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Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7–36)amide, peptide histidine methionine and is responsible for their degradation in human serum

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Peptides of the glucagon/vasoactive-intestinal-peptide (VIP) peptide family share a considerable sequence similarity at their N-terminus. They either start with Tyr-Ala, His-Ala or His-Ser which might be in part potential targets for dipeptidyl-peptidase IV, a highly specialized aminopeptidase removing dipeptides only from peptides with N-terminal penultimate proline or alanine. Growth-hormone-releasing factor(1–29)amide and gastric inhibitory peptide/glucose-dependent insulinotropic peptide (GIP) with terminal Tyr-Ala as well as glucagon-like peptide-1(7–36)amide/insulinotropin [GLP-1(7–36)amide] and peptide histidine methionine (PHM) with terminal His-Ala were hydrolysed to their des-Xaa–Ala derivatives by dipeptidyl-peptidase IV purified from human placenta. VIP with terminal His-Ser was not significantly degraded by the peptidase. The kinetics of the hydrolysis of GIP, GLP-1(7–36)amide and PHM were analyzed in detail. For these peptides K_m values of 4–34 μM and V_{max} values of 0.6–3.8 μmol · min^{-1} · mg protein^{-1} were determined for the purified peptidase which should allow their enzymic degradation also at physiological, nanomolar concentrations. When human serum was incubated with GIP or GLP-1(7–36)amide the same fragments as with the purified dipeptidyl-peptidase IV, namely the des-Xaa–Ala peptides and Tyr-Ala in the case of GIP or His-Ala in the case of GLP-1(7–36)amide, were identified as the main degradation products of these peptide hormones. Incorporation of inhibitors specific for dipeptidyl-peptidase IV, 1 mM Lys-pyrrolidide or 0.1 mM diprotin A (Ile-Pro-Ile), completely abolished the production of these fragments by serum. It is concluded that dipeptidyl-peptidase IV initiates the metabolism of GIP and GLP-1(7–36)amide in human serum. Since an intact N-terminus is obligate for the biological activity of the members of the glucagon/VIP peptide family [e. g. GIP(3–42) is known to be inactive to release insulin in the presence of glucose as does intact GIP], dipeptidyl-peptidase-IV action inactivates these peptide hormones. The relevance of this finding for their inactivation and their determination by immunoassays is discussed.

Dipeptidyl-peptidase IV (DPP IV) is a highly specialized aminopeptidase removing dipeptides from bioactive peptides and synthetic peptide substrates provided that proline or alanine are the penultimate N-terminal residues (Mentlein, 1988, for review). Small peptides or chromogenic substrates with proline in this position are far better hydrolysed than those with alanine (Heins et al., 1988). DPP IV occurs in human serum, as an ectoenzyme on the surface of capillary endothelial cells, at kidney brush-border membranes, on the surface of hepatocytes (here termed also GP110 or OX-61 antigen), on the surface of a subset of T-lymphocytes and thymocytes (here termed CD 26, or thymocyte-activating molecule) and other sites (Loijda, 1979; Nausch and Heymann, 1985; Mentlein et al., 1984; McCaughan et al., 1990). The enzyme has been shown to be responsible for the degradation and inactivation of circulating peptides with penultimate proline, like substance P (Heymann and Mentlein, 1978; Ahmad et al., 1992), but also for growth-hormone-releasing factor (GRF) with penultimate alanine (Frohman et al., 1989; Kubiak, 1989; Boulanger et al., 1992). [Ala[15]]GRF(1–29)amide with penultimate Ala is even a comparably good substrate as a synthetic Pro[2]-containing derivative for purified DPP IV (Bongers et al., 1992). This suggests that the conformation or chain length may greatly influence the cleavage of peptides with penultimate proline/alanine-residues by DPP IV.

We therefore evaluated whether or not other peptide hormones related to GRF might be substrates for DPP IV, and whether this probable proteolytic degradation might be of relevance in the circulation. GRF belongs to the glucagon/

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Abbreviations. DPP IV, dipeptidyl-peptidase IV; GIP, gastric inhibitory polypeptide or glucose-dependent insulinotropic polypeptide; GLP-1(7–36)amide, glucagon-like peptide-1(7–36)amide or insulinotropin or preproglucagon(78–107)amide; GLP-2, glucagon-like peptide-2 or preproglucagon(126–159); GRF, growth-hormone-releasing factor/hormone; PHI, peptide histidine isoleucine; PHM, peptide histidine methionine; VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate-cyclase-activating polypeptide.

Enzyme. Dipeptidyl peptidase IV (EC 3.4.14.5).

5							10			
Tyr	Ala	Asp	Ala	Ile	Phe	Thr	Asn	Ser	Tyr	...-29	*h* GRF(1-29)amide
–	–	Glu	Gly	Thr	–	Ile	Ser	Asp	–	...-42	*h* GIP
His	–	Glu	Gly	Thr	–	Thr	Ser	Asp	Val	...-30	*h* GLP-1(7-36)amide
His	–	–	Gly	Ser	–	Ser	Asp	Glu	Met	...-34	*h* GLP-2
His	–	–	Gly	Val	–	–	Ser	Asp	Phe	...-27	*h* PHM-27
His	–	–	Gly	Val	–	–	Ser	Asp	–	...-27	*r* PHI-27
His	Ser	–	–	Val	–	–	Asp	Asn	–	...-28	*h* VIP
His	Ser	–	Gly	Thr	–	–	Ser	Glu	Leu	...-27	*h* Secretin
His	Ser	Gln	Gly	Thr	–	–	Ser	Asp	–	...-29	*h* Glucagon
His	Ser	–	Gly	–	–	–	Asp	–	–	...-38	*h* PACAP-38

Fig. 1. N-terminal sequences of peptides related to growth hormone-releasing factor (GRF). Penultimate alanine and serine residues are in bold; N-terminal tyrosine and histidine residues are underlined; (–) identity to GRF. *h*, Human sequences; *r*, rat sequence.

secretin/vasoactive-intestinal-peptide(VIP) peptide family (Fig. 1) which share N-terminal sequences of considerable similarity. A number of them begin with Tyr-Ala, namely GRF and gastric inhibitory polypeptide/glucose-dependent insulinotropic peptide (GIP), or with His-Ala, namely glucagon-like peptide-1(7–36)amide/insulinotropin [GLP-1(7–36)amide], glucagon-like peptide-2 (GLP-2), peptide histidine methionine (PHM) and peptide histidine isoleucine (PHI, the rat counterpart of human PHM), whereas others have terminal His-Ser (VIP and others). For biological activity the N-terminal moiety is supposed to be the determinant for transducing the ligand message and the C-terminal moiety for playing the major role in specific binding (Christophe et al., 1989, for review). Thus, proteolytic truncation of the N-terminus of the members of the glucagon/VIP family by DPP IV should inactivate them.

EXPERIMENTAL PROCEDURES

Peptides, inhibitors and enzymes

Synthetic peptide hormones (human sequences) were obtained from Saxon Biochemicals, dipeptides and diprotin A were purchased from Bachem. Purity of peptides was checked by HPLC; their amino acid compositions were analyzed by the manufacturer. Lys-pyrrolidide was a gift from Dr. Mike Schutkowski, Martin-Luther-Universität Halle/Saale, Germany. Dipeptidyl-peptidase IV was purified from human placenta and free of contaminating proteases (Püschel et al., 1982).

Degradation assays with purified enzyme

5 nmol of the peptides (5 µl of a 1 mM solution in water) were incubated at 37°C with 0.1 µg peptidase in 50 mM triethanolamine/HCl, pH 7.8, for 10–60 min in 500 µl (final peptide concentration 10 µM) or less (other peptide concentrations). Enzymic reactions were terminated by addition of 5 µl 10% trifluoroacetic acid, and the mixtures applied onto a Vydac C_{18} widepore (30-nm pores, 5-µM particles) 250 mm × 4.6 mm HPLC column and eluted at a flow rate of 1 ml/min with gradients of acetonitrile in 0.1% trifluoroacetic acid. Either a linear gradient of 0–80% acetonitrile formed within 42 min (GIP degradation), or a stepwise linear gradient of 0–32% acetonitrile formed in 17 min followed by linear gradient of 32–48% acetonitrile formed in 30 min (other peptides) were used for separations. In some HPLC separations, trifluoroacetic acid was replaced by heptafluorobutyric acid. Peptides and their degradation products were monitored by their absorbance at 220 nm (peptide bonds) or 280 nm (aromatic amino acids). They were quantified by integration of their peak areas related to those of standards (synthetic Tyr-Ala or turncated peptides made by complete dipeptidyl-peptidase IV digestion). The concentrations of all peptide solutions were routinely calculated from their absorbance at 280 nm relative to their content of Trp and Tyr (using additively the known absorption coefficients).

Activities were determined from estimations with less than 10% substrate turnover. Catalytic constants were calculated according to the statistical method of Wilkinson (1961).

Degradation of peptides in serum

200 µl serum of healthy males were incubated with 10 µl 1 mM peptide solution in water (final concentration 20 µM) for 60 min at 37°C. Inhibitors were added as 10 mM or 100 mM stock solutions in water. Enzymic reactions were terminated by addition of 20 µl 10% trifluoroacetic acid. Samples were centrifuged (5 min 13000 × *g*), and the supernatant liquids applied to a C_{18} reverse-phase Sep-Pak cartridge (Millipore-Waters) that had been previously activated and washed with 10 ml each of methanol, 80% acetonitrile in 0.1% trifluoroacetic acid and finally 0.1% trifluoroacetic acid. After washing of the serum-loaded cartridges with 20 ml 0.1% trifluoroacetic acid, peptides were eluted with 2 ml 80% acetonitrile in 0.1% trifluoroacetic acid. Acetonitrile eluates were lyophilized, dissolved in 100 µl 0.1% trifluoroacetic acid and analyzed as described above. Non-bound supernatants and washings were combined, lyophilized, reacted with 4-dimethylaminoazobenzene-4-sulphonyl chloride and separated by reverse-phase HPLC as described by Stocchi et al. (1985) for amino acids.

Peptide chemistry and other assays

Fragments separated by HPLC were collected and lyophilized for chemical determinations. Amino acid composition was determined by acid hydrolysis (6 M HCl *in vacuo* at 100°C for 24 h) followed by lyophilisation, reaction with 4-dimethylaminoazobenzene-sulphonyl-chloride and HPLC separation of derivatized amino acids (Stocchi et al., 1985). N-terminal amino acids were determined by manual microsequencing with 4-*N*,*N*-dimethylaminoazobenzene-4′-isothiocyanate (Wittmann-Liebold et al., 1986).

Hydrolysis of 0.5 mM Gly-Pro-4-nitranilide at pH 8.6 and at 37°C was monitored as described (Mentlein and Struckhoff, 1989).

RESULTS

Digestion of peptides by purified DPP IV

DPP IV purified from human placenta liberated Tyr-Ala from GRF(1–29)amide and GIP, and His-Ala from GLP-1(7–36)amide and PHM (Table 1, Fig. 2). No further proteolytic cleavage of these peptides was observed indicating the high specificity of the DPP IV for N-terminal Xaa-Ala (and Xaa-Pro) and the absence of contaminating proteases in the enzyme preparation. Liberated Tyr-Ala (Fig. 2) was identified by its retention time and co-chromatography with a synthetic standard. His-Ala was adsorbed to the C_{18} column

Table 1. Cleavage rates for proteolysis of peptides from the GRF/VIP family by DPP IV purified from human placenta. Data are means of three determinations, variations were less than 10%.

Peptide	Concentration	Cleavage rate
	μM	μmol · min^{-1} · mg^{-1}
GRF(1−29)amide	20	4.4
	150	5.0[a]
GRF(1−44)amide	150	4.5[a]
GIP	20	1.4
	100	2.9
GLP-1(7−36)amide	20	0.79
	100	0.35
PHM	20	0.47
	100	0.58
VIP	20	<0.02
	100	<0.02

[a] Data taken from Bongers et al. (1992).

only with heptafluorobutyric acid as ion-pairing reagent (Table 2) which, however, resulted in a relatively high background. Therefore, liberated His-Ala was also identified as its 4-dimethylaminoazobenzene-sulphonyl-derivative (obtained also with a synthetic dipeptide standard). Moreover, the truncated peptides could be separated from the non-degraded ones in reverse-phase HPLC (Fig. 2, Table 2).

Highest initial velocities for DPP-IV degradation at micromolar peptide concentrations were found for GRF(1−29)amide, whereas those for other members of the VIP/glucagon-related peptides with penultimate Ala were lower (Table 1). No significant cleavage was observed with VIP tested as a representative member of this peptide family with N-terminal His-Ser. DPP IV hydrolysed GIP, GLP-1(7−36)amide and PHM with K_m values in the range 4−34 μM (Table 3). These values are of the same order of magnitude as those determined earlier for the cleavage of other bioactive peptides with N-terminal Xaa-Pro or Xaa-Ala by DPP IV. K_m values in the micromolar range have been generally found for other peptide-degrading proteases. Therefore, degradation rates at physiological peptide concentrations in the nanomolar ranges are given by the rate (specificity) constants k_{cat}/K_m. High rate constants indicate high cleavage rates at nanomolar concentrations (below K_m value). k_{cat}/K_m values of about $10^5\,M^{-1}\cdot s^{-1}$ for GIP, GLP-1(7−36)amide and PHM (Table 3) are lower than those determined earlier for good DPP-IV substrates like substance P, but still high enough to ensure a physiological action.

Degradation of GIP and GLP-1(7–36)amide by human serum

When human serum was incubated with 20 μM GIP, two major degradation products were observed (Fig. 3): one eluting at the position of Tyr-Ala, the other at that of des-Tyr−Ala-GIP. Identity of these peaks was ensured by identical retention times with standards (prepared by digestion with pure DPP IV) as well as by amino-acid analysis of the Tyr-Ala peak and determination of the N-terminal amino acid of the GIP (3−42)-peak, both collected after separation. Moreover, addition of the DPP-IV inhibitors 1 mM Lys-pyrrolidide or 0.1 mM diprotin A abolished the generation of both GIP fragments by human serum nearly completely (residual areas <5%). Hydrolysis of 0.5 mM Gly-Pro-4-nitranilide (an established chromogenic substrate of DPP IV) in the same serum sample was reduced to 2% in the presence of 1 mM Lys-pyrrolidide and to 9% after addition of 0.1 mM diprotin A. Lys-pyrrolidide (Lys-tetrahydropyrrole), a substrate analog, and diprotin A (Ile-Pro-Ile), a bad, but high-affinity (K_m = 4 μM) substrate (Rahfeld et al., 1991a), are competitive inhibitors specific (as far tested) for DPP IV. Concluded from their influence and from the fragments generated, GIP

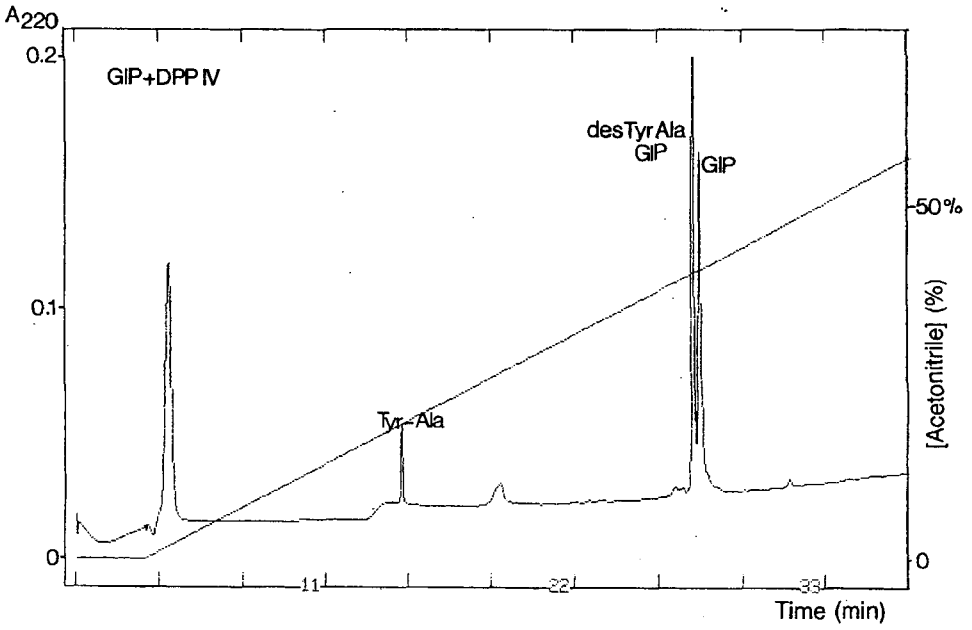

Fig. 2. Reverse-phase HPLC separation of an incubations of GIP with DPP IV purified from human placenta. The positions of liberated Tyr-Ala and of the truncated peptide hormone are indicated. The C_{18} HPLC column was eluted with a gradient of 0−80% acetonitrile in 0.1% trifluoroacetic acid as described in Experimental Procedures. Peptides were monitored in the eluate by their absorbance at 220 nm.

Table 2. Separation of DPP IV cleavage products of gastric inhibitory polypeptide (GIP), glucagon-like peptide-1(7–36)amide [GLP-1(7–36)amide] and peptide histidine methionine (PHM) by reverse-phase HPLC on a C_{18} column. For conditions see Experimental Procedures, retention times varied ±0.3 min. The first 20 min of gradients are identical.

Peptide	Retention time	Gradient
	min	
GIP	27.4	0–3 min 0% + 3–45 min 0–80% acetonitrile in 0.1% trifluoroacetic acid
GIP(3–42)	27.1	
Tyr-Ala	14.3	
His-Ala	3.8	
	18.2	0–3 min 0% + 3–45 min 0–80% acetonitrile in 0.1% heptafluorobutyric acid
GLP-1(7–36)amide	40.7	0–3 min 0% + 3–20 min 0–32% + 20–50 min 32–48% acetonitrile in 0.1% trifluoroacetic acid
GLP-1(9–36)amide	41.7	
PHM	44.1	
PHM(3–27)	44.8	
VIP	35.2	
His-Ala	3.8	

Table 5. Catalytic constants for the degradation of bioactive peptides by human DPP IV. Assays were performed in 50 mM triethanolamine/HCl, pH 7.8, at 37°C. Values of k_{cat} were calculated using a molecular mass of 120 kDa for one identical subunit of the human placental DPP IV dimer (Püschel et al., 1982). GLP-1(7–36)amide shows substrate inhibition above 50 µM, catalytic constants (±SD) were calculated from the linear ranges of Lineweaver-Burk plots.

Peptide	N-terminus	S_0	No. of runs	K_m	V_{max}	k_{cat}	k_{cat}/K_m	Reference
		µM		µM	µmol · min^{-1} · mg^{-1}	s^{-1}	$M^{-1} \cdot s^{-1}$	
GIP	YA-E...	1–100	7	34 ± 3	3.8 ± 0.2	7.6	$0.22 \cdot 10^6$	this study
GLP-1(7–36)-amide	HA-E...	5–100	7	4.5 ± 0.6	0.97 ± 0.05	1.9	$0.43 \cdot 10^6$	this study
PHM	HA-D...	5–100	6	6.5 ± 0.5	0.62 ± 0.03	1.2	$0.19 \cdot 10^6$	this study
[Ala^{15}]GRF(1–29)-amide	YA-D...	2–350	12	4.7 ± 0.3	4.7 ± 0.1	9.5	$2.0 \cdot 10^6$	Bongers et al., 1992
β-Casomorphin	YP-F...	20–500		59	90	180	$3.1 \cdot 10^6$	Nausch et al., 1990
Substance P	RP-K...	25–200		22	10	20	$0.91 \cdot 10^6$	Nausch et al., 1990

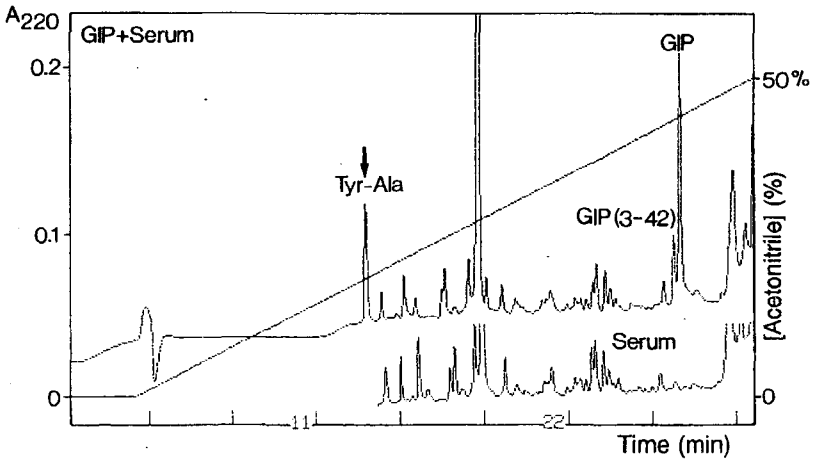

Fig. 3. Reverse-phase HPLC analysis of an incubation assay of 20 µM gastric GIP with human serum (GIP + Serum) compared to a serum blank (Serum, inset). Positions of GIP and its degradation products Tyr-Ala and GIP(3–42) are indicated. Experimental conditions as in Fig. 2.

is metabolized by DPP IV activity of human serum mainly to Tyr-Ala and GIP(3–42).

Incubation of human serum with 20 µM GLP-1(7–36)amide yielded one degradation product at the position of the des-His–Ala-peptide after reverse-phase HPLC (not shown). This fragment was identified by identical retention time with a standard (obtained with pure DPP IV, Table 2) and by determination of the N-terminal amino acid. His-Ala

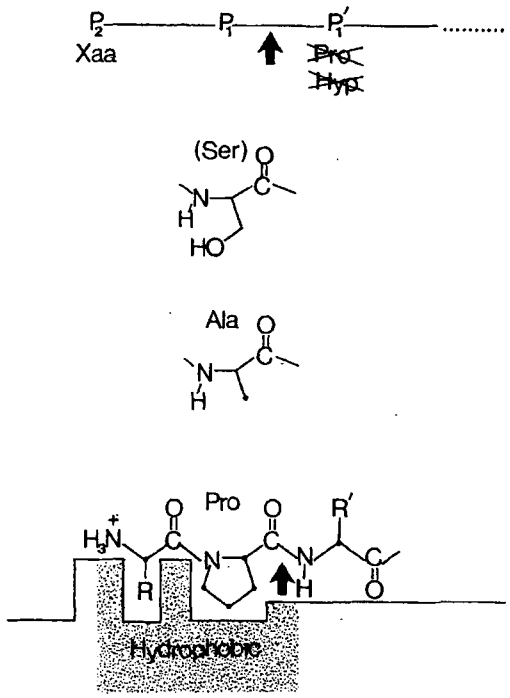

Fig. 4. Schematic representation of the substrate-binding and substrate-cleaving (arrow) sites of DPP IV. Proline and alanine fit in the hydrophobic P_1-substrate-binding pocket, whereas serine appears to be too hydrophilic to yield appreciable binding. In the P_2 position bulky amino acids with an obligate free amino group are preferred. Peptides with Pro or Hyp in the P_1' position are not cleaved by DPP IV. Preferential amino acids for the P_1' position are not known.

as further degradation product could be identified after derivatisation with 4-dimethylaminoazobenzene-sulphonyl-chloride (see Experimental Procedures) by identical retention time and co-chromatography with a derivatized, synthetic His-Ala standard. Again, in the presence of Lys-pyrrolidide (1 mM) and diprotin A (0.1 mM), the generation of the des-His−Ala-fragment was abolished (<5%). Thus, as concluded from specific inhibition and generation of His-Ala and the des-His−Ala-peptide GLP-1(7−36)amide is cleaved by human serum mainly by action of DPP IV.

In sera of healthy males we measured a mean activity of 55 ± 12 μmol · min^{-1} · l^{-1} ($n = 6$) with the chromogenic substrate 0.5 mM Gly-Pro-4-nitranilide for DPP IV. No significant differences were found for the peptidase activities in preprandial an postprandial sera ($n = 3$). In a serum with an activity of 50 μmol · min^{-1} · l^{-1} for Gly-Pro-4-nitranilide, we estimated degradation rates of about 0.3 μmol · min^{-1} · l^{-1} for Tyr-Ala liberation from 20 μM GIP and 0.4 μmol·min^{-1} · l^{-1} for His-Ala liberation from 20 μM GLP-1(7−36)amide.

DISCUSSION

Members of the VIP/glucagon peptide family with N-terminal penultimate alanine are good substrates for DPP IV. GRF(1−29)amide or GRF(1−44)amide as analyzed here and by Bongers et al. (1992), GIP, GLP-1(7−36)amide and PHM are cleaved to their des-Tyr−Ala or des-His−Ala derivatives by the highly purified human enzyme. In contrast, VIP with N-terminal His-Ser was not significantly degraded. This fits well with the known, preferential specificity of DPP IV for penultimate proline or alanine residues (Fig. 4). Almost no other naturally occurring amino acid is accepted in this position. Replacement of penultimate Ala in a GRF(1−29)amide derivative by hydrophilic Ser or Gly resulted in dipeptidyl-peptidase-IV substrates of far lower k_{cat} and higher K_m values (Bongers et al., 1992). In contrast, substrates with synthetic hydrophobic derivatives of the proline ring (oxa- or thia derivatives) or short, unbranched hydrophobic alkyl derivatives in the P_1 position are good substrates for DPP IV (Rahfeld et al., 1991b; Schutkowski, 1991). This indicates a hydrophobic substrate (P_1) recognition site for DPP IV where Ser is less well (or not) bound than Ala or Pro (Fig. 4). Moreover, a bulky N-terminal amino acid with free amino group (P_2 position) as with Tyr or His in the peptides investigated here is optimal for high DPP-IV activity. This together with effects of the C-terminal part of the peptides might account for the relatively low K_m and high k_{cat} values of DPP IV for the 29−42 residue hormones GRF, GIP, GLP-1(7−36)amide and PHM as compared to those found earlier for small chromogenic substrates with penultimate Ala (Heins et al., 1988).

GIP released postprandially into the blood from intestinal endocrine K cells inhibits the secretion of gastric acid and stimulates insulin release from pancreatic β-cells in the presence of elevated glucose levels. Schmidt et al. (1986, 1987) have clearly shown that N-terminal Tyr-Ala is absolutely required for the insulin-releasing activity (the main physiological effect) of GIP. Pure des-Tyr−Ala-GIP (3−42) unlike intact GIP did not increase insulin secretion in the presence of 16.7 mM glucose from rat pancreatic islets at physiological or higher concentrations even up to 250 nM. Therefore, truncation of GIP by DPP IV results in its inactivation with respect to its major physiological, the insulinotropic, action.

Cleavage products and influence of specific inhibitors clearly show that dipeptidyl peptidase IV is the main degradation and, considering the above findings, inactivation enzyme for GIP in human serum. The enzyme should be still more active on this peptide hormone at other sites, e. g. endothelial cells of blood vessels, hepatocytes, kidney brush-border membranes (podocytes of the glomerular basement membrane and proximal tubule cells), lymphocytes, chief cells of gastric glands, or epithelial cells of the intestine, where it is found in high concentrations as an ectoenzyme of the plasma membranes (Loijda, 1979; Hartel et al., 1988; Gossrau, 1979; McCaughan et al., 1990; Mentlein et al., 1984). Active hydrolysis by DPP IV might therefore explain why GIP(3−42) has been isolated as a second component (relative yield about 20−30%) beside intact GIP from porcine intestine and has been found as a contaminant of natural GIP preparations (Jörnvall et al., 1981; Schmidt et al., 1987).

GLP-1(7−36)amide is a product of the tissue-specific post-translational processing of the glucagon precursor. It is released postprandially from intestinal endocrine L cells and stimulates insulin secretion. Gallwitz et al. (1990) have shown that the C-terminal fragment of the peptide is important for receptor binding of the hormone, but is not sufficient to transduce a biological action as does the intact peptide (raise in cyclic AMP levels in rat insulinoma RINm5F cells). It appears that as in the case of glucagon (Unson et al., 1989), of GIP (Schmidt et al., 1986, 1987) and of other members of the VIP/glucagon peptide family (Christophe et al., 1989; Robberecht et al., 1992) also for GLP-1(7−36)amide an intact N-terminus is needed for signal transduction and biological action. Provided this, action of DPP IV inactivates GLP-1(7−36)amide.

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