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The metabolism of neuropeptides

Hydrolysis of peptides by the phosphoramidon-insensitive rat kidney enzyme 'endopeptidase-2' and by rat microvillar membranes

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Endopeptidase-2, the second endopeptidase in rat kidney brush border [Kenny & Ingram (1987) Biochem. J. **245**, 515–524] has been further characterized in regard to its specificity and its contribution to the hydrolysis of peptides by microvillar membrane preparations. The peptide products were identified, after incubating luliberin, substance P, bradykinin and angiotensins I, II and III with the purified enzyme. The bonds hydrolysed were those involving a hydrophobic amino acid residue, but this residue could be located at either the P_1 or P_1' site. Luliberin was hydrolysed faster than other peptides tested, followed by substance P and bradykinin. Human α-atrial natriuretic peptide and the angiotensins were only slowly attacked. Oxytocin and [Arg^8]vasopressin were not hydrolysed. No peptide fragments were detected on prolonged incubation with insulin, cytochrome *c*, ovalbumin and serum albumin. In comparison with pig endopeptidase-24.11 the rates for the susceptible peptides were, with the exception of luliberin, much lower for endopeptidase-2. Indeed, for bradykinin and substance P the ratio $k_{cat.}/K_m$ was two orders of magnitude lower. Since both endopeptidases are present in rat kidney microvilli, an assessment was made of the relative contributions to the hydrolysis of luliberin, bradykinin and substance P. Only for the first named was endopeptidase-2 the dominant enzyme; for bradykinin it made an equal, and for substance P a minor, contribution.

INTRODUCTION

The brush border of renal proximal tubules is particularly rich in peptidases. In pig, rabbit and human kidney microvilli there is just one endopeptidase, endopeptidase-24.11 (EC 3.4.24.11), which is complemented by a group of exopeptidases (for reviews, see Kenny, 1986; Kenny *et al.*, 1987). In the renal brush borders of rat, however, a second endopeptidase, characterized by its insensitivity to inhibition by phosphoramidon, has been identified, namely 'endopeptidase-2' (Kenny *et al.*, 1981). The molecular and catalytic properties of this rat enzyme have recently been described (Kenny & Ingram, 1987). It is a zinc metallo-endopeptidase, and is unusual among this group of ectoenzymes in having SS (disulphide)-linked subunits. Apart from hydrolysing the standard assay substrate, ^{125}I-insulin B chain, it also hydrolysed azocasein and *N*-benzoyl-L-tyrosyl-*p*-aminobenzoic acid ('PABA-peptide'). The last named is a typical substrate for 'PABA-peptide' hydrolase present in the human intestinal brush border (Sterchi *et al.*, 1982, 1983) and indicated the ability of endopeptidase-2 to hydrolyse bonds involving the carboxy function of tyrosine, in line with the observation that the Tyr^3-Glu^4 bond of neurotensin was cleaved. Another metallo-endopeptidase, 'meprin', has been found in kidneys of some strains of mice (Beynon *et al.*, 1981) and was later shown to be a brush-border enzyme (Bond *et al.*, 1983). It contains both Zn^{2+} and Ca^{2+} and exists as an SS-linked tetramer (Bond *et al.*, 1986; Butler *et al.*, 1987) and clearly has a number of properties in common with rat endopeptidase-2 (Kenny & Ingram, 1987). The specificity has been studied recently by Butler *et al.* (1987), who used oxidized insulin B-chain, angiotensin II and bradykinin as substrates. A variety of peptide bonds were hydrolysed, but the results indicated that meprin had a preference for bonds flanked by hydrophobic or neutral amino acid residues. An exception to this was the hydrolysis of the Gly^{20}–Glu^{21} bond of insulin B-chain.

We have recently reported on the manner in which pig kidney microvillar membranes initiate the hydrolysis of a number of neuropeptides (Stephenson & Kenny, 1987*a*,*b*). By exploiting some specific inhibitors we showed that the hydrolysis of substance P, bradykinin, the angiotensins, human α-atrial natriuretic peptide (α-hANP) and oxytocin was mainly initiated by endopeptidase-24.11 in microvilli from pig kidney. Microvilli from human kidney contain the same complement of peptidases (Abbs & Kenny, 1983), hence these data are also relevant to this species. However, since the rat is a commonly used model in studying the fate of peptides, it has become important to define any differences in peptide metabolism by rat microvilli which might arise from the presence of endopeptidase-2.

In the present study we have investigated the peptide-bond specificity of endopeptidase-2 using luliberin (LHRH), substance P, bradykinin and the angiotensins. We have shown that, like meprin, endopeptidase-2 has a preference for peptide bonds flanked by hydrophobic amino acids. Endopeptidase-2 is largely responsible for

Abbreviations used: α-hANP, α-(human) atrial natriuretic peptide; CM-, carboxymethyl; Dip-F, di-isopropyl fluorophosphate; LHRH, luliberin (luteinizing-hormone releasing hormone); NMec, 7-amino-4-methylcoumarylamide.

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the hydrolysis of luliberin by rat kidney microvillar membranes and also plays a role in the hydrolysis of bradykinin and, to a lesser extent, substance P. The hydrolysis of α-hANP was predominantly due to endopeptidase-24.11.

EXPERIMENTAL

Materials

Angiotensins I, II and III, bradykinin, [Arg^8]-vasopressin, hippuryl-L-histidyl-L-leucine and L-histidyl-L-leucine were obtained from Sigma Chemical Co. LHRH, substance P and oxytocin were obtained from Cambridge Research Biochemicals (Harston, Cambridge, U.K.). α-hANP was purchased from Peptide Institute (Scientific Marketing Associates, London N1 4RH, UK). Marker peptide fragments of LHRH were synthesized, and kindly donated, by Dr. George Flouret, Department of Physiology, Northwestern University, Chicago, IL, U.S.A. Phosphoramidon was obtained from Peptide Institute. Amastatin and Dip-F were purchased from Sigma Chemical Co. Captopril (SQ14225) was a gift from the Squibb Institute for Medical Research, Princeton, NJ, U.S.A. Bovine serum albumin (code A7638), ovalbumin (grade V) and cytochrome *c* (code C7752) were from Sigma Chemical Co.

Purification of endopeptidase-2

Endopeptidase-2 was purified as described previously (Kenny & Ingram, 1987). The specific activity of the enzyme was 87 nmol/min per mg of protein assayed with ^{125}I-insulin B-chain as substrate. As with previous preparations, SDS/polyacrylamide-gel electrophoresis showed two bands migrating as 80 and 74 kDa polypeptides. Only small traces of other peptidase activities (μmol/min per mg of protein) were present: aminopeptidase N, 0.006; aminopeptidase A, 0.10 and dipeptidyl peptidase IV, 0.023 [assayed with Ala-NMec, α-Glu-NMec and Gly-Pro-NMec respectively as described by Fulcher & Kenny (1983)]. No endopeptidase-24.11 activity was detected.

Purification of endopeptidase-24.11

Endopeptidase-24.11 was isolated by immunoaffinity chromatography as described by Gee *et al.* (1983), except that the monoclonal antibody was GK4A9 (Gee & Kenny, 1985).

Microvillar membrane preparations

These were prepared from rat and pig kidneys as previously described (Booth & Kenny, 1974).

Use of inhibitors

All incubations with endopeptidase-2 included inhibitors to block contaminating peptidases (Stephenson & Kenny, 1987*a*). Final concentrations and preincubation periods at 20 °C (in parentheses) were: amastatin (1 μM, 15 min) to inhibit aminopeptidases N and A; captopril (1 μM, 15 min) to inhibit peptidyl dipeptidase A; Dip-F (0.1 mM, 1 h) to inhibit dipeptidyl peptidase IV. Phosphoramidon (1 μM, 15 min) was also included. Reactions with rat and pig kidney microvillar membranes were performed in the presence or absence of the group of inhibitors as described above. All incubations with endopeptidase-24.11 included amastatin, captopril and Dip-F as described above.

Hydrolysis of peptides

The incubation mixture (volume 100 μl) contained 100 mM-Tris/HCl, 150 mM-NaCl, pH 7.4 (at 37 °C), 250 μM-peptide (except for α-hANP, which was 50 μM) and either 100 ng of rat kidney endopeptidase-2 or pig kidney endopeptidase-24.11, or 1 μg of microvillar-membrane protein, unless otherwise stated. Samples were incubated in the presence of the four inhibitors (see above) at 37 °C for various times from 0 to 360 min. Incubations with purified peptidases were terminated by heating to 100 °C, and those with microvillar membranes were stopped by the addition of 30 μl of 30% (v/v) acetic acid. Samples were centrifuged before h.p.l.c. analysis, which was performed as described previously (Matsas *et al.*, 1983), except that the acetonitrile gradient used was 4.05–45% (v/v). Amino acid analyses of pooled peak fractions were performed as described above.

Hydrolysis of proteins

Insulin (100 μM), cytochrome *c* (40 μM), ovalbumin (10 μM) and albumin (10 μM) were incubated for 24 h as described above for peptides. The reaction was terminated by the addition of 30 μl of 30% (v/v) acetic acid and centrifuged. The acetonitrile gradient was 6–60% (v/v) for 30 min and 10 min at final conditions.

Kinetic determinations

Endopeptidase-2 (50 ng) was incubated at 37 °C with LHRH (15 min), bradykinin (120 min) and substance P (30 min) in 100 mM-Tris/HCl/150 mM-NaCl, pH 7.4, including the inhibitors amastatin, captopril, phosphoramidon and Dip-F as described above. The incubation times were such that approximately similar proportions of the peptides were degraded. Peptide concentrations were varied over a 15-fold range (20–300 μM), and hydrolysis rates were assessed by the disappearance of the substrate peak, which did not exceed 20% as assessed by h.p.l.c. The values are means for two separate experiments. The values for $k_{cat.}$ were calculated by assuming a catalytic subunit of 80 kDa.

RESULTS

Rates of hydrolysis of peptides by endopeptidase-2 compared with endopeptidase-24.11

Peptides were incubated with each of the two endopeptidases and specific activities calculated from the initial rates using the linear part of the progress curves (Table 1). For rapidly hydrolysed peptides and for those yielding a complex mixture of products, the rates were assessed by disappearance of the substrate peak monitored by h.p.l.c. However, in the case of endopeptidase-2 hydrolysing the angiotensins at the Tyr–Ile bond (see below), the release of the *N*-terminal fragment (peptide 1 in each case; see Fig. 2) was quantified (Stephenson & Kenny, 1987*a*). LHRH was the most rapidly hydrolysed peptide in the group, followed by substance P and bradykinin. α-hANP and the angiotensins were more slowly degraded. As a group, the angiotensins were degraded at a comparable rate when the attack at the Tyr-Ile bond was considered. However, angiotensin I was also subjected to a more rapid attack at the *C*-terminal His–Leu bond [rate of formation of peptide-2 (Fig. 2 below) was 0.14

Table 1. Comparison of the specific activities of rat kidney endopeptidase-2 and pig kidney endopeptidase-24.11 with various peptides

See the Experimental section for details; note that α-hANP was used at 50 μM, whereas other peptides were used at 250 μM. Specific activities were calculated from the initial (i.e. linear) rates of hydrolysis, assessed from disappearance of substrate peak, except for those marked with an asterisk (*), where formation of product was used. Endopeptidase-2 was incubated with the peptides at 37 °C for 0, 5, 10, 15, 30, 60, 120 and 360 min. Inhibitors of other peptidases were included in the assay, namely 1 μM-phosphoramidon, 1 μM-captopril, 1 μM-amastatin and 0.1 mM-Dip-F. A similar experiment with endopeptidase-24.11 omitted phosphoramidon from the incubation. n.d., no hydrolysis detectable.

Peptide	Specific activities (μmol/min per mg)	
	Endopeptidase-2	Endopeptidase-24.11
LHRH	2.44	1.15
Substance P	0.74	12.30
Bradykinin	0.48	11.50
α-hANP	0.10	1.86
Angiotensin II	0.066*	5.00
Angiotensin I	0.064*	2.06
Angiotensin III	0.043*	1.61
Oxytocin	n.d.	0.14
[Arg^8]vasopressin	n.d.	n.d.

μmol/min/mg of protein]. This is atypical of endopeptidase-2 and (see below) is likely to be due to a contaminating carboxypeptidase, which distorted the specific activity when assessed by substrate disappearance. Endopeptidase-24.11 had a much higher specific activity than endopeptidase-2 with all the peptides studied, except LHRH, for which the value was half that for endopeptidase-2.

Bonds in various peptides hydrolysed by endopeptidase-2

Fig. 1 shows the h.p.l.c. patterns after incubation of LHRH, substance P, bradykinin and the angiotensins with endopeptidase-2. The individual peak fractions were collected from several 6 h incubations, pooled and subjected to amino acid analysis. The amino acid compositions of each peak fraction, and hence the identities and yields of the fragments, were determined and are shown in Table 2. The sites of hydrolysis of each of the peptides predicted from these analyses can be seen in Fig. 2.

LHRH. LHRH was hydrolysed at three bonds: Trp^3–Ser^4, Ser^4–Tyr^5 and Tyr^5–Gly^6, the first being the preferred site. The identities of peaks 3, 4 and 5 were confirmed by their being co-eluted with the marker peptides 1-4, 1-3 and 1-5 respectively. The analysis of peptide 2 failed to reveal the expected content of tyrosine, the recovery of which was occasionally low in other peptides. However, the detection of serine along with the other amino acids served to identify the bond hydrolysed as Trp^3–Ser^4. The amino acid analysis of the material in Peak 1 was inconclusive, but the peak was co-eluted with

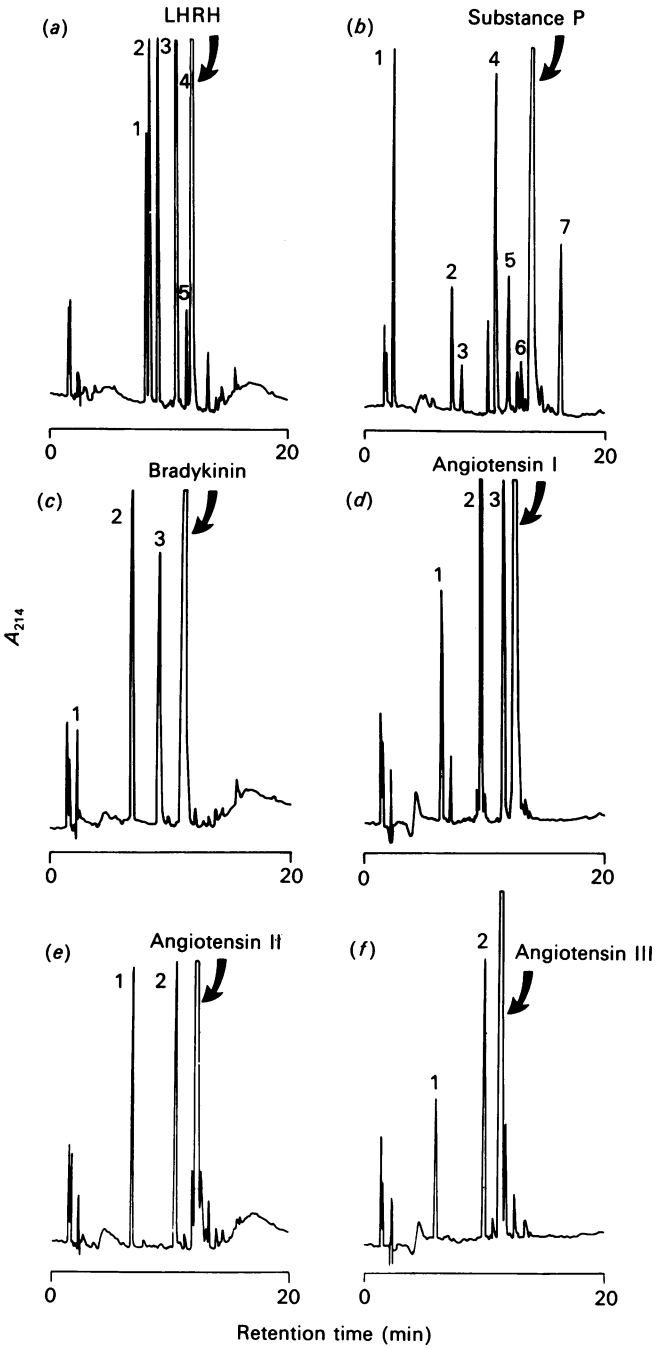

Fig. 1. H.p.l.c. analysis of peptide products formed by incubation of peptides with rat kidney endopeptidase-2

See the Experimental section for details. The product peaks are numbered in order of elution; they correspond to the numbering in Table 2 and Fig. 2. The incubation mixture contained 100 ng of endopeptidase-2 and 250 μM-peptide, incubated for the following times: LHRH, 30 min; substance P and bradykinin, 60 min; angiotensins, 360 min.

a product of a reaction of LHRH with endopeptidase-24.11 which had been identified as LHRH fragment 5-10. Fragment 6-10 was not observed.

Substance P. This peptide was hydrolysed at Gln^6–Phe^7, Phe^7–Phe^8 and Phe^8–Gly^9. Peaks 3, 5, 6 and 7 were also identified by their being co-eluted with

Table 2. Identification of peptides released by incubation for 6 h with rat kidney endopeptidase-2

See the Experimental section for details. Peaks (similar to those shown in Fig. 1) from several 6 h incubations were pooled and subjected to amino acid analysis. The yields were determined from single 6 h incubations which contained 25 nmol of peptide.

Substrate	Peak no.	Retention time (min)	Yield (nmol)	Amino acid composition (molar ratio, in parentheses)	Identity of fragment
LHRH	1	8.02	4.3	Gly (2.53), Leu (1.13), Tyr (0.69), Arg (1.61), Pro (1.00)	5-10
	2	8.32	10.0	Ser (0.58), Gly (2.16), Leu (0.87), Arg (0.81), Pro (1.00)	4-10
	3	9.00	4.0	Ser (1.39), Glu (1.00), His (0.80)	1-4
	4	10.56	8.3	Glu (1.00), His (1.01)	1-3
	5	11.42	0.2	Identified by being co-eluted with marker peptide	1-5
Substance P	1	2.26	6.1	Glu (2.02), Lys (1.14), Arg (1.00), Pro (2.35)	1-6
	2	7.16	2.4	Glu (2.14), Phe (0.99), Lys (1.00), Arg (0.90), Pro (1.96)	1-7
	3	7.98	10.4	Gly (1.11), Met (0.70), Leu (1.00)	9-11
	4	10.96	4.0	Glu (2.12), Phe (2.05), Lys (1.00), Arg (0.94), Pro (2.03)	1-8
	5	11.96	1.5	Phe – Identified by co-elution with marker peptide	7-8
	6	12.84	2.3	Gly (1.00), Met (0.83), Leu (0.87), Phe (1.03)	8-11
	7	16.28	4.0	Gly (1.00), Met (0.96), Leu (1.01), Phe (1.92)	7-11
Bradykinin	1	2.36	4.3	Gly (1.00), Arg (0.86), Pro (2.00)	1-4
	2	6.78	13.8	Ser (1.13), Phe (1.00) Arg (0.99), Pro (1.14)	6-9
	3	9.00	13.3	Gly (1.00), Phe (0.91), Arg (0.92), Pro (2.21)	1-5
Angiotensin I	1	6.54	1.9	Asp (1.23), Val (1.00), Tyr (0.96), Arg (0.95)	1-4
	2	9.82	3.0	Asp (1.15), Val (1.04), Ile (0.90), Tyr (1.00) Phe (1.00), His (2.03), Arg (0.97), Pro (1.18)	1-9
	3	11.78	2.0	Ile (0.79), Leu (1.00), Phe (1.06), His (2.00), Pro (1.00)	5-10
Angiotensin II	1	6.70	2.6	Asp (1.19), Val (0.82), Tyr (0.32), Arg (1.00)	1-4
	2	10.36	3.0	Ile (0.90), Phe (1.25), His (1.00), Pro (1.50)	5-8
Angiotensin III	1	5.84	1.2	Val (0.99), Tyr (1.00), Arg (1.10)	1-3
	2	10.16	1.6	Ile (0.96), Phe (1.21), His (1.00), Pro (1.23)	4-7

marker peptides for fragments 9-11, 7-8, 8-11 and 7-11 respectively.

Bradykinin. The primary attack on this peptide was at Phe[5]–Ser[6]. Peptide 1-4 was produced more slowly, indicating a secondary attack at the Gly[4]–Phe[5] bond. Peptide 5-9 was not identified, although after 6 h incubation a peak that was eluted close to the substrate was observed, which may have been Phe[5]–Arg[9].

Angiotensins I, II and III. These three peptides were all hydrolysed at the Tyr–Ile bond. Angiotensin I, however, was also hydrolysed at His[9]–Leu[10], which may be due to a contaminating carboxypeptidase, and this cleavage proceeded at about twice the rate of the attack on the Tyr[4]–Ile[5] bond. Hippuryl (Hip)-L-histidyl-L-leucine and L-histidyl-L-leucine were tried as potential inhibitors of this carboxypeptidase attack, but no inhibition was seen even at concentrations as high as 5 mM (His-Leu) and 10 mM (Hip-His-Leu).

Incubation of native proteins with endopeptidase-2

Insulin, cytochrome *c*, ovalbumin and serum albumin were incubated for 24 h with endopeptidase-2 under the same conditions as those used for studying the hydrolysis of peptides. No peptide fragments from these proteins were detected by h.p.l.c. analysis. Broad substrate peaks attributable to insulin, cytochrome *c* and serum albumin, but none for ovalbumin, were observed. These did not differ between the 0 and 24 h incubations.

Kinetics of hydrolysis of LHRH, bradykinin and substance P by endopeptidase-2

The values of K_m and $k_{cat.}$ are given in Table 3, together with published values for endopeptidase-24.11 hydrolysing the same three peptides. It is noteworthy that K_m values for bradykinin and substance P are about 3-fold higher for endopeptidase-2, whereas that for LHRH is 3-fold lower. The specificity constants ($k_{cat.}/K_m$) are very low for endopeptidase-2 (0.3–2.5 $min^{-1} \cdot \mu M^{-1}$). Only in the case of LHRH does the value exceed that of endopeptidase-24.11, those for bradykinin and substance P being two orders of magnitude lower.

Contribution of endopeptidase-2 to the hydrolysis of LHRH, bradykinin, substance P and α-hANP by rat kidney microvillar membranes

The hydrolysis of LHRH, bradykinin and substance P by rat and pig kidney microvillar membranes (the essential difference being the presence and absence of endopeptidase-2) was followed for periods of up to 30 min by the disappearance of the substrate peak by h.p.l.c. analysis. This was necessary because the rates of hydrolysis of these peptides differed for the two preparations and it was desirable to assess the contribution of endopeptidase-2 under conditions where the extent of hydrolysis was equivalent. From these progress curves, time points were selected for rat and pig kidney microvilli such that the extent of hydrolysis for a particular peptide was the same. These were: for LHRH, 12 min and 30 min; for bradykinin, 16 min and 10 min; and for substance P, 12 min and 10 min respectively. The

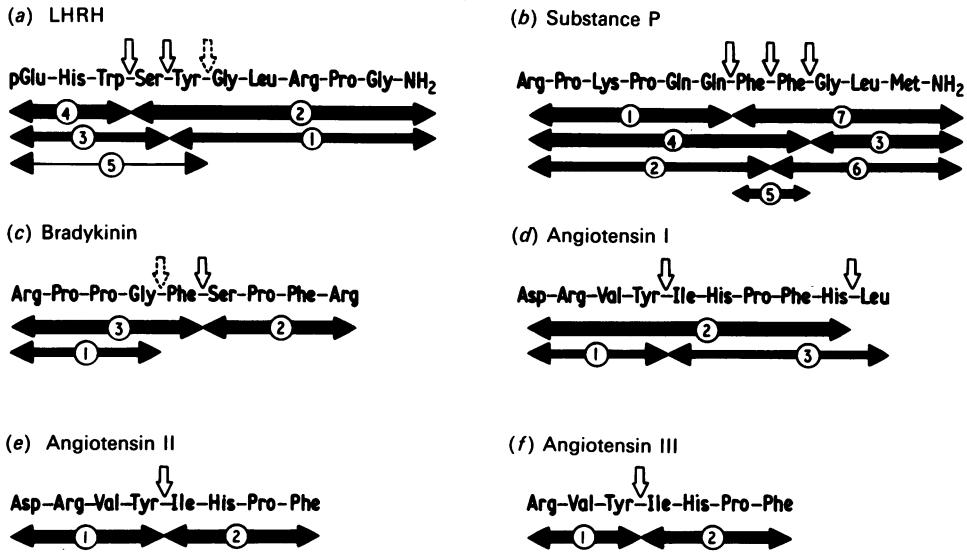

Fig. 2. Identities of the products formed by incubation of peptides with rat kidney endopeptidase-2

See the Experimental section for details. The numbered peptides correspond to the h.p.l.c. peaks in Fig. 1 and were identified by determining the amino acid composition and referring to the parent compound. The thickness of the lines designating the products refers to their yields (Table 2). Arrows indicate the bonds attacked, the 'broken' ones indicating those only slowly attacked.

Table 3. Comparison of kinetic constants of LHRH, bradykinin and substance P with rat kidney endopeptidase-2 and pig kidney endopeptidase-24.11

Results for endopeptidase-2 are presented as the means of two separate determinations (see the Experimental section for details). Kinetic constants for endopeptidase-24.11 are from Matsas *et al.* (1984).

Peptide	Endopeptidase-2			Endopeptidase-24.11		
	K_m (μM)	$k_{cat.}$ (min^{-1})	$k_{cat.}/K_m$ ($min^{-1} \cdot \mu M^{-1}$)	K_m (μM)	$k_{cat.}$ (min^{-1})	$k_{cat.}/K_m$ ($min^{-1} \cdot \mu M^{-1}$)
LHRH	270	669	2.48	755	840	1.1
Bradykinin	325	87.7	0.27	92	6360	69
Substance P	86	180	2.09	32	5060	159

same experiment was performed in parallel in the presence of peptidase inhibitors (phosphoramidon, captopril, amastatin and Dip-F). The effect of inhibitors was then examined at these defined time points. The results are shown in Fig. 3.

LHRH was hydrolysed in these experiments more slowly than the other two peptides. However, although its breakdown by pig kidney microvilli was completely blocked by the inhibitors, its hydrolysis by rat kidney microvilli was suppressed by only 25%. This implies that endopeptidase-2 may play a dominant role in the hydrolysis of LHRH by rat renal microvillar membranes. The patterns of hydrolysis of LHRH by endopeptidase-2 and the rat membranes, as seen by h.p.l.c., were similar (results not shown), suggesting that this is the case. Bradykinin was hydrolysed rapidly. The inhibitors wholly suppressed its breakdown by pig kidney microvilli, but only inhibited hydrolysis by rat microvilli, by approx. 50%. The h.p.l.c. traces from incubations with rat microvilli and endopeptidase-2 had some peaks in common. Thus endopeptidase-2 may be partly responsible for bradykinin breakdown by rat renal microvillar membranes. Substance P was also hydrolysed rapidly and, in the case of pig microvilli, wholly blocked when

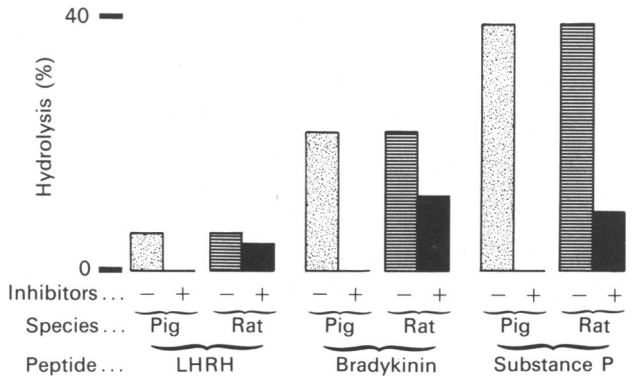

Fig. 3. Comparison of the hydrolysis of peptides by rat and pig kidney microvillar membranes

See the Experimental section for details. The results are shown as hydrolysis (%) in the presence (+) or absence (−) of inhibitors (phosphoramidon, captopril, amastatin and Dip-F). The conditions were such that, in the absence of inhibitors, the hydrolysis rates by rat and pig microvillar membranes were equal. The residual hydrolysis when inhibitors were present is an indication of the contribution of endopeptidase-2.

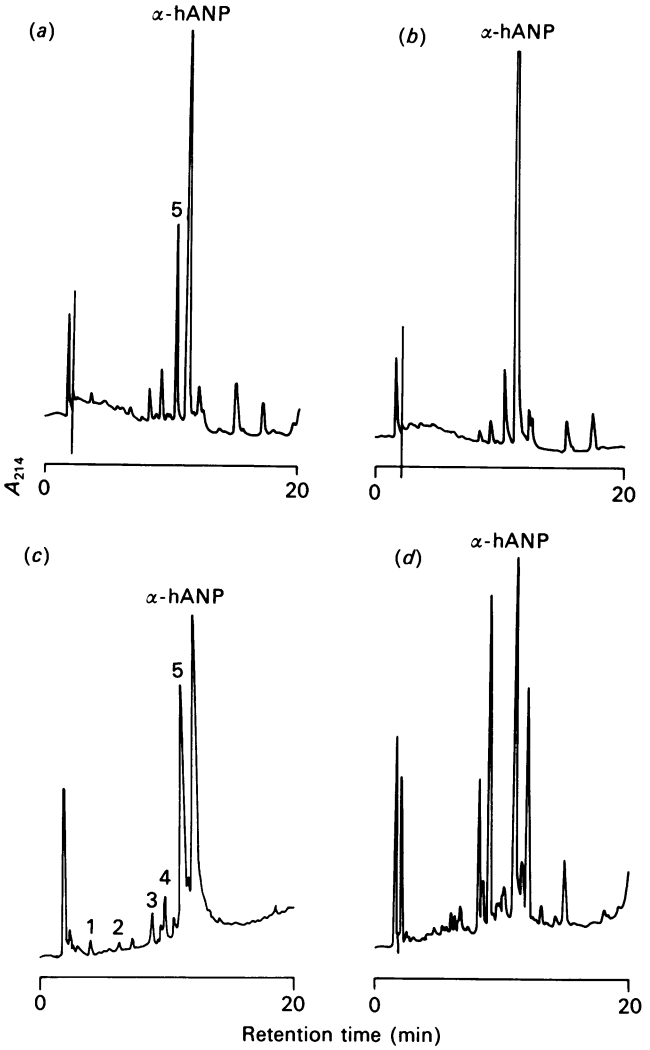

Fig. 4. Hydrolysis of α-hANP by microvillar membranes and by purified endopeptidase-2

See the Experimental section for details. H.p.l.c. traces are shown of the peptide products after incubation at 37 °C of 50 μM-α-hANP with: (*a*) 2 μg of rat kidney microvillar membranes (15 min); (*b*), as (*a*), with 1 μM-phosphoramidon; (*c*) 2 μg of pig kidney membranes (15 min); (*d*) 100 ng of endopeptidase-2 (6 h). Peaks, numbered 1–5 in (*c*), are those reported for pig kidney microvilli (Stephenson & Kenny, 1987*b*); the main product (peptide 5) was also generated by rat kidney microvilli (*a*).

inhibitors were included in the incubation; however, 25% residual hydrolysis persisted with rat microvilli. The pattern of hydrolysis by rat microvilli was very similar to that observed with pig membranes, in which hydrolysis has been attributed to the action of endopeptidase-24.11 and dipeptidyl peptidase IV (Stephenson & Kenny, 1987*a*). Endopeptidase-2 may, therefore, play only a small role in the hydrolysis of substance P by rat kidney microvillar membranes.

The pattern of hydrolysis of α-hANP (50 μM) by rat kidney microvillar membranes (Fig. 4*a*) was similar to that observed with pig kidney microvillar membranes (Fig. 4*c*) and endopeptidase-24.11 (Stephenson & Kenny, 1987*b*). The inclusion of phosphoramidon (Fig. 4*b*) in the incubation resulted in almost complete inhibition of hydrolysis by rat membranes, the substrate peak at 15 min being 90% of the initial value. The products formed when α-hANP was incubated with 100 ng of endopeptidase-2 were strikingly different from those produced by rat microvillar membranes. Fig.4(*d*) shows the pattern after 6 h incubation, when about 35% of the α-hANP remained unhydrolysed. Hence, the dominant enzyme in the hydrolysis of α-hANP by rat kidney microvillar membranes is endopeptidase-24.11.

DISCUSSION

Specificity

The peptide-bond specificity of endopeptidase-2 was defined with a range of peptides as substrates. When LHRH, substance P, bradykinin and the angiotensins were substrates, hydrolysis was seen to occur on both sides of hydrophobic (particularly aromatic) residues. Hydrolysis occurred on the carboxy side of aromatic residues: tryptophan and tyrosine (LHRH), phenylalanine (substance P and bradykinin) and tyrosine (the angiotensins). Cleavage was also observed on the amino side of tyrosine (LHRH) and phenylalanine (substance P and bradykinin). Angiotensin I was also hydrolysed by an apparent carboxypeptidase attack at the His^9–Leu^{10} bond. Changaris *et al.* (1986, 1987) have reported such an activity attacking angiotensin I to be present in the cytosol of rat kidney and brain, and it is likely that our preparation was contaminated by a similar carboxypeptidase which was not susceptible to any of the inhibitors used.

Chain length also influences the attack on peptides. This appeared to be the explanation for the slow attack on [Met]enkephalin-Arg^6-Phe^7 and the absence of hydrolysis of [Leu]enkephalin-Arg^6, [Leu]enkephalinamide, and [Leu]- and [Met]-enkephalins (Kenny & Ingram, 1987). The somewhat slower attack on angiotensin III compared with I and II observed here is consistent with this phenomenon.

Preferred peptide substrates for endopeptidase-2

Of the peptides studied LHRH, substance P and bradykinin were the most rapidly hydrolysed, whereas others, such as α-hANP and the angiotensins, were only slowly attacked. This is a different preference from that of endopeptidase-24.11, by which LHRH is poorly hydrolysed, whereas α-hANP and the angiotensins are relatively good substrates (Matsas *et al.*, 1984; Stephenson & Kenny, 1987*a*,*b*). When the specific activities of the two endopeptidases were directly compared for a group of peptides, only LHRH was attacked more efficiently by endopeptidase-2 than by endopeptidase-24.11, though only by a factor of two. In regard to the other peptides in the group, endopeptidase-2 was an order of magnitude less effective. The kinetic constants for three peptides also showed that only for LHRH was the specificity constant higher with endopeptidase-2, whereas those for bradykinin and substance P were lower by two orders of magnitude. This may be consistent with the view that endopeptidase-2 hydrolyses longer peptides more efficiently. The K_m for ^{125}I-insulin B-chain was 16 μM, considerably lower than those for these three neuropeptides, yet the ratio $k_{cat.}/K_m$ was only 1.2 $min^{-1} \cdot \mu M^{-1}$ (Kenny & Ingram, 1987). It is clear that we have not yet identified a peptide substrate which is attacked

with the same efficiency that endopeptidase-24.11 exhibits towards its best substrates.

We failed to detect any evidence that endopeptidase-2 hydrolysed native proteins when insulin, cytochrome *c*, ovalbumin and serum albumin were incubated for 24 h. The analytical conditions used would have been expected to reveal peptide fragments. Although we cannot exclude the possibility of limited proteolytic attack such that any products would be co-eluted with the protein, we have no evidence that endopeptidase-2 has activity towards native proteins.

Assessment of the contribution of endopeptidase-2 in hydrolysis of neuropeptides by rat microvillar membranes

Our conclusions from experiments with pig kidney microvilli were that endopeptidase-24.11 was the essential enzyme for initiating hydrolysis of α-hANP, substance P, bradykinin, the angiotensins and oxytocin (Stephenson & Kenny, 1987*a*,*b*). Comparable experiments with rat kidney microvilli are hampered by the lack of a specific inhibitor to endopeptidase-2. Its contribution to the hydrolysis of LHRH, bradykinin and substance P was therefore assessed as the residual activity in the presence of inhibitors for endopeptidase-24.11, peptidyl dipeptidase A, dipeptidyl peptidase IV and aminopeptidases N, A and W. In experiments in which the conditions were adjusted so that the rates of hydrolysis in the absence of inhibitors of each peptide were identical for pig and rat microvilli, only LHRH was hydrolysed predominantly by endopeptidase-2; for bradykinin the contributions of the two endopeptidases were equal and for substance P endopeptidase-24.11 was dominant. The strong inhibitory effect of phosphoramidon on the hydrolysis of α-hANP by rat kidney microvilli indicates that endopeptidase-24.11 and not endopeptidase-2 is the principal enzyme hydrolysing this peptide (for a review, see Kenny & Stephenson, 1988).

Comparison of endopeptidase-2 with meprin

Our studies on endopeptidase-2 have shown it to have a peptide-bond specificity generally similar to that of meprin. Both enzymes have been shown preferentially to hydrolyse bonds flanked by hydrophobic residues, and in the case of endopeptidase-2, no other classes of bond were attacked, although meprin appears to have a broader specificity, hydrolysing Gly^{20}–Glu^{21} in insulin B-chain (Butler *et al.*, 1987). However, the similarities in specificity when considered along with those of structure and topology (Kenny & Ingram, 1987) argue strongly for these two rodent microvillar enzymes being very closely related.

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REFERENCES

Abbs, M. T. & Kenny, A. J. (1983) Clin. Sci. **65**, 551–559
Beynon, R. J., Shannon, J. D. & Bond, J. S. (1981) Biochem. J. **199**, 591–598
Bond, J. S., Shannon, J. D. & Beynon, R. J. (1983) Biochem. J. **209**, 251–255
Bond, J. S., Butler, P. E. & Beynon, R. J. (1986) Biomed. Biochim. Acta **45**, 1515–1521
Booth, A. G. & Kenny, A. J. (1974) Biochem. J. **142**, 575–581
Butler, P. E., McKay, M. J. & Bond, J. S. (1987) Biochem. J. **241**, 229–235
Changaris, D. G., Miller, J. J. & Levy, R. S. (1986) Biochem. Biophys. Res. Commun. **138**, 573–579
Changaris, D., Miller, J., Levy, R. & Lesousky, N. (1987) Fed. Proc. Fed. Am. Soc. Exp. Biol. **46**, 484
Fulcher, I. S. & Kenny, A. J. (1983) Biochem. J. **211**, 743–753
Gee, N. S. & Kenny, A. J. (1985) Biochem. Soc. Trans. **13**, 101–102
Gee, N. S., Matsas, R. & Kenny, A. J. (1983) Biochem. J. **214**, 377–386
Kenny, A. J. (1986) Trends Biochem. Sci. **11**, 40–42
Kenny, A. J. & Ingram, J. (1987) Biochem. J. **245**, 515–524
Kenny, A. J. & Stephenson, S. L. (1988) FEBS Lett. **232**, 1–8
Kenny, A. J., Fulcher, I. S., Ridgwell, K. & Ingram, J. (1981) Acta Biol. Med. Germ. **40**, 1465–1471
Kenny, A. J., Stephenson, S. L. & Turner, A. J. (1987) in Mammalian Ectoenzymes (Kenny, A. J. & Turner, A. J., eds.), pp. 169–210, Elsevier/North-Holland Biomedical Press, Amsterdam
Matsas, R., Fulcher, I. S., Kenny, A. J. & Turner, A. J. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 3111–3115
Matsas, R., Kenny, A. J. & Turner, A. J. (1984) Biochem. J. **223**, 433–440
Stephenson, S. L. & Kenny, A. J. (1987*a*) Biochem. J. **241**, 237–247
Stephenson, S. L. & Kenny, A. J. (1987*b*) Biochem. J. **243**, 183–187
Sterchi, E. E., Green, J. R. & Lentze, M. J. (1982) Clin. Sci. **62**, 557–560
Sterchi, E. E., Green, J. R. & Lentze, M. J. (1983) J. Pediat. Gastroenterol. Nutr. **2**, 539–547