinal homogenates and enterocyte cytosol

Jejunal fluid from rats and humans was collected as described previously (Krondahl et al 1997; Lindahl et al 1997). For the preparation of the intestinal homogenates, the rats (weighing 270–340 g) were anaesthetised by an intraperitoneal injection of Inaktin BYK (thiobutabarbital sodium, 120–150 mg kg^{-1}), the abdomen was opened and the whole intestinal tract removed and put in ice-cold saline (0·9%). All subsequent procedures were performed at 4°C. The intestine was cut into various segments. The first 8 cm of the small intestine was regarded as the duodenum, the next 35 cm as the jejunum and 25 cm proximal from the cecum as the ileum (Bai 1994a). The colon was the region located distal to the cecum. Each segment was flushed with ice-cold saline, opened and the mucosa scraped off with the back of a razor blade. After the intestinal mucosa from different rats (three or more) had been pooled and weighed, 5 mL of 70 mM phosphate buffer (pH 6·5) was added to 1 g of the mucosa, homogenised in a Potter Elvehjem homogeniser and then immediately frozen. Small intestinal homogenate (prepared in phosphate buffer, pH 7·0) was subjected to ultracentrifugation (L5-65 Ultracentrifuge, Beckman) at 100 000 *g* for 60 min (4°C). The supernatant from this centrifugation was designated enterocyte cytosol. The cytosol was frozen immediately after preparation. All preparations were stored at −80°C. Protein concentrations in representative intestinal homogenates and in enterocyte cytosol were determined by the method of Lowry (Lowry et al 1951).

Metabolism studies

Degradation of the peptides in the presence of the pure enzymes was examined using 1-mL incubation mixtures containing 10 μM trypsin, chymotrypsin, elastase, CP-A, CP-B or carboxylesterase in phosphate buffer (pH 7·4) or 1 U mL^{-1} AP-N in phosphate buffer (pH 6·5). The enzyme solution was preincubated for 5 min at 37°C before the peptide solution was added to give a final concentration of 0·1 mg mL^{-1} of the peptide. Samples

(100 μL) were taken at predetermined times, mixed with 100 μL acetic acid (50%) to stop the enzymatic reaction, immediately put on ice and then frozen.

Incubations with rat jejunal fluid (pooled fluid from two rats) and with human jejunal fluid (pooled fluid from three humans) were performed as previously described (Krondahl et al 1997). Samples were withdrawn from the incubation mixtures, added to the same volume of acetic acid (50%) and centrifuged, and the supernatants were frozen for later metabolite identification. TArPP was also incubated in human jejunal fluid in the presence of 1 mM EDTA (ethylenediaminetetraacetic acid) and the samples were treated as above.

Incubations of the peptides in intestinal homogenates, with and without inhibitors, were performed at pH 6·5 and 37°C as previously described (Krondahl et al 1997). Briefly, the incubation mixtures (2 mL) consisted of homogenate (1 mL), peptide-phosphate buffer solution (0·8 mL) and phosphate buffer, including the inhibitors, if any (0·2 mL). The incubation mixtures were preincubated at 37°C for 15 min, with or without inhibitors, before the peptide solution was added (the final peptide concentration was 0·1 mg mL^{-1}). The final protein concentrations in these mixtures were about 12 (duodenal), 11 (jejunal) and 10 (ileal and colonic incubations) mg mL^{-1}. Samples (100 μL) were taken, mixed with the same volume of acetic acid (50%) and centrifuged at 10 000 *g* for 5 min, and then the supernatants were frozen. The experiments with enterocyte cytosol were performed in a similar way to the homogenate incubations, except that a different pH (7·0) and a lower protein concentration (about 6 mg mL^{-1}) were used.

Control incubations of the peptides were performed in the pure phosphate buffers and in the presence of pure chymotrypsin or jejunal homogenate mixed with acetic acid (both experiments using the same concentration of acetic acid as usually used to terminate the enzymatic reaction). All incubation samples were stored at −80°C pending analysis.

Measurement of intact peptide by high-performance liquid chromatography

The peptides were assayed by a high-performance liquid chromatography (HPLC) method described previously (Krondahl et al 1997). The samples were diluted 11 times with the mobile phase and injected (20 μL) onto a reversed-phase column (Symmetry C8, 3·9 × 150 mm, Waters) with a guard column (Symmetry C8, 3·9 × 20 mm, Waters). The mobile phase (flow rate 1 mL min^{-1}) consisted of a sodium phosphate buffer (pH 2·0, I = 0·05) and 11, 16·5 and 25% (v/v) acetonitrile for TArPN, TArPP and TAPP respectively. The retention time for each peptide was around 6·5 min, the UV detection wavelength was 220 nm and the limits of quantitation were 2·1, 2·1 and 2·3 μg mL^{-1} for TArPN, TArPP and TAPP respectively.

Identification of metabolites by LC–MS

Mass analysis of the tetrapeptides (P) and their metabolites (M1–M5) was performed using HPLC with electrospray ionisation mass spectrometry (Table 2). The HPLC system consisted of a binary pump (HP 1100 G 1312A, Hewlett-Packard) with an autosampler (HP 1100 G 1313A , Hewlett-Packard). Separation was achieved on a reversed-phase column (Symmetry C8, 1·0 × 150 mm, Waters, Milford, Mississippi, USA) with a guard column (Opti-Guard, 1·0 × 10 mm, Alltech Associates, Deerfield, Illinois, USA). The mass spectrometer was a Quattro II (Micromass, Man-

Table 2. Amino acid sequences, m/z for single protonised ions and retention times (min) of the parent tetrapeptides (P) and their five metabolites (M1–M5) monitored by LC–MS. Nva, Norvaline; CV, cone voltage (manually selected).

Tetrapeptide/metabolite	TArPN	TArPP	TAPP	CV (V)
P	Tyr-D-Arg-Phe-Nva-NH_2 583·4, 30·2	Tyr-D-Arg-Phe-Phe-NH_2 631·4, 34·4	Tyr-D-Ala-Phe-Phe-NH_2 546·4, 40·9	40
M1	Tyr-D-Arg-Phe-Nva-OH 584·4, 31·6	Tyr-D-Arg-Phe-Phe-OH 632·4, 35·5	Tyr-D-Ala-Phe-Phe-OH 547·4, 42·2	40
M2	Tyr-D-Arg-Phe-OH 485·3, 27·7	Tyr-D-Arg-Phe-OH 485·3, 28·0	Tyr-D-Ala-Phe-OH 399·3, 30·4	35
M3	D-Arg-Phe-Nva-NH_2 420·3, 28·0	D-Arg-Phe-Phe-NH_2 468·3, 32·3	D-Ala-Phe-Phe-NH_2 383·3, 36·1	35
M4	D-Arg-Phe-Nva-OH 421·3, 29·8	D-Arg-Phe-Phe-OH 469·3, 33·8	D-Ala-Phe-Phe-OH 384·3, 38·1	35
M5	D-Arg-Phe-OH 322·2, 25·6	D-Arg-Phe-OH 322·2, 25·9	D-Ala-Phe-OH 237·2, 29·5	30

chester, UK) with an electrospray ion source and a cross-flow counter electrode.

A gradient program was used to elute the peptides and their metabolites. The mobile phases consisted of: A, 0·03% (v/v) trifluoroacetic acid in water; B, 0·03% (v/v) trifluoroacetic acid in water–acetonitrile (50 : 50, v/v). The mixture of the mobile phases was as follows: time 0, 10% B; 1 min, 26% B; 2 min, 34% B; 3 min, 38% B; 34–50 min, 100% B. The flow rate was 0·04 mL min^{-1} and the injection volume was 5 μL. Before analysis, the samples were thawed and transferred to autosampler vials. No further sample treatment took place.

Using selected ion monitoring, mass chromatograms for the protonated molecular ions from the original tetrapeptide (P) and the five main peptide metabolites (M1–M5) that were expected from previous studies were obtained (Table 2). TArPN, TArPP and TAPP have several amino acids in common. This is reflected in a very similar retention time pattern for their metabolites. For most of them, the identification was supported by comparison with reference compounds and tandem mass spectra (MS-MS), which always contained the expected immonium ions from the aromatic amino acids. For TArPP, the relative response (RR) in LC–MS was determined. The peak area per unit weight of monohydrochloride sample, relative to TArPP (triple determinations), was found to be: P, RR = 1; M1, RR = 1·0; M2, RR = 0·22; M3, RR = 2·7; M4, RR = 3·9; M5, RR = 4·8.

Data analysis

Degradation rate constants (k) and half-lives ($t^{1}/_{2}$) in the presence of pure enzymes were calculated by linear regression of first-order plots of parent peptide concentration versus time during the first 60 min. Data from 0–30 min were used in the calculation of k and $t^{1}/_{2}$ for the peptides in the intestinal homogenates. The percentage inhibition of metabolism in the jejunal homogenate was estimated by comparing the k values obtained with and without the enzyme inhibitors.

Possible regional differences in intestinal metabolism rates were evaluated by a robust one-way analysis of variance, Welch's test (StatView, Abacus Concepts, Inc., Berkeley, California, USA). $P < 0{\cdot}05$ was considered significant. Scheffé's F-test was used to identify significant differences between the groups. All data are presented as mean ± s.d. unless otherwise stated.

In order to determine which of the metabolites formed can be regarded as the main metabolite, we compared the peak area of each metabolite with the total peak area of all the presented components after 60 min of incubation.

Results

Metabolism in the presence of pure enzymes

No degradation of the three peptides was observed in pure phosphate buffer. TArPN was the peptide that was found to be most resistant to hydrolysis in the presence of the pure enzymes. Only chymotrypsin and CP-A metabolised TArPN to any extent (Figure 1). The half-life of TArPN in the presence of chymotrypsin was 144 ± 13 min, and LC-MS analysis revealed that two metabolites were formed: Tyr-D-Arg-Phe-Nva-OH and Tyr-D-Arg-Phe-OH (Figure 2A). In the presence of CP-A the half-life of TArPN was estimated to be 85·5 ± 7·4 min, and the tripeptide Tyr-D-Arg-Phe-OH was formed by this enzyme (Figure 2a). Almost no degradation was found for TArPN in the presence of the other pure enzymes ($t^{1}/_{2} > 360$ min).

TArPP and TAPP were very rapidly hydrolysed by chymotrypsin at the C-terminal amide to M1 (Table 2), with half-lives of 0·93 ± 0·04 and 1·49 ± 0·05 min respectively (Figures 1 and 2A). In contrast, no degradation was observed of any of the peptides in the control incubations (120 min) when acetic acid was included in the chymotrypsin incubation mixture. Trypsin also formed the deamidated tetrapeptides (M1) with disappearance half-lives of 17·7 ± 1·0 and 26·2 ± 0·2 min for TArPP and TAPP respectively. The initial concentration–time profiles for these two peptides in the presence of CP-A appeared not to follow first-order kinetics and therefore no half-lives were calculated. However, approximately 40 and 30% of TArPP and TAPP, respectively, remained after incubation for 60 min (Figure 1). The tripeptide Tyr-D-Arg-Phe-OH was formed from TArPP by CP-A. Although TAPP disappeared rather quickly in the presence of CP-A, none of the five metabolites examined (M1–M5) was found. Both peptides were almost stable in the presence of CP-B and elastase ($t^{1}/_{2} > 360$ min). The hydrolysis of TAPP was somewhat more rapid with AP-N and carboxylesterase ($t^{1}/_{2} = 175 \pm 19$ and 318 ± 75 min respectively) than that of TArPN ($t^{1}/_{2} > 360$ min for both enzymes) and TArPP ($t^{1}/_{2} = 343 \pm 42$ and >360 min respectively) (Figure 1). AP-N cleaved the tetrapeptides at the N-terminal Tyr (giving M3), while small amounts of the deamidated tetrapeptides (M1) were found to be formed by carboxylesterase.

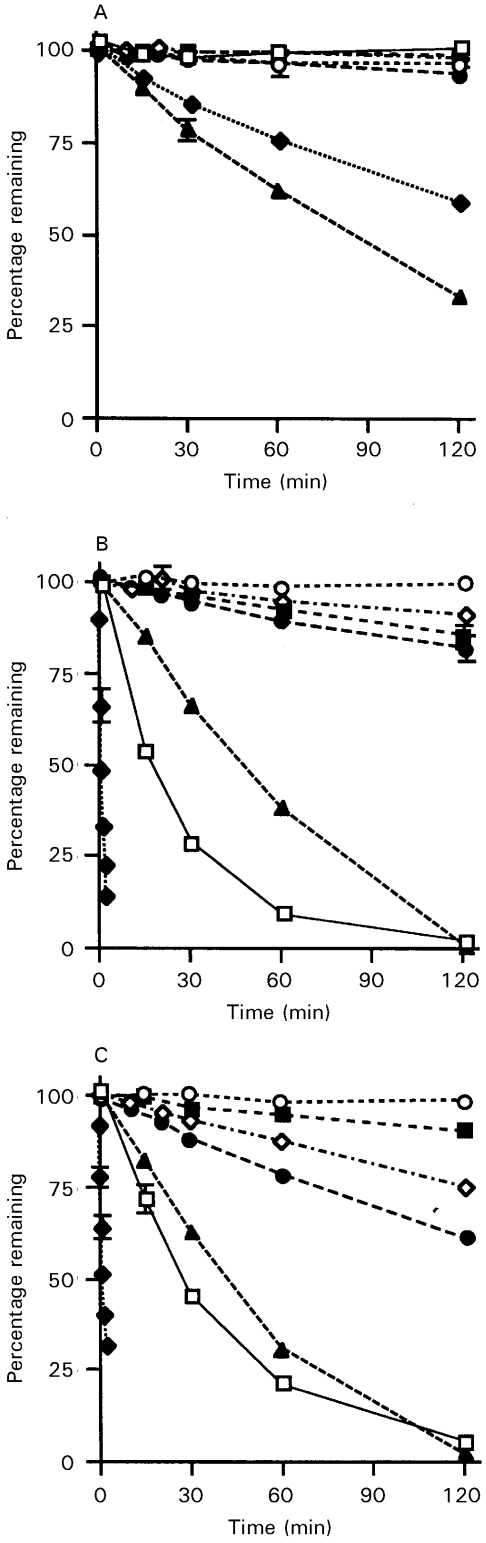

Figure 1. Time courses of degradation of TArPN (A), TArPP (B) and TAPP (C) (0·1 mg mL^{-1}) in the presence of 10 μM trypsin (□), chymotrypsin (◆), elastase (○), carboxypeptidase A (▲), carboxypeptidase B (■), and carboxylesterase (◇) at pH 7·4, or 1 U mL^{-1} aminopeptidase N (●) at pH 6·5. Data are means ± s.d. of three incubations.

Metabolism in rat and in human jejunal fluid

By means of LC–MS we identified the deamidated tetrapeptide (M1) as a metabolite for each of the peptides in rat jejunal fluid (Figure 2B). TArPP was also metabolised to Tyr-D-Arg-Phe-OH and D-Arg-Phe-Phe-OH. In human jejunal fluid, however, no deamidated tetrapeptides were detected. Instead, the M2 tripeptides with a cleaved C-terminal amino acid were formed. In addition, D-Arg-Phe-Phe-OH (from TArPP) and D-Ala-Phe-OH (from TAPP) were found. When TArPP was incubated in human jejunal fluid in the presence of 1 mM EDTA (to inhibit CP-A), the half-life was prolonged from about 3 min to 13 min. However, no deamidated TArPP was observed.

Metabolism in homogenates from various intestinal regions and in enterocyte cytosol from rats

Significant regional differences in intestinal metabolism rates were found for all three peptides ($P < 0{\cdot}001$). The highest metabolic activity was observed in rat jejunal and/or colonic homogenates (Table 3). The deamidated tetrapeptides were identified by LC–MS as the major metabolites in the rat jejunal homogenate (Figure 3). A small amount of the M4 tripeptides was also detected for all three peptides. The M2 tripeptide Tyr-D-Arg-Phe-OH was formed from both TArPN and TArPP, but the corresponding M2 tripeptide from TAPP was not found. Instead, a small amount of the M5 dipeptide, D-Ala-Phe-OH, was identified. No degradation was observed when the peptides were incubated for 120 min in the jejunal homogenate in the presence of acetic acid.

The deamidated tetrapeptides (M1) were to some extent formed in the enterocyte cytosol. The half-lives of the parent peptides were estimated to be 170 ± 18, 71·3 ± 4·8 and 58·3 ± 3·5 min for TArPN, TArPP and TAPP respectively.

Metabolism in jejunal homogenate in the presence of various inhibitors

Various types of enzyme inhibitors (Table 1) were screened for metabolic inhibition in the jejunal homogenate for each peptide. Degradation was inhibited by more than 90% for TArPN in the presence of ZPCK (*N*-CBZ-L-phenylalanine chloromethyl ketone) and the Complete Mini tablet (Figure 4). Both inhibitors prolonged $t_{1/2}$ in the jejunal homogenate from about 12 to 165 min. Furthermore, TLCK (*N*α-*p*-tosyl-L-lysine chloromethyl ketone), TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone) and E-64 (*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane)

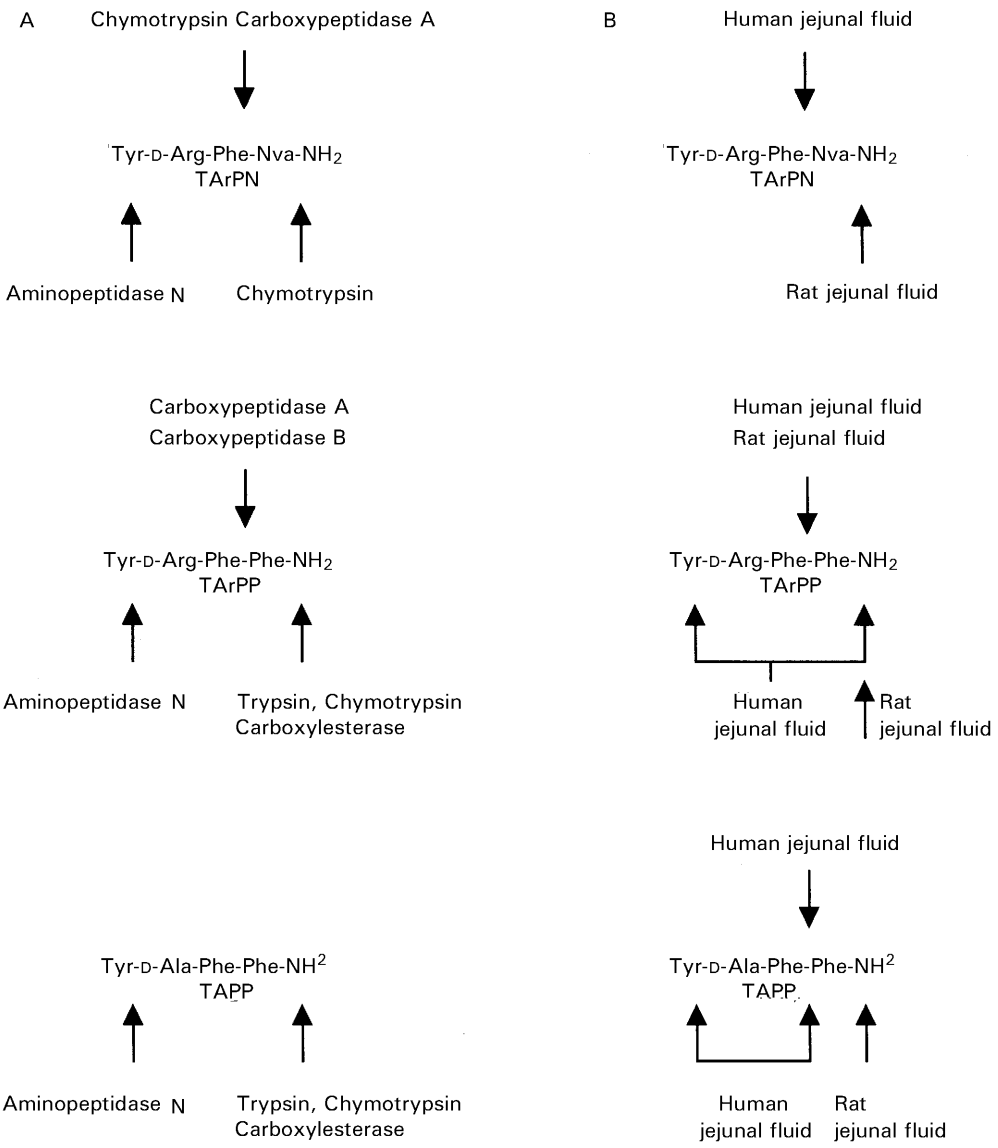

Figure 2A. Cleavage sites of the peptides by pure enzymes at 60 min identified by LC–MS (single determination). B. Cleavage sites of the peptides by enzymes in rat jejunal fluid and human jejunal fluid at 60 min identified by LC–MS (single determination).

showed pronounced inhibition of the metabolism of TArPN (~80%).

For TArPP and TAPP, the highest inhibition, ~80%, was seen with a cocktail consisting of Pefabloc SC, bestatin and thiorphan (Figure 4) (Krondahl et al 1997). Pefabloc SC alone, ZPCK, paraoxon and the Complete Mini tablet all inhibited over 50% of the metabolism of these two peptides. For these peptides, the inhibition seen in the presence of TLCK and TPCK was smaller than for TArPN, and E-64 had no effect at all.

Both the esterase inhibitors, paraoxon and BNPP (bis(*p*-nitrophenyl)phosphate), inhibited metabolism of TArPN by 30% (Figure 4). For TArPP and TAPP, paraoxon was far more potent than BNPP (65–78% inhibition by paraoxon compared with about 20% by BNPP). The other inhibitors used had minor or no inhibitory effects on the metabolism of the peptides in the jejunal homogenate (Figure 4).

Discussion

Identification of possible cleavage sites of the three opioid peptides TArPN, TArPP and TAPP by pancreatic proteases in the intestinal lumen took

Table 3. Half-lives of TArPN, TArPP and TAPP in homogenates from different regions of the rat intestine at pH 6·5 and 37°C.

	Half-life (min)		
Intestinal region	TArPN	TArPP	TAPP
Duodenum (D)	20·4 ± 5·5	46·9 ± 4·0	34·6 ± 2·7
Jejunum (J)	12·2 ± 1·4	30·9 ± 2·2	25·3 ± 3·9
Ileum (I)	18·6 ± 1·7	33·8 ± 2·2	29·8 ± 5·2
Colon (C)	16·9 ± 0·5	23·1 ± 2·8	28·0 ± 2·1

Values are mean ± s.d. of six or more incubations using homogenates prepared within at least two different occations. The following significant regional differences ($P < 0·05$) were found by Scheffé's F-test: TArPN C–J, D–J, I–J; TArPP C–D, C–I, C–J, D–I, D–J; TAPP C–D, D–J.

place by incubation of the peptides with pure pancreatic enzymes and jejunal fluid from rats and humans. Samples from these incubation mixtures were then analysed for metabolites by LC–MS. Thus we demonstrated that all three tetrapeptides were deamidated in the presence of pure chymotrypsin. TArPP and TAPP were also deamidated by trypsin, although less rapidly. Chymotrypsin is known to cleave mainly peptide bonds near hydrophobic amino acids, for example Phe, Tyr and Trp, while trypsin usually prefers to cleave peptide bonds near basic amino acids such as Arg and Lys (Lee 1988). Both TArPP and TAPP have Phe-NH_2 at the C-terminus, and this amide bond is apparently very sensitive to deamidation by chymotrypsin.

Surprisingly, the peptides were also hydrolysed by CP-A to various degrees, despite the protected C-terminus. CP-A usually hydrolyses peptide bonds at the C-terminus of neutral aliphatic or aromatic amino acids of peptides with a free carboxylic group (Lee 1988).

As previously reported, species differences in the jejunal lumenal metabolism of these tetrapeptides seem to exist between rat and man (Krondahl et al 1997). Based on the results from the incubations with the pure pancreatic enzymes, we suggest that the deamidation of the opioid peptides in rat jejunal fluid is most probably performed predominantly by chymotrypsin. Chymotrypsin and trypsin are the major proteases found in pancreatic juice from the rat (Scheele 1994). However, in human jejunal fluid no deamidated tetrapeptides were observed. Instead, the M4 tripeptides Tyr-D-Arg-Phe-OH and Tyr-D-Ala-Phe-OH were detected as the major metabolic products in human jejunal fluid. Such a hydrolysis pattern agrees with the cleavage sites of TArPN and TArPP in the presence of pure CP-A. However, 1 mM EDTA only prolonged the half-life

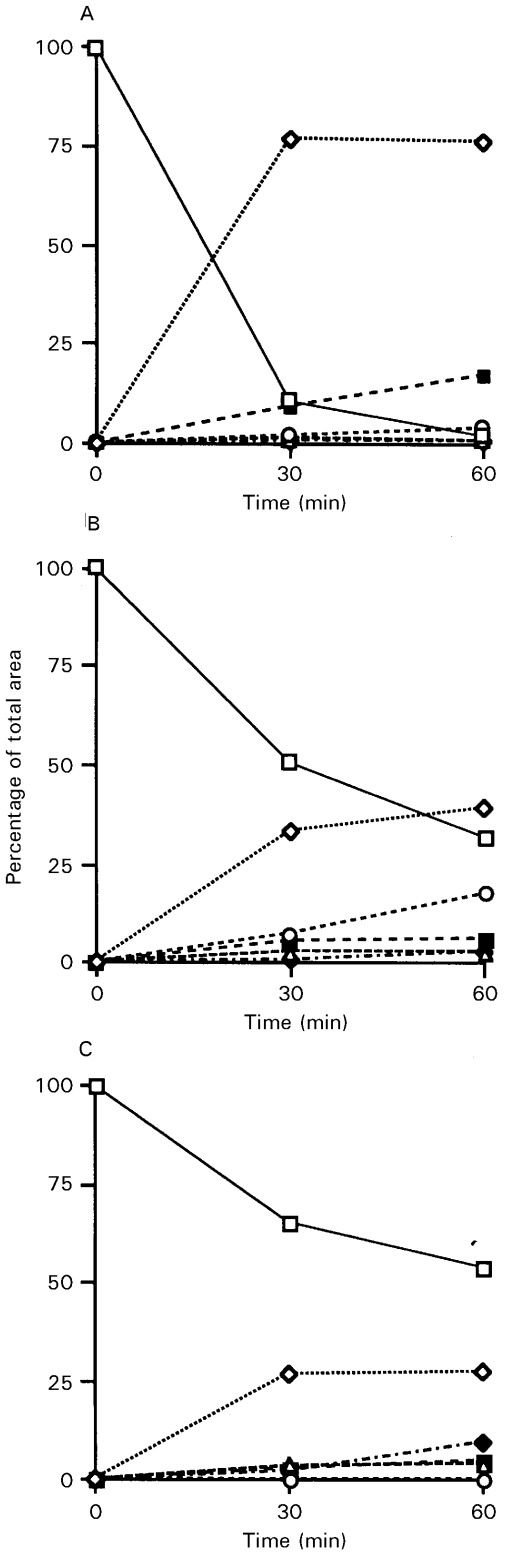

Figure 3. Disappearance of TArPN (A), TArPP (B) and TAPP (C) (0·1 mg mL^{-1}) in rat jejunal homogenate and appearance of their metabolites, identified by LC–MS (single determination). Percentage of total peak area represents the peak area of a specific component compared with the total peak area of all the components presented. P (□), M1 (◇), M2 (○), M3 (△), M4 (■) and M5 (◆).

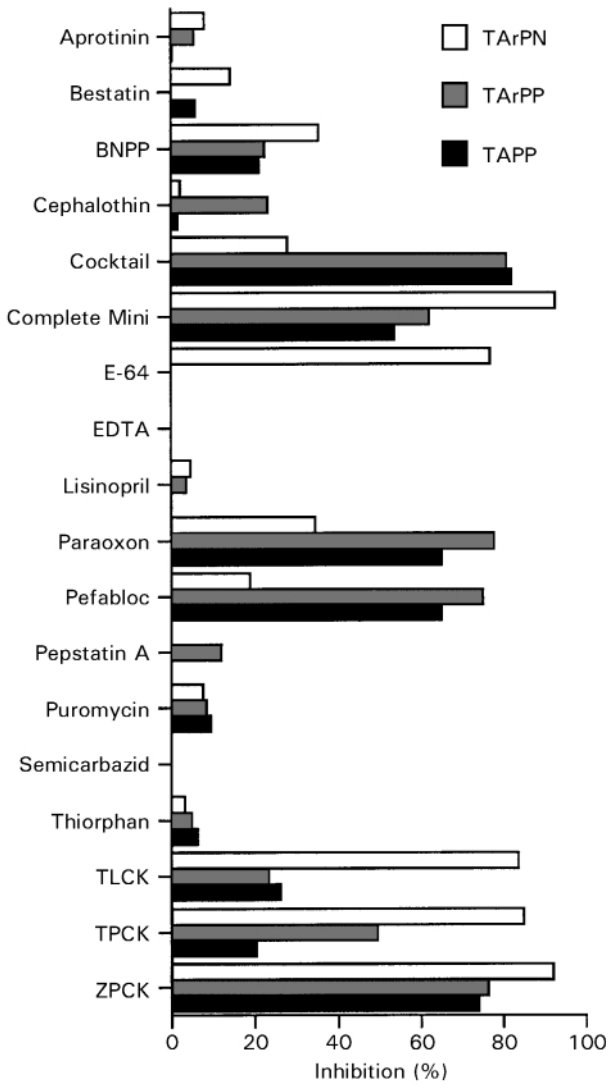

Figure 4. Effect of various protease inhibitors on the metabolism of TArPN, TArPP and TAPP (0·1 mg mL^{-1}) in jejunal homogenate. Each bar represents the mean of at least duplicate experiments. Control experiments without inhibitors were performed in parallel.

of TArPP from 3 to 13 min compared with the mixture of three protease inhibitors (Pefabloc SC, bestatin and thiorphan) used in a previous study, which prolonged the half-life to >180 min (Krondahl et al 1997). Hence, it may be hypothesised that chymotrypsin is also the main metabolising enzyme of TArPP (and of the other two peptides) in human jejunal fluid, although with a different cleavage site from that of rat (or bovine) chymotrypsin.

Regional differences in intestinal metabolism rates have previously been reported for other opioid peptides, for example Leu-enkephalin and D-Ala^2- and D-Leu^2-enkephalin, in various homogenates with a rank order of colon < duodenum < ileum < jejunum (Uchiyama et al 1998). The results were explained as being due to the high activities of jejunal brush-border enkephalinases and aminopeptidases. Regional differences in intestinal metabolism rates were also shown in this study ($P < 0{\cdot}001$), with the jejunal and/or colonic homogenates having the highest and the duodenal homogenate the lowest metabolic activity. The high metabolic activity of the colonic mucosa probably reflects an intracellularly located enzyme (or enzymes) with deamidase activity rather than brush-border activity, as peptide metabolism by brush-border peptidases is more pronounced in the rat small intestine than in the colon (Langguth et al 1994b; Bai et al 1995; Heizmann et al 1996). On the other hand, there are cytosolic enzymes in both the small intestine and colon that have been reported to be capable of metabolising peptide drugs extensively, for example neurotensin (Bai 1994b). We observed some deamidase activity against the tetrapeptides in the enterocyte cytosol. However, it cannot be ruled out that this activity is a contamination of intracellularly located vesicles and/or its contents instead of a deamidase enzyme resident in the cytosol.

Although relatively little attention has been paid to deamidation as an inactivation pathway of biologically active peptides by mammalian enzymes (Skidgel & Erdös 1998), several enzymes in the literature have been reported to hydrolyse C-terminal amides. Calpains, which are Ca^{2+}-dependent, mainly cytosolic, cysteine proteases, have been reported to deamidate substance P, CCK-tetrapeptide and to a small extent [D-Ala^2]met-enkephalin amide (Hatanaka et al 1985). An unidentified enzyme from rat liver microsomes deamidates gastrin tetrapeptide (Walsh & Laster 1973). A peptidase isolated from human platelets, deamidase, was found to deamidate, for example, tachykinins, endothelin I, D-Ala^2-Leu^5- and D-Ala^2-Met^5-enkephalinamide (Jackman et al 1990, 1992). This enzyme is probably also responsible for the reported deamidation of the substance P-based anticancer peptide antagonist G (Jones et al 1995). Deamidase has been reported to be identical to the so-called lysosomal protective protein also known as cathepsin A (EC 3.4.16.5) (Jackman et al 1990; Galjart et al 1991). Endomorphin-1 and endomorphin-2 were recently demonstrated to be hydrolysed at the C-terminal amide group by carboxypeptidase Y, the yeast homologue to the mammalian protective protein/cathepsin A (Péter et al 1999).

We have previously reported that a cocktail consisting of the enzyme inhibitors Pefabloc SC, bestatin and thiorphan protects TArPP and TAPP

from about 80% of metabolism (Krondahl et al 1997). In this study, when Pefabloc SC (a serine protease inhibitor) was used alone at the same concentration, we found almost as pronounced inhibition as seen with the cocktail. This suggests that the inhibitory effect seen with the cocktail is predominantly due to Pefabloc SC. Serine protease inhibitors that were effective for all peptides were the Complete Mini EDTA-free tablet and ZPCK. Aprotinin, another serine protease inhibitor, exhibited almost no inhibition. This may be explained by a different inhibition mechanism to that for the other serine protease inhibitors. The organophosphorus compound paraoxon, which is often used as an inhibitor of carboxyl and cholinesterases, inhibited peptide hydrolysis to a similar extent as Pefabloc SC. As organophosphorus compounds are inhibitors of both serine proteases and serine esterases (Aldridge 1993), the inhibition caused by paraoxon probably reflects inhibition of a serine protease.

The inhibitory profiles of these opioid peptides show similarities with those reported for deamidase (protective protein/cathepsin A). Serine protease inhibitors that react with the active site in serine, for example diisopropylfluorophosphate, are good inhibitors of deamidase (Skidgel & Erdös 1998). However, other serine protease inhibitors such as aprotinin, chelating agents or cysteine inhibitors (e.g. E-64) are not inhibitory (Skidgel & Erdös 1998). Our findings suggest that the three opioid tetrapeptides are metabolised by serine protease(s) in the jejunal homogenate, probably by protective protein/cathepsin A or a similar enzyme. As TArPN seems to be highly protected by the combination of serine and cysteine protease inhibitors, and also by the cysteine protease inhibitor E-64 alone, it may be speculated that more than one enzyme is involved in the deamidation process of this peptide.

Both the aminopeptidase inhibitors bestatin and puromycin exhibited only minor inhibitory effects on the metabolism rates in the rat jejunal homogenate. LC–MS analysis identified tripeptide metabolites without the amino acid Tyr (M3 and M4) as minor metabolites in the rat jejunal homogenate. Furthermore, the tetrapeptides were rather resistant to enzymatic hydrolysis by pure AP-N at pH 6·5. From these results we can conclude that the first and predominant metabolic pathway of these tetrapeptides in rat jejunal homogenate at pH 6·5 is deamidation of the C-terminal amide, whereas aminopeptidases do not seem to be involved to any major extent. Whether or not the deamidation reaction found in the various intestinal homogenates is important in the in-vivo situation is still an unresolved question. In intestinal homogenates all enzymes of the intestinal cell are mixed and the observed metabolism represents metabolism by brush-border membrane peptidases, as well as metabolism by intracellularly located cytosolic and lysosomal proteases. Intracellular intestinal metabolism is interesting provided that the peptides are transcellularly transported. If they are transported through the intestinal membranes solely via the paracellular route, only metabolism by brush-border membrane peptidases and not intracellular metabolism is important. However, as the transport mechanism/pathway through the gut wall is not known for these tetrapeptides, transcellular transport, and hence intracellular metabolism, for example by the deamidase enzyme, cannot be ruled out in the in-vivo situation.

In conclusion, this study focused on the enzymatic barrier of the intestine in limiting oral drug absorption of small opioid peptides. The D-amino acid in position 2 of the tetrapeptides seemed largely to protect them from N-terminal aminopeptidase cleavage, while in contrast the C-terminal amide did not fully stabilise them against deamidase and carboxypeptidase activities. Significant hydrolysis of the peptides was seen in the presence of the pure pancreatic serine proteases chymotrypsin and trypsin, by pancreatic CP-A, as well as in mucosal homogenates from both the small intestine and colon of the rat. A species difference in lumenal metabolism seems to exist between rat and man since deamidation was not observed for any of the peptides in human jejunal fluid. Finally, the marked protection from enzymatic degradation in the jejunal homogenate in the presence of some serine protease inhibitors further highlights the overall importance of serine enzymes in the metabolism of these opioid peptides.

Acknowledgements

We would like to thank Masoud Nekoei and Karin Sturman for skilful technical assistance during the incubations.

References

Abbott, A. (1999) A post-genomic challenge: learning to read patterns of protein synthesis. Nature 402: 715–720

Akil, H., Watson, S. J., Young, E., Lewis, M. E., Khachaturian, H., Walker, J. M. (1984) Endogenous opioids: biology and function. Ann. Rev. Neurosci. 7: 223–255

Alari, L., Martel, R. (1996) Antinociceptive effects of LEF553, a peripherally acting opioid peptide in mice. (8th) World Congress on Pain, IASP Press, p. 455

Aldridge, W. N. (1993) The esterases: perspectives and problems. Chem. Biol. Interact. 87: 5–13

Bai, J. P. F. (1994a) Distribution of brush-border membrane peptidases along the rat intestine. Pharm. Res. 11: 897–900

Bai, J. P. F. (1994b) Subcellular distribution of proteolytic activities degrading bioactive peptides and analogues in the rat small intestinal and colonic enterocytes. J. Pharm. Pharmacol. 46: 671–675

Bai, J. P. F., Chang, L.-L., Guo, J.-H. (1995) Targeting of peptide and protein drugs to specific sites in the oral route. Crit. Rev. Ther. Drug Carrier Syst. 12: 339–371

Barber, A., Gottschlich, R. (1992) Opioid agonists and antagonists: an evaluation of their peripheral actions in inflammation. Med. Res. Rev. 12: 525–562

Dass, C., Mahalakshmi, P. (1996) Phosphorylation of enkephalins enhances their proteolytic stability. Life Sci. 58: 1039–1045

Dodda Kashi, S., Lee, V. H. L. (1986) Enkephalin hydrolysis in homogenates of various absorptive mucosae of the albino rabbit: similarities in rates and involvement of aminopeptidases. Life Sci. 38: 2019–2028

Duthie, D. J. R., Nimmo, W. S. (1987) Adverse effects of opioid analgesic drugs. Br. J. Anaesth. 59: 61–77

Galjart, N. J., Morreau, H., Willemsen, R., Gillemans, N., Bonten, E. J., d'Azzo, A. (1991) Human lysosomal protective protein has cathepsin A-like activity distinct from its protective function. J. Biol. Chem. 266: 14754–14762

Hardy, G. W., Lowe, L. A., Yih Sang, P., Simpkin, D. S. A., Wilkinson, S., Follenfant, R. L., Smith, T. W. (1988) Peripherally acting enkephalin analogues. 1. Polar pentapeptides. J. Med. Chem. 31: 960–966

Hardy, G. W., Lowe, L. A., Mills, G., Yih Sang, P., Simpkin, D. S. A., Follenfant, R. L., Shankley, C., Smith, T. W. (1989) Peripherally acting enkephalin analogues. 2. Polar tri- and tetrapeptides. J. Med. Chem. 32: 1108–1118

Hatanaka, M., Sasaki, T., Kikuchi, T., Murachi, T. (1985) Amidase-like activity of calpain I and calpain II on substance P and its related peptides. Arch. Biochem. Biophys. 242: 557–562

Heizmann, J., Langguth, P., Biber, A., Oschmann, R., Merkle, H. P., Wolffram, S. (1996) Enzymatic cleavage of thymopoietin oligopeptides by pancreatic and intestinal brush-border enzymes. Peptides 17: 1083–1089

Höllt, V. (1986) Opioid peptide processing and receptor selectivity. Ann. Rev. Pharmacol. Toxicol. 26: 59–77

Jackman, H. L., Tan, F., Tamei, H., Beurling-Harbury, C., Li, X.-Y., Skidgel, R. A., Erdös, E. G. (1990) A peptidase in human platelets that deamidates tachykinins. Probable identity with the lysosomal 'protective protein'. J. Biol. Chem. 265: 11265–11272

Jackman, H. L., Morris, P. W., Deddish, P. A., Skidgel, R. A., Erdös, E. G. (1992) Inactivation of endothelin I by deamidase (lysosomal protective protein). J. Biol. Chem. 267: 2872–2875

Jones, D. A., Cummings, J., Langdon, S. P., MacLellan, A., Smyth, J. F. (1995) Characterization of the deamidase enzyme responsible for the metabolism of the anticancer peptide: H-Arg-D-Trp-N^{me}Phe-D-Trp-Leu-Met-NH_2. Biochem. Pharmacol. 50: 585–590

Krondahl, E., Orzechowski, A., Ekström, G., Lennernäs, H. (1997) Rat jejunal permeability and metabolism of μ-selective tetrapeptides in gastrointestinal fluids from humans and rats. Pharm. Res. 14: 1780–1785

Langguth, P., Bohner, V., Biber, J., Merkle, H. P. (1994a) Metabolism and transport of the pentapeptide metkephamid by brush-border membrane vesicles of rat intestine. J. Pharm. Pharmacol. 46: 34–40

Langguth, P., Merkle, H. P., Amidon, G. L. (1994b) Oral absorption of peptides: the effect of absorption site and enzyme inhibition on the systemic bioavailability of metkephamid. Pharm. Res. 11: 528–535

Langguth, P., Bohner, V., Heizmann, J., Merkle, H. P., Wolffram, S., Amidon, G. L., Yamashita, S. (1997) The challenge of proteolytic enzymes in intestinal peptide delivery. J. Control. Rel. 46: 39–57

Lee, V. H. L. (1988) Enzymatic barriers to peptide and protein absorption. Crit. Rev. Ther. Drug Carrier Syst. 5: 69–97

Lindahl, A., Ungell, A.-L., Knutson, L., Lennernäs, H. (1997) Characterization of fluids from the stomach and proximal jejunum in men and women. Pharm. Res. 14: 497–502

Lord, J. A. H., Waterfield, A. A., Hughes, J., Kosterlitz, H. W. (1977) Endogenous opioid peptides: multiple agonists and receptors. Nature 267: 495–499

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265–275

Mizuma, T., Ohta, K., Koyanagi, A., Awazu, S. (1996) Improvement of intestinal absorption of leucine enkephalin by sugar coupling and peptidase inhibitors. J. Pharm. Sci. 85: 854–857

Péter, A., Tóth, G., Tömböly, C., Laus, G., Tourwè, D. (1999) Liquid chromatographic study of the enzymatic degradation of endomorphins, with identification by electrospray ionization mass spectrometry. J. Chromatogr. A 846: 39–48

Scheele, G. A. (1994) Extracellular and intracellular messengers in diet-induced regulation of pancreatic gene expression. In: Johnson, L. R. (ed.) Physiology of the Gastrointestinal Tract. Raven Press, New York, pp 1543–1554

Shibanoki, S., Weinberger, S. B., Schulteis, G., Ishikawa, K., Martinez, J. L. Jr (1992) Enkephalin hydrolysis by mouse plasma in-vitro. Life Sci. 50: 667–675

Skidgel, R. A., Erdös, E. G. (1998) Cellular carboxypeptidases. Immunol. Rev. 161: 129–141

Uchiyama, T., Kotani, A., Kishida, T., Tatsumi, H., Okamoto, A., Fujita, T., Murakami, S., Muranishi, M., Yamamoto, A. (1998) Effects of various protease inhibitors on the stability and permeability of [D-Ala^2, D-Leu^5]enkephalin in the rat intestine: comparison with leucine enkephalin. J. Pharm. Sci. 87: 448–452

Walsh, J. H., Laster, L. (1973) Enzymatic deamidation of the C-terminal tetrapeptide amide of gastrin by mammalian tissues. Biochem. Med. 8: 432–449

Weber, S. J., Greene, D. L., Sharma, S. D., Yamamura, H. I., Kramer, T. H., Burks, T. F., Hruby, V. J., Hersh, L. B., Davis, T. P. (1991) Distribution of analgesia of [^{3}H]-[D-Pen^2, D-Pen^5]enkephalin and two halogenated analogues after intravenous administration. J. Pharmacol. Exp. Ther. 259: 1109–1117

Zadina, J. E., Hackler, L., Ge, L.-J., Kastin, A. J. (1997) A potent and selective endogenous agonist for the μ-opiate receptor. Nature 386: 499–502