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Endoproteolysis by isolated membrane peptidases reveal metabolic stability of glucagon-like peptide-1 analogs, exendins-3 and -4

Summary

These in vitro studies aimed to characterize the pattern and the kinetics of endoproteolysis of the insulinotropic hormone glucagon-like peptide-1 (GLP-1) and related peptides by native ectopeptidases. Peptides were incubated with isolated rat or pig kidney brush-border microvilli membranes, which are a rich source of the ectopeptidases that are responsible for the post-secretory metabolism of peptide hormones. The proteolytic products were separated by reversed-phase HPLC column chromatography and characterised by molecular mass and primary structure. The relative importance of specific peptidases was established by measuring the effects of specific peptidase inhibitors on the kinetics of proteolysis. Dipeptidyl-peptidase-IV was found to be rate-limiting in the endoproteolysis of GLP-1. GLP-1 homologs, exendins-3 and -4, exhibited exceptional stability in the presence of isolated kidney microvilli membranes. Our finding that exendin-4 is several orders of magnitude more stable than GLP-1 and Ser-8-GLP-1 is especially noteworthy given this peptide's widely reported insulinotropic potency.

Key words
Glucagon-like peptide-1 · Exendin-3 · Exendin-4 · Membrane ectopeptidases · Endoproteolysis

Introduction

The post-secretory metabolism of peptide hormones involves their endoproteolysis by a small group of plasma membrane-bound enzymes, which have their active sites orientated towards the extracellular space and are thus refered to as ectopeptidases (Kenny and Booth, 1991; Medeiros and Turner, 1994; Turner and Tanzawa, 1997). Most of these ectopeptidases exhibit broad substrate specificity and a wide tissue distribution (Turner and Tanzawa, 1997). Differences in the rates of post-secretory processing of peptide hormones must reflect the variable susceptibility of peptide hormones to the combined effects of the various peptidases that they encounter within the body's extracellular compartment. Identification of the peptidases involved in the post-translational processing of a peptide hormone is of particular interest for those peptides of potential therapeutic value.

Here, we describe in vitro analysis of the endoproteolysis of the peptide hormone, glucagon-like peptide (GLP-1) by brush-border microvillar membranes (BBMM) isolated from rat and pig renal proximal tubules (Booth and Kenny, 1976). This in vitro approach allows a detailed biochemical characterisation of the pattern of post-secretory processing of this peptide, the therapeutic potential of which is hindered by a very short circulatory half-life (Kreymann, et al., 1987). BBMM contain high levels of all the major membrane ectopeptidase in their native form (Booth and Kenny, 1976) and allow direct comparison of their relative importance. Thus, which peptidases are most crucially involved in the post-secretory processing of a given peptide hormone can be readily identified. This involves quantification and comparison of the effectiveness of peptidase-specific inhibitors, the isolation and characterisation of proteolytic products as well as kinetic analysis. Additionally, the comparative metabolic stabil-

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ity of peptide homologs and analogs can be accurately assessed in vitro.

GLP-1 is an intestinal hormone that is released from the intestinal L-cell post-prandially and its major function appears to be to limit post-prandial hyperglycemia (Kieffer and Habener, 1999). It causes a glucose-dependent increase in insulin secretion (Holst, et al., 1987; Kreymann et al., 1987) (Ritzel et al., 1995) and also delays gastric emptying. It is a hormone that has attracted considerable interest due to it's possible therapeutic value for Type-II diabetes (Fehmann et al., 1992; Nauck et al., 1993; Kieffer and Habener, 1999). Studies of the post-secretory processing of GLP-1 have led to identification of the possible peptidases involved (Hupe-Sodmann et al., 1995; Kieffer et al., 1995; Deacon et al., 1998) (Mentlein et al., 1993). Dipeptidyl peptidase (DPP)-IV has been identified as a key enzyme in the post-secretory processing of GLP-1 and DPP-IV-resistant analogs of GLP-1 do exhibit improved metabolic stability (Chou et al., 1997; Deacon et al., 1998; Deacon et al., 1998; Ritzel et al., 1998; Gallwitz et al., 2000; Larsen et al., 2001).

Here we aimed to demonstrate the effectiveness of using isolated BBMMs for the in vitro analysis of the post-secretory processing of GLP-1. We aimed to identify which peptidases are most effective at endoproteolysis of GLP-1 and to compare the rate of endoproteolysis with that of two other gut peptide hormones, glucose-dependent insulinotropic peptide (GIP) (Pederson, 1994) and peptide-YY (PYY) (Taylor, 1993), and of the non-mammalian GLP-1 homologs, exendins-3 and -4 whose metabolic stability have not been previously tested systematically. The primary sequences of human GLP-1, human GIP, human PYY, exendin-3 and exendin-4 are shown in Table **1**.

Materials and methods

Reagents

All peptides, except exendins-3 and -4, were purchased from Polypeptide Laboratories GmbH (Wolfenbuettel, Germany). Exendins-3 and -4 were purchased from Peninsula Ltd (St Helens, UK). Human recombinant neutral endopeptidease (NEP) 24.11 (EC 3.4.24.11) was kindly provided by Genentech (San Francisco, USA). Dipetidyl peptidase (DPP) IV (EC 3.4.14.5), isolated from human placenta, was obtained from Calbiochem (Bad Soden, Germany). For the assay of NEP 24.11, the fluorogenic substrate glutaryl-alanyl-alanyl-phenylalanyl-4-methoxy-naphthyl-NH_2 (GAAF-4-MNA) and aminopeptidase N (EC 3.4.11.2) ("leucine aminopeptidase type VI") were obtained from Sigma Chemicals (Deisenhofen, Germany). The following peptidase inhibitors, thiorphan (inhibits NEP 24.11), 1,10-ortho-phenanthroline (inhibits metalloproteases) and pepstatin A, were purchased from Sigma Chemicals (Deisenhofen, Germany), phosphoramidom (inhibits NEP 24.11) and phosphomethyl-sulphonyl fluoride (inhibits "serine proteases") from Serva (Heidelberg, Germany) and captopril (a specific inhibitor of peptidyl dipeptidase-15.1 (EC 3.4.15.1) ("angiotensin-converting enzyme") was a gift from Schwartz GmbH (Meppenheim, Germany).

Kidney brush-border microvilli membrane (BBMM) preparation

Kidney BBMM membranes were prepared from pig and from rat renal cortex, according to the sequential differential centrifugation procedure described by Booth (Booth and Kenny, 1976). The yield and degree of purification was monitored at each step by colorimetric assay of the activity of standard subcellular marker enzymes and by fluorometric assay of the membrane peptidase, neutral endopeptidase (NEP) 24.11. These methods have been described in detail previously (Hupe-Sodmann et al., 1997).

High pressure liquid chromatography

The rpHPLC was performed using a 4 × 150 mm (5 μ and WP300) endcapped Lichrosher C-8 column (Merck, Darmstadt, Germany), a Pharmacia (Freiburg, Germany) gradient former and single "2248" pump HPLC system, a Whisp 710B Autosampler (Millipore-Waters, Germany). The products of the incubations were eluted with a linear gradient of acetonitrile, in 0.1% TFA, that increased in concentration from 0 to 35% in 50 minutes and was followed by a further linnear gradient increase to 70% in 10 minutes. The products of the incubations were detected by their UV-absorbance which was measured on-line using SP4 UV detector (Gyncotec, Germany). The detector was set at 215 nm for optimal detection of eluted peptides and the chart recordings were made at two sensitivity settings.

Structural analysis of peptide fractions

For GLP-1, UV-absorbing fractions were collected by hand and further processed for analysis of their primary structure and molecular mass. The mass of each incubation product was determined using a BIOJon 20 plasma desorption mass spectroscope (Applied Biosystems, Weiterstadt, Germany). Their primary structures were determined by solid-phase Edmann degradation using a 447A 120A amino acid sequencer (Applied Biosystems, Weiterstadt, Germany).

Incubations and peptide analyses

30 μg BBMM membrane preparation was incubated with 30 μM of peptide in 100 μl of 50 mM Hepes buffer, 50 Mm NaCl, pH 7.4. Some experiments, were performed in the presence of selected peptidase inhibitors. After selected time intervals, the incubations were terminated by cooling on ice, before boiling for 10 minutes. The incubation mixture was then centrifuged for 10 minutes at 10,000 g, before analysis by reversed phase high-pressure liquid chromatography (rpHPLC).

Kinetic analysis of endoproteolysis

The rate of hydrolysis of each substrate peptide was determined as previously described (Hupe-Sodmann et al., 1997). Each peptide substrate was incubated at different time intervals (5, 15, 30, and 60 minutes), in duplicate, with isolated BBMMs. The time taken for a 10% reduction in the average height of the substrate UV_{215} peak, as measured by on-line detection following HPLC separation, was used to calculate the initial reaction rate of the proteolysis.

Results

Kidney BBMM membrane preparation

The assay of the BBMM ectopeptidase, NEP 24.11, indicated an enrichment factor of greater than 20 for the final BBMM membrane protein preparation as compared to the original homogenate. Reduced contamination with lysomal and endoplasmic reticulum membranes was indicated by the low level (less than 20%) of the activity of the respective marker enzymes (β-glucuronidase and glucose-6-phosphatase) in the BBMM membranes compared to the original homogenate. This compares very favourably with the quality of preparation reported in the original description of the method by Booth and Kenny (Booth and Kenny, 1976).

Endoproteolysis of GLP-1 by BBMM membranes/DPP-IV

Control experiments in which differing known amounts of synthetic GLP-1 were analysed by rpHPLC under identical conditions to those used for the analysis of the proteolytic experiments, showed that the height of the UV-absorbing peaks is linearly related to the quantity of peptide applied to the HPLC column (data not shown). Following incubation of GLP-1 with either porcine or rat BBMM, there was a time-dependent reduction in the height of the GLP-1 UV-absorbing peak as detected following elution from the HPLC column (Fig. **1**). The data points are average values of duplicate incubations at each time interval with the same membrane preparation This data indicate that the rate of endoproteolysis by the rat BBM preparation was greater than that by the porcine BBM preparation. Since identical amounts of BBM protein was tested in each experiement, this reflects that the yield of peptidases in the rat BBMs was greater than that in the porcine BBMs. This was also reflected in the relative amounts of NEP 24.11 measured in the two preparations by the fluorometric assay (data not shown).

Figure **2** shows the results of rpHPLC analysis of GLP-1 following incubation in the presence and absence of BBMM. It is seen here that the GLP-1 peak is reduced in the presence of rat BBMM membranes and that there is a concomitant appearance of additional product peaks. Also shown in Figure **2**, is the profile obtained following incubating GLP-1 with rat BBMM membranes together with a cocktail of peptidase inhibitors. The presence of inhibitors preserves the GLP-1 substrate peak which indicates that the effect of the presence of BBMM membranes is due to peptidase activity. Similar HPLC profiles were obtained with reaction mixtures obtained with porcine BBMM membranes (data not shown) with the exception that the major peak eluting at 59 minutes that is seen in rat profiles is absent from the porcine profiles. Incubating GLP-1 with DPP-IV generates (Fig. **2**) one major product peak that elutes at 63.4 minutes which is later than the GLP-1 substrate. A product peak with an identical elution time is also generated by both rat and porcine BBMM membranes.

All the major peaks that were resolved following incubation with rat and porcine BBMM membranes and with DPP-IV were collected and analysed for mass and partial amino terminal primary structure. The results are shown in Table **1**. These

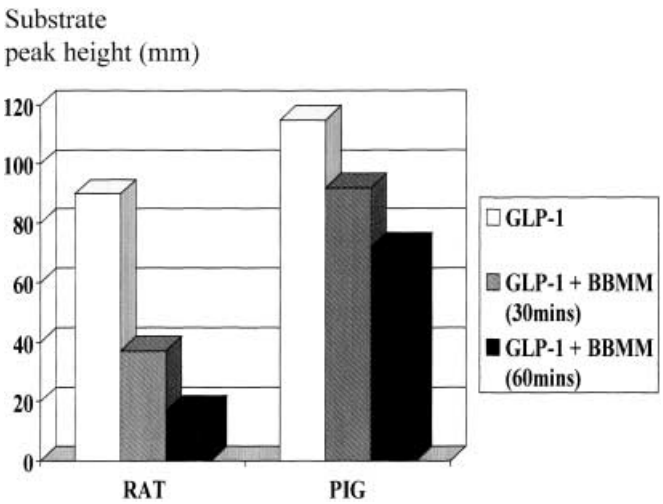

Fig. **1** Time-dependent decrease in the GLP-1 substrate peak (deteced by rpHPLC with on-line UV-detection) in the presence of rat and pig BBMM. Each histogram value is the average of two incubations for each time interval using the same rat or porcine membrane preparation

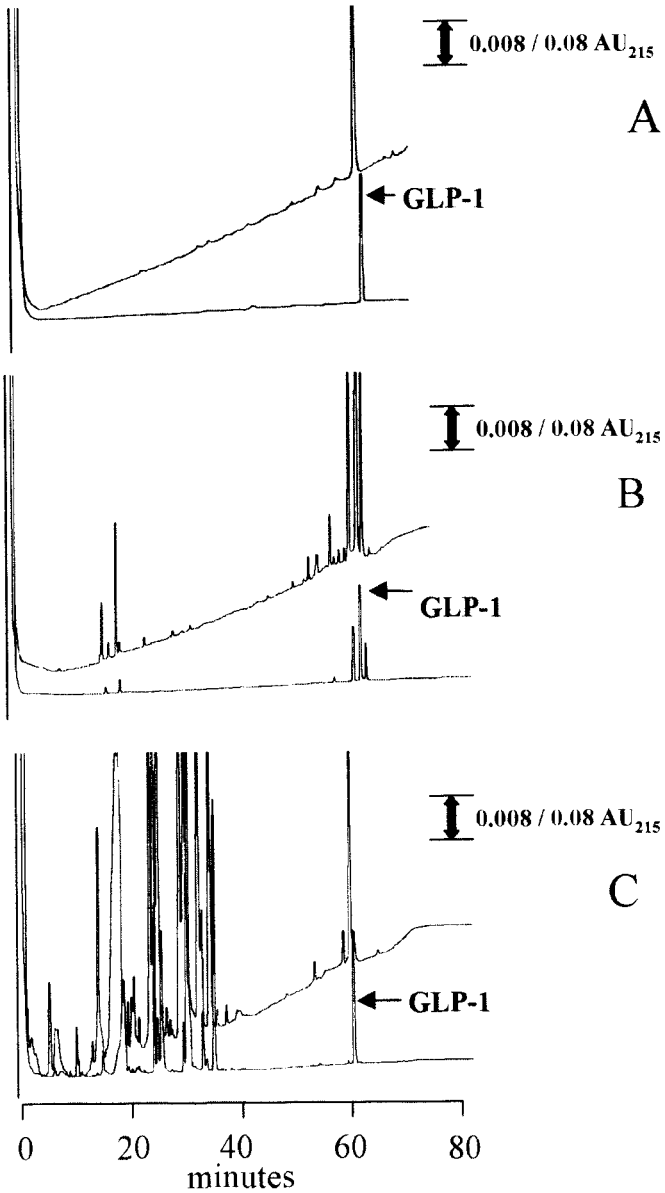

Fig. **2** UV-profiles (215 nm) of GLP-1 (30 µg) (**A**), and of reaction mixtures following incubation of GLP1 (30 µg) with rat BBMM (0.5 µg protein) (**B**), and with rat BBMM (0.5 µg protein) in the presence of a cocktail of peptidase inhibitors (10^{-3} M of each of diprotonin A, captopril, phosphoramidon and bestatin) (**C**). The double-headed arrow indicates the scale of absorbtion units. For each analysis, two profiles are shown each obtained with a different sensitivity setting

Table **1** Primary structures of human GLP-1, human GIP, human PYY, exendin-3 and exendin-4.

GLP-1	HAEGTFTSDVSSYLGQAAKEF IAWLVKGR
GIP	YEGTFISDYSIAMOKJHQQDFVNWLLAQKGKKNDWKHNITQ
EXENDIN-3	HSDGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS
EXENDIN-4	HGEGTFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS
PYY	YPSKPDNPGEDAPAEDMARYYSALRHYINLITRQRY

indicate that both BBMM membranes and DPP-IV generate GLP-1 (3–30) (peak that elutes at 63.4 minutes) which clearly suggests that BBMM membranes contain DPP-IV which acts on GLP-1 identically to the isolated enzyme. This is further confirmed by the effectiveness of diprotin A in inhibiting the generation of this product by both the BBMM membranes and DPP-IV.

In order assess which peptidase is the most rate-limiting in the proteolysis of GLP-1 by BBMM membranes, high concentrations of specific inhibitors were tested individually for their effectiveness in increasing the recovery of GLP-1 following incubation with BBMM membranes. The data shown in Table **3** indicate that the peptidase DPP-IV is rate-limiting in the endoproteolysis of GLP-1 by BBMM membranes. This is indicated by the significantly greater effectiveness of the DPP-IV inhibitor, diprotin A, in increasing the recovery of the GLP-1 substrate following incubation with BBMM membranes. Inhibition of NEP 24.11 activity, through addition of phosphoramidon, had little effect on

Table **2** Molecular characteristics and corresponding deduced truncated GLP-1 structures of the UV-absorbing rpHPLC fractions collected at the stated retention times.

Peptidase preparation	*HPLC fraction*	*Retention time [min]*	*Molecular weight [D]*	*Amino-terminal partial sequence*	*Identity of GLP-1 partial sequence*
Pig BBMM	1	60	3297,5	H, E, A...	GLP-1(7–36)
	2	61	3092	E, G, T...	GLP-1(9–36)
Rat BBMM	1	59	2467	D, V, S...	GLP-1(15–36)
	2	60	3297,5	H, E, A...	GLP-1(7–36)
	3	61	3092	E, G, T...	GLP-1(9–36)
DPP-IV	1	60	3297,5	H, E, A...	GLP-1(7–36)
	2	61	3092	E, G, T...	GLP-1(9–36)

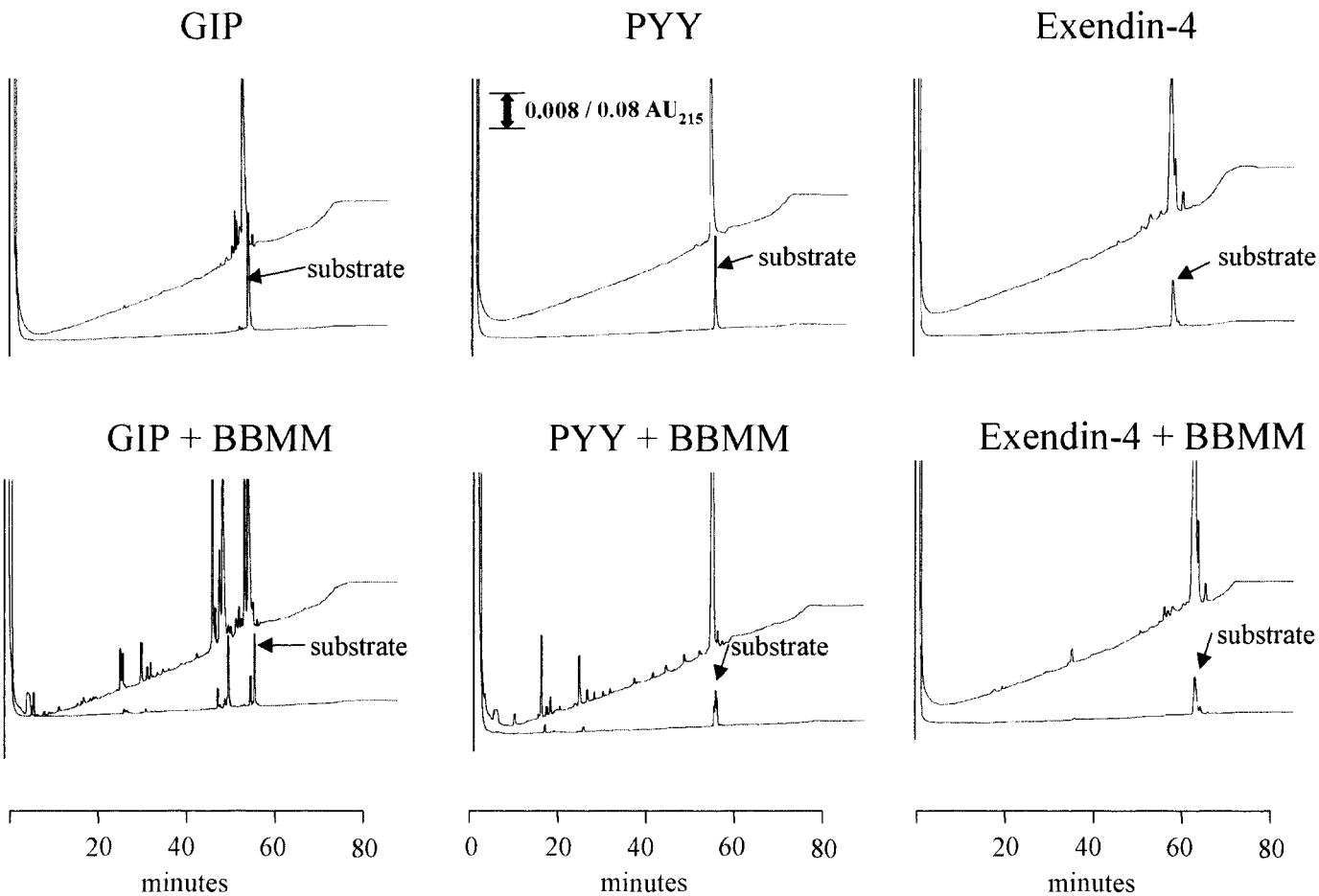

Fig. **3** UV profiles obtained following rpHPLC of synthetic versions of the peptide hormones, GIP, PYY and exendin-4 following 60 minutes incubation of each peptide (10 µg) in reaction buffer (upper profiles) and in the presence of 0.5 µg of BBMM protein (lower profiles). The double-headed arrow indicates the scale of absorbtion units. For each analysis, two profiles are shown each obtained with a different sensitivity setting

Table **3** The mean percentage inhibition of BBM-mediated endoproteolysis of GLP-1 by the stated peptidase inhibitors.

Inhibitor	*Inhibitor concentration*	*Mean percent inhibition of GLP-1 proteolysis by BBM*	*Number of Experiments*
Diprotin A	10^{-3} M	65.1	6
Captopril	10^{-3} M	20.3	4
Phosphoramidon	10^{-3} M	−4.7	8
Bestatin	10^{-3} M	5.9	4
Mixture of all four inhibitors	10^{-3} M of each	91.1	2

Table **4**

Peptide	*Initial rate of proteolysis [μM/min/mg BBMM protein] [mean of 3–4 experiments ± standard deviation]*
GLP-1(7–36) amide	655 ± 106
GIP	600 ± 85
PYY	576 ± 80
(Ser-8)-GLP-1(7–36) amide	280 ± 50
Exendin-4	1 ± 0.26
Exendin-3	0.82 ± 0.3

the recovery of GLP-1, whereas captopril and bestatin each had some effect.

Comparison of rates of endoproteolysis of GLP-1, GIP, PYY and exendin-4 by kidney BBMM

Figure **3** shows the rpHPLC analysis of the peptide hormones, GIP, PYY and exendin-4 following incubation in the presence and absence of BBMM. Only the profiles obtained following 60 minutes incubation are shown. As observed for GLP-1, there was a clear, time-dependant reduction in the GIP and in the PYY substrate peaks in the presence of rat BBMM membranes and a concurrent appearance of additional product peaks. This was much less evident with exendin-4. Table **4** shows the calculated initial rates of proteolysis of the four peptides in the presence of BBMM. These calculations were performed as described in the methods.

Discussion

We have employed an in vitro model to characterise the stability of GLP-1 and other peptides on exposure to the combined effects of ectopeptidases present in isolated BBMMs, with the aim of assessing whether this provides a convenient means of characterising the pattern of post-secretory processing of peptide hormones. We have used GLP-1 as a model peptide for which there is some knowledge of its post-secretory processing in vivo. Following incubation of GLP-1 with isolated BBMMs, we were able to detect, using rpHPLC with online UV-detection, the generation of UV-absorbing fractions that accompanied a reduction in the recover of the substrate peptide fraction. That this represented proteolysis of the peptide hormone was indicated by the absence of these additional fractions and the total recovery of the substrate fraction by the addition of a cocktail of peptidase inhibitors.

Mild conditions were chosen in order to achieve only partial endoproteolysis of GLP-1 by BBMM and to allow identification of the major initial products and of the peptidases responsible for the initial proteolysis. Under these conditions, both rat and porcine BBMM generate 3–30 fragment of GLP-1, which is a product of the action of DPP-IV (Mentlein et al., 1993). This study also demonstrates that DPP-IV is rate-limiting in the rapid endoproteolysis of GLP-1 that occurs in the presence of BBMMs, and substantiates previously published evidence that the enzyme, DPP-IV, is critically involved in the post-secretory processing of GLP-1. GLP-1 has been previously shown to be a substrate for this enzyme (Medeiros and Turner, 1994; Deacon et al., 1995; Kieffer et al., 1995; Pauly et al., 1996). A number of DDPP-IV-resistant analogs of GLP-1 have been generated (Chou et al., 1997; Ritzel et al., 1998; Gallwitz et al., 2000; Larsen et al., 2001) and we tested one of these, (Ser-2)-GLP-1, (Ritzel et al., 1998) and found that its proteolysis by BBMM is slower than that of GLP-1, further confirming the importance of DPP-IV in the post-secretory processing of GLP-1.

The rat, but not the porcine BBMM, generate the 9–30 fragment of GLP-1. Which enzyme is responsible for generating this fragment is unclear but it may be meprin (also known as *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid (PABA-peptide)hydrolase or PHH) (EC 3.4.24.18)(Wolz and Bond, 1995). Meprin is an ectopeptidase that has been identified in several mammalian species, including human, but its presence in the microvilli of the renal proximal tubule appears to be unique to rodents (Wolz and Bond, 1995) and is also capable of the proteolysis of several regulatory peptides (Sterchi et al., 1988). Although no specific inhibitor is known for this enzyme, its action has been shown to be suppressed by captopril (Sterchi et al., 1988). Therefore, our finding that captopril reduces the recovery of GLP-19–30 following incubation with rat BBMM, favours the possibility that the difference between rat and porcine BBMM in the pattern of proteolysis of GLP-1 is due to the action of meprin in the rat preparation. Further studies are needed to address whether, like DPP-IV, meprin is also a key enzyme involved in the post-secretory processing of GLP-1.

We have previously reported that GLP-1 is a good substrate for NEP 24.11 (EC 3.4.24.11) (Hupe-Sodmann et al., 1995). However, our data suggests that this peptidase is less important that others in the post-secretory processing of GLP-1. We found here that the recovery of GLP-1 following 60 minutes incubation with rat BBMM was significantly increased by specific inhibition of either DPP-IV (with diprotinin A), possibly meprin (by captopril), and aminopeptidases (with bestatin), whereas inhibition of NEP 24.11 (with phosphoramidon) was without effect.

The results of our kinetic studies indicate that GIP (Pederson, 1994), PYY (Taylor, 1993) and GLP-1 differ little in their susceptibility to proteolysis by these ectopeptidases. This contrasts with the report circulating half-lifes that differ for these peptide hormones. Therefore, it may not be easy to extrapolate our in vitro kinetic data to in vivo pharmacokinetics. Our in vitro studies cannot take into account certain factors such as trans-

portation to the location of the ectopeptidases, that may be rate-limiting in post-secretory processing of peptide hormones in vivo. Conversely, measurements of peptide hormone pharmacokinetics are predominantly derived from radioimmunoassays and, therefore, represent rates of decrease in concentration of immunoreactivity. Such analyses may over-estimate metabolic stability, since some metabolic fragments are likely to be immunreactive.

We have shown here that the non-mammalian GLP-1 homologs, exendins-3 and -4 (Eng, 1992) exhibit remarkable stability in the presence of mammalian ectopeptidases. They are both greater than 50% homologous to GLP-1 but exhibit considerably lower rates of endoproteolysis in the presence of BBMMs. Both exendins share the biological properties of GLP-1. In particular, exendin-4 exhibits a specific and high affinity for the GLP-1 receptor (Goke et al., 1993; Thorens et al., 1993), is much more potently insulinotropic than GLP-1 (Greig et al., 1999; Zhou et al., 1999; Szayna et al., 2000) and its therapeutic potential is attracting much interest. The data presented here is the first direct evidence that exendin-4 exhibits high metabolic stability, which is already suggested by its very long-acting effects in vivo.

The exendins possesses DPP-IV-resistant primary structures, but this does not explain their remarkable resistance to proteolysis. They are each several orders of magnitude more stable than GLP-1 and the DPP-IV-resistant Ser-8-GLP-1. This suggests that conformational aspects of the molecular structure of the exendins may be important for imparting metabolic stability and may be the basis for the development of novel metabolically-stable analogs. Our findings indicate that, in addition to providing a systematic means of characterising the endoproteolysis by native ectopeptidases, this in vitro approach is also convenient for identifying metabolically-stable peptide analogs.

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