Metabolism of neuropeptides

Hydrolysis of the angiotensins, bradykinin, substance P and oxytocin by pig kidney microvillar membranes

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Microvillar membranes derived from the brush border of the renal proximal tubule are very rich in peptidases. Pig kidney microvilli contain endopeptidase-24.11 associated with a battery of exopeptidases. The manner by which some neuropeptides are degraded by the combined attack of the peptidases of this membrane has been investigated. The contribution of individual peptidases was assessed by including inhibitors (phosphoramidon, captopril, amastatin and di-isopropyl fluorophosphate) with the membrane fraction when incubated with the peptides. Substance P, bradykinin and angiotensins I, II and III and insulin B-chain were rapidly hydrolysed by kidney microvilli. Oxytocin was hydrolysed much more slowly, but no products were detected from [Arg⁸]vasopressin or insulin under the conditions used for other peptides. The peptide bonds hydrolysed were identified and the contributions of the different peptidases were quantified. For each of the susceptible peptides, the main contribution came from endopeptidase-24.11 (inhibited by phosphoramidon). Peptidyl dipeptidase A (angiotensin-I-converting enzyme) was of less importance, even in respect of angiotensin I and bradykinin. When [2,3-³H]bradykinin was also investigated at a lower concentration (20 nM), the conclusions in regard to the contributions of the two peptidases were unchanged. The possibility that endopeptidase-24.11 might attack within the six-residue disulphide-bridged rings of oxytocin and vasopressin was examined by dansyl(5-dimethylaminonaphthalene-1-sulphonyl)ation and by reduction and carboxymethylation of the products after incubation. Additional peptides were only observed after prolonged incubation, consistent with hydrolysis at the Tyr²-Ile³ and Tyr²-Phe³ bonds respectively. These results show that a range of neuropeptides are efficiently degraded by microvillar membranes and that endopeptidase-24.11 plays a key role in this process.

INTRODUCTION

No other plasma membrane is known to contain such an abundance of peptidases as that of the renal brush border. Yet we have little understanding of the roles of these enzymes in this particular location in the proximal tubule, nor even the nature of their normal substrates. Those that have been studied in regard to their topology are known to be ectoenzymes, i.e. they are integral membrane proteins with their active sites exposed at the luminal surface (for a review, see Kenny & Maroux, 1982). Their potential substrates are those that are filtered at the glomerulus. These might include plasma proteins whose size and charge permit passage through the glomerulus, but it is generally agreed that proteins are metabolized by a route involving pinocytosis by the proximal-tubule cells and digestion within the lysosomal system (for a review, see Emmanouel *et al.*, 1983). Moreover, the microvillar peptidases seem to have little or no proteinase activity: in pig kidney microvilli there is only one endopeptidase, endopeptidase-24.11 (EC 3.4.24.11), whose action is limited to peptides (Kerr & Kenny, 1974). This is also the only endopeptidase in human and rabbit kidney brush borders (Abbs & Kenny, 1983), but in rats and mice second endopeptidases have

been distinguished, referred to as 'neutral endopeptidase-2' (Kenny *et al.*, 1981) and 'meprin' (Beynon *et al.*, 1981) respectively. The latter enzyme has been assayed with azocasein as substrate and may therefore exhibit some proteinase activity. But this possibility apart, it seems likely that the peptidases of the renal brush border are involved in the hydrolysis of peptides present in the glomerular filtrate.

There is a good deal of information on the specificity of the microvillar peptidases from studies with purified enzymes. Endopeptidase-24.11 cleaves bonds involving the amino groups of hydrophobic-amino-acid residues (Kerr & Kenny, 1974), but the interaction of other nearby residues with the active site greatly influences the efficiency of hydrolysis (Almenoff & Orlowski, 1983; Matsas *et al.*, 1984; Pozsgay *et al.*, 1986). Although peptidyl dipeptidase A (PDP, EC 3.4.15.1, 'angiotensin-I-converting enzyme') is, by definition, an exopeptidase, it has been shown to possess endopeptidase activity towards substance P and luliberin (Cascieri *et al.*, 1983; Yokosawa *et al.*, 1983; Skidgel & Erdős, 1985). Hence pig kidney microvilli contain two endopeptidases complemented by a variety of exopeptidases, including aminopeptidase N (EC 3.4.11.2), aminopeptidase A (EC 3.4.11.7), aminopeptidase W (Gee & Kenny, 1985b),

Abbreviation used: Dip-F, di-isopropyl fluorophosphate; DPP-IV, dipeptidyl peptidase IV; PDP, peptidyl dipeptidase A ('angiotensin-converting enzyme'); -NMec, 7-amino-4-methylcoumarylamide derivative of amino acids and peptides; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl; Bz-, benzoyl-; IC₅₀, concentration causing half-maximal inhibition.

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aminopeptidase P (EC 3.4.11.9; Kenny *et al.*, 1977), dipeptidyl peptidase IV (EC 3.4.14.5; Macnair & Kenny, 1979), peptidyl dipeptidase A and carboxypeptidase P (EC 3.4.17.-; Hedeager-Sørensen & Kenny, 1985). Microvillar membranes therefore provide a unique opportunity to study the way in which a group of peptidases might act in concert to metabolize some defined neuropeptides. To do this, the experiments need to be designed so that the initial steps of hydrolysis can be observed by an appropriate analytical system, a requirement that necessitates substrate concentrations greatly in excess of those found *in vivo*. By employing appropriate inhibitors, it is also possible to assess the contributions of some of the peptidases. Phosphoramidon is a specific inhibitor of endopeptidase-24.11 (Kenny, 1977) and captopril of peptidyl dipeptidase A (Ondetti *et al.*, 1977). There are no inhibitors specific for each of the aminopeptidases, but amastatin is a potent inhibitor of aminopeptidase N and also effective against aminopeptidases A (Aoyagi *et al.*, 1978) and W (N. S. Gee & A. J. Kenny, unpublished work). Dipeptidyl peptidase IV is the only serine enzyme in kidney microvilli, and hence di-isopropyl fluorophosphate (Dip-F) may be regarded as specific in this instance (Kenny *et al.*, 1976).

In the present paper we report some results using these strategies. We show that endopeptidase-24.11 plays a dominant role in the initial attack of all the susceptible neuropeptides studied. In the case of bradykinin a similar conclusion was reached when [^3H]bradykinin was studied at concentrations nearer to those existing physiologically.

EXPERIMENTAL

Materials

Pig kidneys were kindly given by ASDA Farm Stores, Lofthousegate, Wakefield, West Yorkshire, U.K. Kidneys were removed within a few minutes of death, packed on ice and brought back to the laboratory. Cortical tissue was frozen in batches at -70°C until required.

The fluorogenic substrates Ala-NMec, α -Glu-NMec and Gly-Pro-NMec were purchased from Bachem, Hauptstrasse 144, CH-4416 Bubendorf, Switzerland. Angiotensin I, angiotensin II and bradykinin were obtained from Sigma Chemical Co. Angiotensin III, oxytocin and [Arg^8]vasopressin were obtained from Cambridge Research Biochemicals (Harston, Cambridge, U.K.). [2,3- $\text{Pro}^{3,4,5}\text{H}$]Bradykinin was obtained from New England Nuclear (Code NET-706). Insulin was obtained from Novo Research Institute, DK-2800, Bagsvaerd, Denmark. Reduced carboxymethylated B-chain of insulin was prepared as described previously (Kenny, 1977). Phosphoramidon was obtained from Protein Research Foundation, Osaka, Japan. 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.

Methods

Purified peptidases. Endopeptidase-24.11 was isolated by immunoaffinity chromatography as described by Gee *et al.* (1983), except that the monoclonal antibody was GK4A9 (Gee & Kenny, 1985a). The preparation contained no detectable activity against Ala-NMec or

Gly-Pro-NMec. Aminopeptidase N was the same preparation described previously (Matsas *et al.*, 1985). PDP was prepared by affinity chromatography using Lisinopril-Sephrose (Bull *et al.*, 1985) and was generously given by Mr. N. M. Hooper, of this Department. DPP-IV was prepared as described by Kenny *et al.* (1976).

Microvillar membranes. These were prepared as described by Booth & Kenny (1974) and were washed once by resuspending in the mannitol/Tris buffer, pH 7.1, containing 250 mM-NaCl. The pellet obtained after centrifugation was resuspended in mannitol/Tris buffer (5 mg of protein/ml) and was stored in batches at -70°C .

Enzymic assays. Aminopeptidase N was assayed with Ala-NMec, aminopeptidase A with α -Glu-NMec and DPP-IV with Gly-Pro-NMec as described by Fulcher & Kenny (1983). Endopeptidase-24.11 was assayed with [^{125}I]insulin B-chain (Fulcher & Kenny, 1983) and PDP with Bz-Gly-His-Leu as substrate (Hooper *et al.*, 1985).

Hydrolysis of neuropeptides. The incubation mixture (vol. 100 μl) contained 100 mM-Tris/HCl, 150 mM-NaCl, pH 7.4 (at 37°C), 250 μM -peptide (except where otherwise stated) and 1–5 μg of microvillar-membrane protein or 50–100 ng of purified peptidases. Samples were incubated at 37°C for 15–30 min. Incubation was terminated by heating to 100°C for 4 min and samples centrifuged before analysis.

Hydrolysis of [^3H]bradykinin. The incubation conditions were as described above, except that 0.2 μg of microvillar membrane and 20 nM-[^3H]bradykinin were used. At the end of the incubation (15 min) a sample (80 μl) of another incubation mixture of unlabelled bradykinin with microvillar membranes (as above) was added and the combined samples immediately heated to 100°C for 4 min.

Inhibitor experiments. Phosphoramidon, captopril and amastatin were used at a final concentration of 1 μM and were preincubated for 15 min at 20°C with membranes or enzyme preparation before addition of 10 μl of the peptide solution. Dip-F was used at 0.1 mM and preincubated for 1 h at 20°C .

Analysis of peptide products by h.p.l.c. This was performed as previously described (Matsas *et al.*, 1983), except that the acetonitrile gradients used were 4.5–30% (v/v) (oxytocin and vasopressin); 4.05–45% (v/v) (bradykinin, the angiotensins and substance P) and 4.8–60% (v/v) for insulin and insulin B-chain. The products were identified by co-elution with marker peptides (when available) or by collection of the individual peaks followed by amino acid analyses (Matsas *et al.*, 1983). When [^3H]bradykinin products were analysed, 1 ml (0.5 min) fractions were collected throughout the run. Radioactivity was assayed by liquid-scintillation counting using Cocktail T (BDH, Poole, Dorset, U.K.).

Reduction and carboxymethylation of oxytocin and vasopressin. The incubation mixture contained 0.4 μg of endopeptidase-24.11 and 0.1 mM-peptide (vol. 200 μl).

After terminating the incubation by heating, the samples, after removal of the protein precipitate, were treated with dithiothreitol (to 20 mM) and incubated for 2 h at 37 °C. Iodoacetic acid was added (to 40 mM) and the incubation continued for 15 min. These reagents were dissolved in 0.3 M-Tris/HCl, pH 8.6, and the steps were performed under N₂. The samples were analysed as described above.

Dansylation. Oxytocin and [Arg⁸]vasopressin (0.2–0.5 mM) were incubated with 200 ng of endopeptidase-24.11 in 25 mM-Pipes/100 mM-NaCl, pH 7.0 (total vol. 100 µl) at 37 °C. After suitable intervals, the incubation was stopped as described above; 85 µl of the supernatant was freeze-dried and 85 µl of 0.2 M-NaCO₃ was then added; the sample was again freeze-dried. Dansylation, hydrolysis of peptide bonds and analysis of the dansylated amino acids were performed essentially as described by Hartley (1970).

Quantification of products analysed by h.p.l.c., monitored at 214 nm. Amino acids absorbing at this wavelength (namely histidine, phenylalanine, tyrosine and tryptophan), alanine- and glycine-containing peptides (Ala-Ala, Ala-Ala-Ala, Ala-Ala-Ala-Ala, Ala-Gly, Ala-Gly-Gly, Gly-Ala-Ala) and proline-containing peptides (Gly-Pro, Gly-Pro-Ala, Ala-Pro-Gly, Gly-Gly-Pro, Pro-Gly) were injected (10 nmol) and the peak areas recorded.

RESULTS

Quantification of products of peptide hydrolysis

Ideally, synthetic peptide standards should enable each peptide peak to be quantified in molar terms. In practice, these standards were only occasionally available and in our studies an approximate conversion was made on the basis of the amino acid composition of the peptide products. The peak areas/nmol are shown in Table 1. The validity of the values obtained was checked against the peak areas observed with some of the peptide substrates studied. Thus, for bradykinin, angiotensins I

and II and vasopressin, the observed and calculated values were within 5, 10, 3 and 1% respectively.

Specific activity of peptidases in kidney microvillar membranes

The enrichment of the peptidases was approx. 10-fold. Specific activities (nmol) of peptide hydrolysed/min per mg of protein) were: aminopeptidase N, 480; DPP-IV, 450; aminopeptidase A, 90; endopeptidase-24.11, 8.0 and PDP, 90.

Hydrolysis of peptides by microvillar membrane peptidases

The h.p.l.c. traces, monitored at 214 nm, of the products after incubation of six peptides with microvillar membranes are shown in Fig. 1. The conditions were such that hydrolysis of the peptides was less than 25% except for angiotensin III, which was 50%. No apparent hydrolysis of vasopressin was detected. The three angiotensins and bradykinin yielded multiple products, whereas the much slower attack on oxytocin gave only one additional peak.

The products were collected, dried *in vacuo*, hydrolysed and subjected to amino acid analysis. The identities of the peptides or amino acids are shown in Fig. 2 and from this information the bonds cleaved were identified and are shown as open arrows.

The identifications were complemented by experiments in which peptides (0.1 mM) were incubated with purified aminopeptidase N, DPP-IV, PDP and endopeptidase-24.11. Angiotensin I was hydrolysed at Tyr⁴-Ile⁵ and Pro⁷-Phe⁸ by endopeptidase-24.11 and at Phe⁸-His⁹ by PDP. Angiotensins I and III were hydrolysed by endopeptidase-24.11 at the same Tyr-Ile bond, whereas the N-terminal arginine residue of angiotensin III was removed by aminopeptidase N. Bradykinin was hydrolysed at the Pro⁷-Phe⁸ bond by both PDP and endopeptidase-24.11; in these conditions, no hydrolysis was observed at the Gly⁴-Phe⁵ bond. The hydrolysis of substance P by endopeptidase-24.11 has been reported previously (Matsas *et al.*, 1983). In these experiments,

Table 1. Quantification of amino acids and peptides monitored by h.p.l.c. at 214 nm

See the Experimental section for details. * indicates that the value was obtained by subtraction of assumed value (105) for α-peptide bond.

Amino acid or peptide	Peak area/nmol	Peak area/α-peptide bond	Peak area/imido bond
Tryptophan	4750	—	—
Tyrosine	1200	—	—
Phenylalanine	975	—	—
Histidine	800	—	—
Ala-Ala	118	118	—
Ala-Ala-Ala	224	112	—
Ala-Ala-Ala-Ala	368	123	—
Ala-Gly	82	82	—
Ala-Gly-Gly	164	82	—
Gly-Ala-Ala	233	117	—
Pro-Gly	98	98	—
Gly-Pro	619	—	619
Gly-Pro-Ala	742	—	637*
Ala-Pro-Gly	705	—	600*
Gly-Gly-Pro	714	—	609*

105 ± 6.5
(mean ± S.E.M.)

616 ± 8
(mean ± S.E.M.)

Fig. 1. Analyses by h.p.l.c. of peptide products formed by incubation of peptides with kidney microvillar membranes

See the Experimental section for details. Some minor peaks which were eluted very early in the gradient were also observed in the blank samples. The product peaks are numbered in the order of elution; they correspond to the numbering in Fig. 2.

DPP-IV was shown to hydrolyse the Pro²-Lys³ and Pro⁴-Gln⁵ bonds. Endopeptidase-24.11 hydrolysed oxytocin at the Pro⁷-Leu⁸ bond, but no apparent hydrolysis of [Arg⁸]vasopressin was observed. The bonds hydrolysed of bradykinin, angiotensins I and II, and oxytocin by pig endopeptidase-24.11 are in agreement with those previously reported for the human enzyme (Gafford *et al.*, 1983; Johnson *et al.*, 1984).

Rate of hydrolysis of peptides by kidney microvillar membranes

The relative rates, as $t_{1/2}$ values, are given in Table 2. Two of the peptides studied, [Arg⁸]vasopressin and insulin, were essentially resistant to attack under the conditions employed, although some degradation of vasopressin was noted after 24 h. Oxytocin was only slowly hydrolysed, with a $t_{1/2}$ of about 8 h, whereas the other peptides were very rapidly hydrolysed with $t_{1/2}$ values of 5–34 min. Substance P and bradykinin were attacked considerably faster than any other peptide in the group.

Effect of inhibitors on the pattern of hydrolysis of peptides by kidney microvillar membranes

Some indication of the contributions of the various peptidases in the hydrolysis of peptides was obtained by using four selectively acting inhibitors. Table 3 shows these effects as well as the yields of the products of hydrolysis. Phosphoramidon inhibits endopeptidase-24.11; captopril, PDP; amastatin, aminopeptidases, while Dip-F inhibits DPP-IV.

Angiotensin I. The main product (peptide 4) resulting from hydrolysis of Pro⁷-Phe⁸ was 83% suppressed by phosphoramidon and unaffected by other inhibitors. The other fragment from this attack (peptide 6, Phe-His-Leu) was similarly affected by the inhibitors. His-Leu (peptide 1) was, rather surprisingly, 40% suppressed by phosphoramidon and only 16% by captopril, but the explanation is that His-Leu is generated by both a primary attack by PDP and an aminopeptidase attack, inhibited by amastatin, liberating phenylalanine (product 2) from Phe-His-Leu. Amastatin increased the yield of peptide 6 to 132%. Angiotensin II (peptide 7), substantially suppressed by captopril, was only a minor product. Peptides 3 and 5, from attack at Arg²-Val³ and Tyr⁴-Ile⁵, were about 80% suppressed by phosphoramidon. The *N*-terminal dipeptide Asp-Arg was not detected, even in the presence of amastatin.

Angiotensin II. The main attack occurring at the Tyr⁴-Ile⁵ bond (generating peptides 3 and 6) was wholly suppressed by phosphoramidon. Peptide 5 arose from a carboxypeptidase attack (liberating phenylalanine, product 2) and was not significantly inhibited by any of the reagents. This would be consistent with an attack by carboxypeptidase P, which hydrolyses Pro-Xaa bonds (Hedegaard-Sørensen & Kenny, 1985). The only other bonds shown to be hydrolysed appear to be those subjected to aminopeptidase attack after cleavage at the Tyr⁴-Ile⁵ bond. Peptides 1 and 4 were wholly suppressed by phosphoramidon and substantially by amastatin.

Angiotensin III. Three points of primary attack were revealed. The *N*-terminal arginine residue was removed to yield peptide 5, partially suppressed by amastatin. Phenylalanine was released from the *C*-terminus, and this

Fig. 2. Identities of the products formed by incubation of peptides with kidney microvillar membranes

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 amino acid sequences of the parent compound. The thickness of the lines designating the products relates to their concentration.

Table 2. t_1 values of peptides incubated with kidney microvillar membranes

See the Experimental section for details. The incubations contained 0.5 mM-peptide, except in the case of insulin B-chain and insulin (0.2 mM). The amount of membrane protein used was 2 μ g. Samples were incubated at 37 °C for 0, 1, 5, 10, 15, 30, 60, 120, 180, 240 and 1440 min and the progress of hydrolysis monitored by the disappearance of the substrate peak. The progress curves approximated to first-order decay curves from which the t_1 values were computed.

Peptide	t_1 (min)
Substance P	5
Bradykinin	8
Angiotensin III	19
Angiotensin II	21
Insulin B-chain	26
Angiotensin I	34
Oxytocin	510
[Arg ⁸]Vasopressin	> 24 h
Insulin	No hydrolysis at 24 h

cleavage was unaffected by any of the inhibitors. The main attack was at Tyr³-Ile⁴, yielding peptides 2 and 4 and this was strongly inhibited by phosphoramidon.

Bradykinin. The primary attack on this peptide was at the Pro⁷-Phe⁸ bond, liberating Phe-Arg (peptide 1) and bradykinin-(1-7)-peptide (peptide 3). Two inhibitors were effective: phosphoramidon inhibited 84% and captopril only 8%, which reflects the relative contributions of endopeptidase-24.11 and PDP. The only other fragment detected was phenylalanine released by a secondary aminopeptidase attack and therefore suppressed by amastatin as well as the inhibitors of the primary attack.

Oxytocin. This was hydrolysed at only one bond, Pro⁷-Leu⁸ (yielding peptide 1) and was wholly inhibited by phosphoramidon. The C-terminal fragment (Leu-GlyNH₂) was not detected, probably because it was degraded by aminopeptidase action, although no additional peak was seen in the presence of amastatin.

Vasopressin. This peptide, differing from oxytocin in

Table 3. Effect of inhibitors on the hydrolysis of neuropeptides by pig kidney microvillar membranes

See the Experimental section for details. The products are numbered as in Figs. 1 and 2. The amount of each product formed in the absence of inhibitors is given in nmol. The effect of each inhibitor on the yield of the products is expressed as a percentage of this value. Abbreviation used: n.d., not determined. The data are based on three experiments, each with duplicate incubations, except for oxytocin, which was studied in two experiments. Peptide 1 (His-Pro) from angiotensin II was successfully resolved in only one experiment.

Peak	Peptide	Retention time (min)	Product formed in the absence of inhibitor (nmol)	Inhibitor ...	Peptide remaining (%)			
					Phosphoramidon (1 μ M)	Captopril (1 μ M)	Amastatin (1 μ M)	Dip-F (0.1 mM)
Angiotensin I								
1	His-Leu	2.50	1.93		60	84	46	93
2	Phe	3.82	1.18		8	93	32	87
3	Asp-Arg-Val-Tyr	5.50	1.22		14	105	97	103
4	Asp-Arg-Val-Tyr-Ile-His-Pro	8.34	2.59		17	101	96	104
5	Val-Tyr-Ile-His-Pro	9.96	0.50		26	91	40	140
6	Phe-His-Leu	10.38	1.85		15	87	132	85
7	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	11.50	0.78		124	58	118	110
Angiotensin II								
1	His-Pro	1.88	1.91		0	73	31	31
2	Phe	3.66	1.85		72	116	71	77
3	Asp-Arg-Val-Tyr	5.16	3.96		3	103	97	99
4	His-Pro-Phe	6.30	1.35		5	100	11	131
5	Asp-Arg-Val-Tyr-Ile-His-Pro	8.24	0.96		120	103	102	99
6	Ile-His-Pro-Phe	8.98	2.81		8	100	149	101
Angiotensin III								
1	Phe	3.70	1.57		84	102	105	99
2	Arg-Val-Tyr	4.58	2.90		3	104	108	102
3	Arg-Val-Tyr-Ile-His-Pro	7.74	0.99		130	107	104	102
4	Ile-His-Pro-Phe	9.08	3.54		11	100	104	103
5	Val-Tyr-Ile-His-Pro-Phe	11.94	0.99		120	101	48	101
Bradykinin								
1	Phe-Arg	2.74	6.10		16	92	109	97
2	Phe	3.72	0.83		7	91	6	130
3	Arg-Pro-Gly-Phe-Ser-Pro	8.78	7.22		13	92	102	103
1	Oxytocin	10.92	1.22		9	107	113	n.d.
	Cys-Tyr-Ile-Gln-Asn-Cys-Pro							

the substitution of Phe for Ile³ and Arg for Leu⁸, was not apparently hydrolysed under these conditions.

Substance P. This was rapidly hydrolysed to give a complex pattern of products in the h.p.l.c. trace (results not shown). Phosphoramidon alone did not prevent hydrolysis, nor did Dip-F alone, but in combination these two inhibitors were successful in preserving substance P from attack, the substrate peak being 96% of that at zero time.

Evidence for hydrolysis within the Cys¹-Cys⁶ disulphide-linked rings of oxytocin and vasopressin

The experiments described above gave no indication of hydrolysis of any bonds other than the Pro⁷-Leu⁸ of oxytocin. An endopeptidase attack within the disulphide-linked rings might have been missed if it did not alter the retention time of the peptide products or if it proceeded at a very low rate. One of the peaks in the h.p.l.c. analysis of [Arg⁸]vasopressin after 24 h incubation (result not shown) had the same retention time as phenylalanine. Assuming this identity to be correct, it could have arisen only by an initial hydrolysis by endopeptidase-24.11 followed by an aminopeptidase attack. It was, however, a minor product (5.2% yield in 24 h), and therefore not detectable under the usual conditions. Nevertheless, the possibility of an attack within the ring was further investigated in two ways: first, by reduction and carboxymethylation of peptide(s) present after incubation and secondly by dansylation in order to reveal any new *N*-terminal residues generated by hydrolysis within the ring.

The h.p.l.c. traces after reduction and carboxymethylation of peptides produced by incubation with

endopeptidase-24.11 are shown in Fig. 3. The reagent blank (Fig. 3a) contained a large amount of material absorbing at 214 nm which was eluted early in the gradient. There were, however, additional peaks in the samples containing the peptides (Figs. 3b and 3c). Although three fractions corresponding to some of these peaks were subjected to amino acid analysis, they could not be positively identified or quantified except for peak 1, (Fig. 3b) which was oxytocin-(1-7)-peptide. In these experiments, 400 ng of purified endopeptidase-24.11 was used. The products after incubation with microvillar membranes under normal conditions (results not shown) also yielded additional peaks, some corresponding to those in Figs. 3(b) and 3(c), but were inadequate for further investigation. Incubation of each peptide (0.1–0.2 mM) with 200 ng of endopeptidase-24.11 for 2 and 17 h yielded dansyl derivatives corresponding to only monodansylcystine and *O*-dansyltyrosine, and dansyl-leucine in the case of oxytocin. Thus no evidence of attack within the ring was obtained under these conditions. However, incubation of 0.5 mM-oxytocin with 5 µg of microvillar membranes for 22 h did yield dansylisoleucine, confirming an attack at Tyr²-Ile³.

Hydrolysis of [2,3-³H]bradykinin by pig kidney microvillar membranes and purified enzymes

The experimental conditions in the foregoing experiments were designed to permit monitoring of the products of hydrolysis by their *A*₂₁₄ and the determination of the amino acid composition of the peak fractions. This required the use of 0.2–0.5 mM-peptides in the incubation, concentrations vastly in excess of those likely to occur physiologically. With this in mind, the mode of hydrolysis of [³H]bradykinin was investigated at

Fig. 3. Hydrolysis of oxytocin and [Arg⁸]vasopressin by endopeptidase-24.11: h.p.l.c. analyses after reduction and carboxymethylation

See the Experimental section for details. (a) Reagent blank (no enzyme or peptide); (b) oxytocin; (c) [Arg⁸]vasopressin. In this experiment, (b) and (c) were incubated for 2 h; the fractions containing peaks 1, 2 and 3 were collected and subjected to amino acid analyses.

Fig. 4. Hydrolysis of [2, 3-Pro^{3,4}-³H]bradykinin

See the experimental section for details. The incubations contained 20 nM-[³H]bradykinin. The recovery of radioactivity, relative to the control, is shown in parentheses. (a) Kidney microvilli, $t = 0$ (100%); (b) kidney microvilli, 15 min (79%); (c) kidney microvilli + 1 μ M-phosphoramidon, 15 min (77%); (d) kidney microvilli + 1 μ M-captopril, 15 min (92%); (e) purified PDP, 15 min (89%) (f) purified endopeptidase-24.11, 15 min (81%).

concentrations closer to physiological values (Fig. 4). The possibility of excessive losses due to binding to surfaces at this concentration (20 nM) was minimized by adding a sample derived from a parallel incubation of 100 μ M-bradykinin with microvilli or purified enzymes before the h.p.l.c. analysis. Recovery of radioactivity over the elution was in the range of 77–92%. The [³H]bradykinin eluted with the same retention time as that for unlabelled at zero time (Fig. 4a). After 15 min incubation with microvillar membranes, the [³H]peptide products were eluted at about 2.5 min and 9 min. The latter retention time was consistent with bradykinin-(1–7)-peptide (peptide 3, Table 3). The former is likely to be bradykinin-(1–4)-peptide, but this identity could not be directly determined. The elution pattern on incubation with endopeptidase-24.11 (Fig. 4f) gave the same two radioactive products. The addition of phosphoramidon abolished hydrolysis by microvilli (cf. Fig. 4c with Fig. 4a) and by endopeptidase-24.11 (results not shown). The main [³H]peptide produced by PDP had a retention time

of 8.5 min, with another minor product at 9 min. These are consistent with bradykinin-(1–5)- and -(1–7)-peptides from a sequential peptidyl dipeptidase attack. Captopril abolished PDP hydrolysis (results not shown), but had little effect on the pattern produced by microvilli (Fig. 4d), which closely resembled that obtained without inhibitors (Fig. 4b). Since the label was in proline residues 2 and 3, any fragments lacking these residues were not detected in the analysis.

DISCUSSION

Inhibitors

In trying to understand the way in which neuropeptides are metabolized by tissue fractions the experimenter is often hampered by some degree of ignorance of the enzymes that may be involved. This is not the case in respect of the peptidases of kidney microvilli, which are a very well characterized group of membrane enzymes. This background knowledge gives us some confidence in

attributing the attack to one or more of the peptidases known to be present in pig kidney microvilli. Such conclusions clearly depend on the specificity of the inhibitors employed. By using 1 μM -phosphoramidon and 1 μM -captopril, there was no cross-inhibition of PDP or endopeptidase-24.11 (Matsas *et al.*, 1984). Amastatin is very effective as an inhibitor of aminopeptidase N (Matsas *et al.*, 1985), but cannot be regarded as specific, since it also inhibits aminopeptidase A (Aoyagi *et al.*, 1978) and aminopeptidase W (N. S. Gee & A. J. Kenny, unpublished work). Rich *et al.* (1984) showed that preincubation of amastatin with aminopeptidase N lowered K_i values from 20 μM to 20 nM. We have observed a similar effect on IC_{50} values: a fall from 6 μM to 50 nM when Ala-NMec was used as substrate (results not shown). Of all the peptidases identified in kidney microvilli, DPP-IV is the only serine enzyme. It is rapidly and irreversibly inactivated by 0.1 mM-DipF and, with [^{32}P]Dip-F, it is the only microvillar protein to be labelled (Kenny *et al.*, 1976).

The four inhibitors used did not embrace all of the microvillar peptidases; in particular, two exopeptidases that remove residues attached to proline, namely aminopeptidase P and carboxypeptidase P, would escape inhibition. The latter enzyme was implicated in the inhibitor-resistant release of phenylalanine from angiotensins II and III, but there were no reasons to suspect that the other peptidase played a significant role in the metabolism of any of the peptides studied. However, in this context, it may be important to emphasize that our studies were with pig membranes and that rat and mouse microvilli contain additional endopeptidases not known to be inhibited by any of the reagents used.

Relative rates of hydrolysis

The peptides studied fell into three groups: those rapidly hydrolysed, those slowly hydrolysed and those resistant to hydrolysis. Most were in the first category, with half-lives ($t_{1/2}$) of 0.5 h or less. One, oxytocin, was hydrolysed slowly, whereas insulin was not attacked and [Arg⁸]vasopressin yielded degradation products only after incubation for 24 h. Since insulin B-chain was in the first category, it follows that the more highly ordered structure of insulin, including the disulphide bridges, as well as its secondary, tertiary and possibly quaternary structural aspects, confer an absolute resistance on the molecule.

Sites of attack

It must be emphasized that the experimental conditions were designed to reveal the initial steps in metabolizing

this group of neuropeptides. One should not conclude that the peptide products found at the end of the incubation period would persist on longer exposure to the microvilli *in vitro* or *in vivo*. Indeed, among the rapidly metabolized group of peptides, the pattern shown in Fig. 1 (at 15–30 min) changed markedly when incubation was for 3 or 4 h, by which time most of the material absorbing at 214 nm was eluted very early in the gradient, probably representing free phenylalanine and tyrosine or dipeptides.

The initial attack occurred at relatively few bonds and was mainly the result of endopeptidase action. A primary attack by an aminopeptidase was noted only in the case of angiotensin III (very probably the action of aminopeptidase N at the Arg–Val bond). Carboxypeptidase P attack at the Pro–Phe bond was observed with angiotensins II and III. DPP-IV also degraded the *N*-terminus of substance P, but in all peptides the principal attack was that attributable to endopeptidase-24.11. Even where the bond hydrolysed was susceptible to attack by either endopeptidase-24.11 or PDP, as in bradykinin, the results indicated that endopeptidase-24.11 predominated. The liberation of His–Leu from angiotensin I, typically the action of PDP, depended to a large extent on endopeptidase-24.11 action followed by an aminopeptidase attack.

Substance P was the only peptide in the group to reveal an effective attack by both endopeptidase-24.11 and DPP-IV. None of the other peptides possessed an *N*-terminal sequence that would permit hydrolysis by DPP-IV. The attack by endopeptidase-24.11 is at three bonds (Gln⁶–Phe⁷, Phe⁷–Phe⁸ and Gly⁹–Leu¹⁰) in the *C*-terminal sequence (Matsas *et al.*, 1983). In relation to most of the activities of substance P, the *C*-terminal sequence is of greater importance than that at the *N*-terminus (for a review, see Pernow, 1983). No contribution from PDP was observed.

The relatively feeble contribution to the hydrolysis of these peptides of PDP was not due to a deficiency of the enzyme. Pig kidney microvilli contained substantial PDP activity when determined with the use of a specific substrate, Bz–Gly–His–Leu. From the specific activities of the purified enzyme and membrane fraction, it appears to account for about 0.5–1.0% of the microvillar membrane protein (N. M. Hooper, personal communication). For comparison, endopeptidase-24.11 amounts to about 4% of the membrane protein (Fulcher & Kenny, 1983). In addition to this difference in the abundance of the two enzymes in the membrane, they also show important differences in their kinetic constants, e.g. for bradykinin and substance P (Table 4). K_m values are

Table 4. Comparison of kinetic constants for endopeptidase-24.11 and PDP

Data for (pig) endopeptidase-24.11 (EP-24.11) are from Matsas *et al.* (1984) and (human) PDP from Erdös & Skidgel (1985).

Constant	Substrate ...	Bradykinin		Substance P	
	Enzyme ...	EP-24.11	PDP	EP-24.11	PDP
K_m (μM)		92	0.4	32	25
$k_{\text{cat.}}$ (min^{-1})		6360	500	5060	225
$k_{\text{cat.}}/K_m$ ($\text{min}^{-1} \cdot \mu\text{M}^{-1}$)		69	1250	158	9

lower for PDP, strikingly so in the case of bradykinin, but k_{cat} values are an order of magnitude higher for endopeptidase-24.11. The concentrations used in our experiments were much higher than the K_m values for either substrate, and it is apparent that our observations are consistent with the differences in k_{cat} values.

These considerations led us to examine the hydrolysis of one peptide, bradykinin, at a concentration (20 nM) nearer to the K_m value with PDP. The plasma concentration of bradykinin has been reported to be 1–4 nM (Talamo *et al.*, 1979), but these values may be 'artefactually' high, the true concentration being about 3 pM (Nielsen *et al.*, 1983). However, the radioactive products produced by purified PDP under these conditions were not observed in the elution pattern after incubating with microvillar membranes. Captopril had very little effect, in contrast with phosphoramidon, which strongly inhibited hydrolysis of [^3H]bradykinin. Although quantitative comparisons are difficult in this experiment, the effects of the two inhibitors can be assessed from the disappearance of the substrate. Allowing for differences in recovery of radioactivity, phosphoramidon inhibited 50% and Captopril by 5%. Thus, even at a very low peptide concentration, the activity of endopeptidase-24.11 predominated over PDP.

Endopeptidase-24.11 was the only enzyme capable of initiating the hydrolysis of oxytocin and [Arg⁸]vasopressin. The former was slowly hydrolysed at the Pro⁷–Leu⁸ bond and both were attacked at an even lower rate within the ring, probably at the Tyr²–Ile³ and Tyr²–Phe³ bonds, though the yields of products from this mode of attack were exceedingly low, even under conditions designed to reveal them. In comparison with the hydrolysis of the much more susceptible peptides, oxytocin was a poor substrate and vasopressin was essentially resistant to hydrolysis.

General conclusions

Although kidney microvilli are unusually rich in their complement of peptidases, there was a marked selectivity in their action, both in regard to individual peptides and in respect of contributions of the different peptidases. The resistance to attack of insulin and [Arg⁸]vasopressin and the slow hydrolysis of oxytocin contrast with the rapid hydrolysis of the other peptides studied. The mode of attack on the susceptible peptides revealed a defined pattern in which the action of endopeptidase-24.11 was the major component. This endopeptidase is very widely distributed (Gee *et al.*, 1985), being especially abundant in lymph nodes (Bowes & Kenny, 1986), but also present in the brain (Matsas *et al.*, 1983; Relton *et al.*, 1983). In none of these locations do we yet have a clear idea of its physiological role, nor in these extra-renal sites do we have such a complete picture of the additional peptidases associated with endopeptidase-24.11 in the same membrane. Nevertheless, in brain striatal synaptic membranes, the hydrolysis of substance P was wholly dependent on endopeptidase-24.11 (Matsas *et al.*, 1983), the absence of any detectable DPP-IV activity contrasting with the attack by kidney microvillar membranes. Enkephalin metabolism by synaptic membranes depends on a combined aminopeptidase and endopeptidase-24.11 attack, with the former predominating. Some recent evidence on the co-localization of endopeptidase-24.11 with substance P supports the view that

it may have a physiological role in respect of this neuropeptide (Matsas *et al.*, 1986).

The kidney poses special questions in regard to the function of the brush-border peptidases, in particular the reasons why it is necessary to inactivate some, though not all, peptides which would be otherwise destined for excretion in the urine. The salvage of essential amino acids may be part of this need, but it seems much more likely that the intense peptidase activity at the luminal surface of the upper nephron may be a protection against non-physiological effects of some regulatory peptides as they become more concentrated in the distal and collecting tubules. A recent report has implicated endopeptidase-24.11 in the metabolism of kinins *in vivo* (Ura *et al.*, 1986).

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