The hydrolysis of α-human atrial natriuretic peptide by pig kidney microvillar membranes is initiated by endopeptidase-24.11

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α-Human atrial natriuretic peptide, a 28-amino-acid-residue peptide, was rapidly hydrolysed by pig kidney microvillar membranes *in vitro*, with a $t_{\frac{1}{2}}$ of 8 min, comparable with the rate observed with angiotensins II and III. The products of hydrolysis were analysed by h.p.l.c., the pattern obtained with membranes being similar to that with purified endopeptidase-24.11 (EC 3.4.24.11). No hydrolysis by peptidyl dipeptidase A (angiotensin I converting enzyme, EC 3.4.15.1) was observed. The contribution of the various microvillar membrane peptidases was assessed by including specific inhibitors. Phosphoramidon, an inhibitor of endopeptidase-24.11, caused 80–100% suppression of the products. Captopril and amastatin (inhibitors of peptidyl dipeptidase A and aminopeptidases respectively) had no significant effect. Hydrolysis at an undefined site within the disulphide-linked ring occurred rapidly, followed by hydrolysis at other sites, including the Ser^{25}–Phe^{26} bond.

INTRODUCTION

Atrial natriuretic factor (ANF) comprises a family of peptides with powerful natriuretic, diuretic and hypotensive activity, which are localized in storage granules of atrial muscle (de Bold, 1982). The low-M_r peptides all have a disulphide-linked loop of 17 amino acid residues with short *N*- and *C*-terminal extensions. The circulating form of ANF in the rat appears to be a 28-residue peptide (α-rat atrial natriuretic peptide, α-rANP; Flynn *et al.*, 1983), which is derived from a high-M_r, 152-amino-acid-residue precursor, rat pre-pro ANP (for review, see Flynn & Davies, 1985). Human atria also contain a 28-amino-acid peptide (α-human atrial natriuretic peptide or α-hANP; see Fig. 3) which differs from α-rANP in only one residue, Met-12 for Ile-12 (Kangawa & Matsuo, 1984).

The integrity of the disulphide bridge has been shown to be essential for activity. Reduction and carboxymethylation abolished natriuretic and diuretic actions (Misono *et al.*, 1984) and no inhibitory effect on aldosterone secretion from adrenal glomerulosa cells was observed after performic acid oxidation (Chartier *et al.*, 1984*a*). No structure–activity studies have been reported on the effect of peptidase attack within the disulphide-linked loop, but the linear fragment of α-rANP-(Phe⁸–Arg²⁷) had very little activity compared with α-rANP-(Ser⁵–Arg²⁷) (Schiller *et al.*, 1985). Removal of the *C*-terminal tripeptide (Phe-Arg-Tyr) from α-rANP-(Arg⁴–Tyr²⁸) markedly decreased, but did not abolish, natriuretic and vasorelaxant actions (Sugiyama *et al.*, 1984).

Very little is known about the biological inactivation of ANP-related peptides. Harris & Wilson (1985) have described an atrial enzyme activity which converts atriopeptin II [α-rANP-(Ser⁵–Arg²⁷)] to atriopeptin I [α-rANP-(Ser⁵-Ser²⁵)] by removal of the *C*-terminal dipeptide Phe-Arg. Olins *et al.* (1986) have reported that atriopeptin III [equivalent to α-rANP-(Ser⁵–Tyr²⁸)] is degraded by rabbit brush border membranes and that

hydrolysis occurred at the Ser–Ser, Cys–Phe and Ser–Phe peptide bonds. A very recent report (Murthy *et al.*, 1986) drew attention to the very rapid turnover of α-rANP *in vivo*, but did not define the mode of attack or the nature of the peptidases involved.

The brush border of the renal proximal tubule is very rich in peptidases. They are integral membrane proteins with their active sites facing the lumen of the tubule (for review, see Kenny & Maroux, 1982). We have recently reported that the hydrolysis of bradykinin, angiotensins I, II, III and oxytocin by pig kidney microvillar membranes is mainly initiated by endopeptidase-24.11 (Stephenson & Kenny, 1987). In the present paper we have extended these studies to α-hANP, a more complex peptide than those in the first series. Our results have shown that endopeptidase-24.11 plays a key role in the degradation of α-hANP by kidney microvilli.

EXPERIMENTAL

Peptides and inhibitors

α-hANP was purchased from Peptide Institute, Inc. (Scientific Marketing Associates, London N1 4RH, U.K.) and its purity checked by h.p.l.c. L-Isoleucylglycine, *N*-Boc-L-phenylalanine-*N*-hydroxysuccinimide ester and L-arginyl-L-tyrosine acetate were obtained from Bachem, Bubendorf, Switzerland. Di-isopropylethylamine was obtained from Aldrich Chemical Co. Phosphoramidon was obtained from Protein Research Foundation, Osaka, Japan. Captopril (SQ 14225) was a gift from the Squibb Institute for Medical Research, Princeton, NJ, U.S.A. Amastatin and [Met⁵]enkephalin-Arg⁶-Phe⁷ were purchased from Sigma Chemical Co.

Synthesis of Phe-Arg-Tyr

Boc-L-phenylalanine-*N*-hydroxysuccinimide ester (10 μmol), dissolved in 1 ml of *NN*-dimethylformamide, was added dropwise to 5 ml of a mixture of L-arginyl-L-tyrosine acetate (50 μmol) and di-isopropylethylamine (50 μmol) also dissolved in *NN*-dimethylformamide. The

Table 1. $t_{\frac{1}{2}}$ values of peptides incubated with kidney microvillar membranes

The incubations contained 50 μM-peptide and 2 μg of membrane protein. Samples were incubated at 37 °C for 0, 5, 7.5, 10, 15 and 20 min and the progress of hydrolysis monitored by the disappearance of the substrate. The progress curves approximated to first-order decay curves from which the $t_{\frac{1}{2}}$ values were computed.

Peptide	$t_{\frac{1}{2}}$ (min)
Bradykinin	3.3
Angiotensin III	7.7
α-hANP	8.2
Angiotensin II	10.0

mixture was stirred (20 h, 20 °C) dried under vacuum, dissolved in 0.5 ml of trifluoroacetic acid and again dried under vacuum. The mixture was analysed by h.p.l.c. as previously (Stephenson & Kenny, 1987) using a 4.05–45% (v/v) gradient of acetonitrile. The product, Phe-Arg-Tyr (retention time, 8.32 min), was well resolved from the reactants and its composition was established by amino acid analysis with the following result: arginine, 1.00; phenylalanine, 0.97; tyrosine, 0.92. The yield was 70%.

Incubation with microvillar membranes and purified peptidases

The preparation of pig kidney microvillar membranes, the purification of endopeptidase-24.11 (Gee *et al.*, 1983)

and peptidyl dipeptidase A (Bull *et al.*, 1985) and the conditions of the incubations with α-hANP were those described previously (Stephenson & Kenny, 1987). The incubation mixture (100 μl) contained either 2 μg of microvillar membrane protein or 100 ng of endopeptidase-24.11 or peptidyl dipeptidase A and either 50 or 100 μM-α-hANP.

Analysis of peptide products by h.p.l.c.

This was performed as previously (Stephenson & Kenny, 1987) with a μBondapak C18 column using a gradient of acetonitrile from 4.05% to 45% in 0.08% H_3PO_4, pH 2.5. The products were monitored at 214 nm. Peaks were identified by co-elution with authentic peptides (α-hANP and Phe-Arg-Tyr) or by amino acid analysis when the product was present in sufficient amount. This was performed in a Rank–Hilger Chromaspek J180 analyser, after 24 h hydrolysis at 110 °C with 6 M-HCl containing 0.3% phenol. Other methods were as described (Stephenson & Kenny, 1987).

RESULTS

Comparison of rates of hydrolysis of α-hANP and other neuropeptides by microvillar membranes

α-hANP, bradykinin and angiotensins II and III were incubated with microvillar membranes for various times and the $t_{\frac{1}{2}}$ values determined from the disappearance of the substrate peaks by h.p.l.c. analysis. All peptides were initially present at a concentration of 50 μM. The $t_{\frac{1}{2}}$ values are shown in Table 1. α-hANP was hydrolysed more slowly than bradykinin, but a rate intermediate between
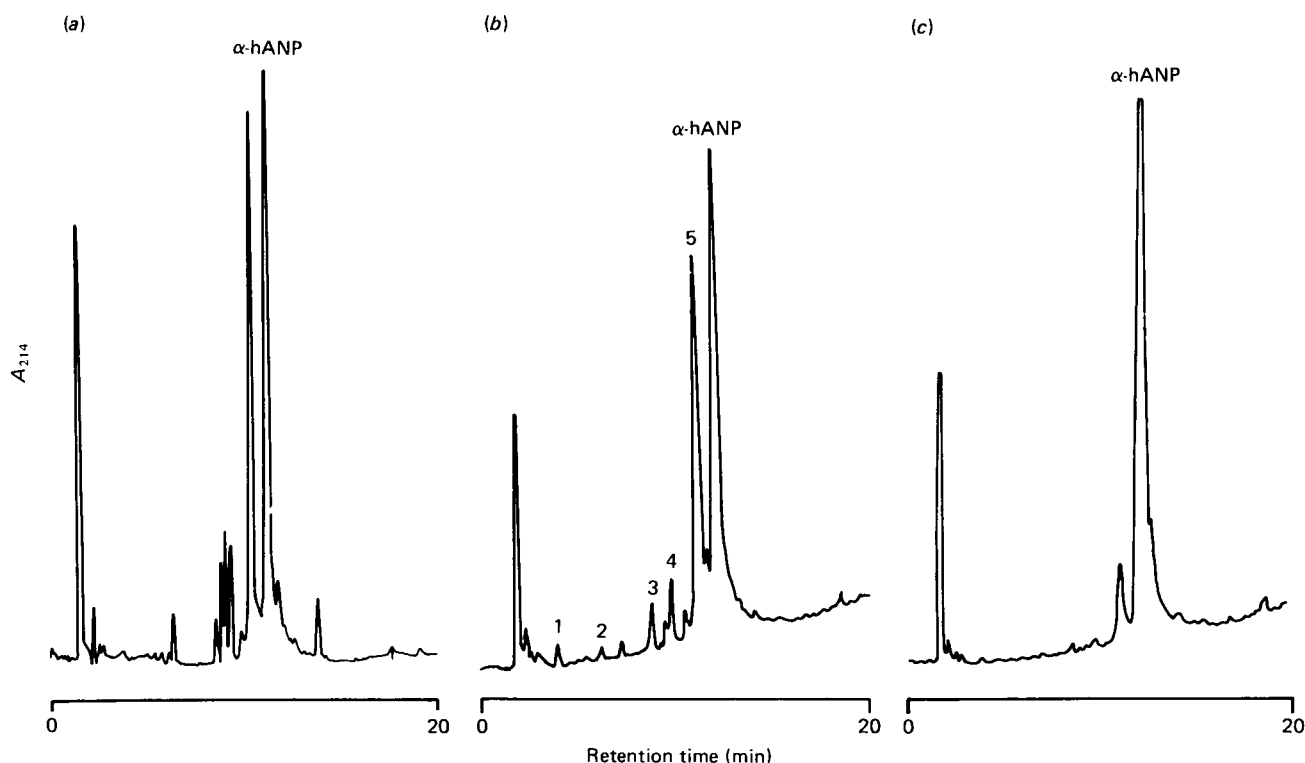
Fig. 1. Analyses of h.p.l.c. of products formed by incubation of α-hANP with microvillar membranes and endopeptidase 24.11

Incubation conditions were 50 μM-α-hANP for 15 min with: (a) 100 ng of endopeptidase-24.11, (b) 2 μg of microvillar membrane protein, or (c) as (b) with 1 μM-phosphoramidon. Elution was by a linear acetonitrile gradient from 4.05 to 45% (v/v) over 20 min. The peaks were numbered as in Table 3.
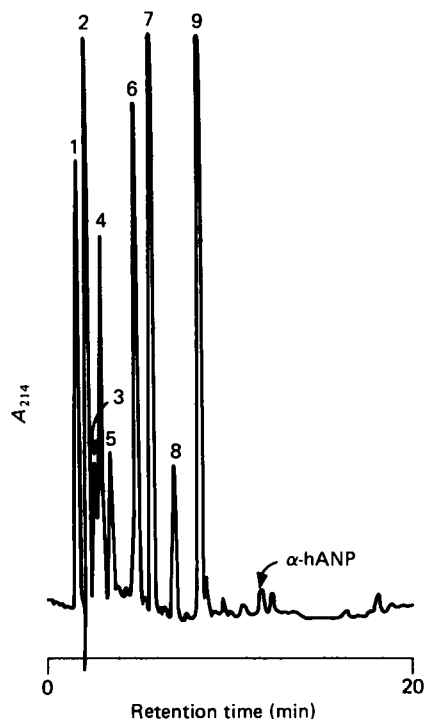
Fig. 2. Analysis by h.p.l.c. of products formed by incubation of α-hANP with endopeptidase-24.11

Incubation conditions were $100\,\mu$M-α-hANP, 24 h, 100 ng of endopeptidase-24.11. Elution was as in the legend to Fig. 1. The peaks are numbered as in Table 2.

Fig. 3. Sequence of α-hANP and identification of points of hydrolysis by endopeptidase-24.11

The peptides are numbered as in Fig. 2 and Table 2.

those of angiotensins II and III. The h.p.l.c. pattern after incubation of α-hANP for 24 h (not shown) was consistent with hydrolysis to free amino acids and dipeptides.

Hydrolysis of α-hANP by endopeptidase-24.11

Incubation of $50\,\mu$M-α-hANP with endopeptidase-24.11 for 15 min yielded one major product and several minor products (Fig. 1a). Prolonged incubation (24 h, $100\,\mu$M-α-hANP) yielded a somewhat different pattern, but gave products in sufficient quantity for collection and amino acid analysis (Fig. 2, Table 2) and this enabled the points of hydrolysis to be identified. The identity of peak 9 was also confirmed by co-elution with the marker peptide Phe-Arg-Tyr.
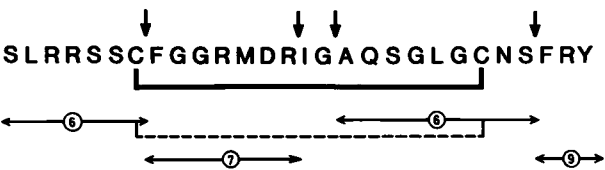
α-hANP was not hydrolysed by pig kidney angiotensin-converting enzyme

Angiotensin-converting enzyme (100 ng) was incubated with $50\,\mu$M-α-hANP for 15 min at 37 °C. No products were detected under these conditions. Prolonged incubation (24 h) with $100\,\mu$M-α-hANP also failed to yield any products. In contrast, incubation of the enzyme in the same conditions with $100\,\mu$M-[Met⁵]enkephalin-Arg⁶-Phe⁷ for 15 min yielded 3.0 nmol of Arg-Phe/min per pg of enzyme.

Hydrolysis of α-hANP by pig kidney microvillar membranes

Incubation of $50\,\mu$M-α-hANP with microvillar membranes for 15 min yielded one major and several minor peaks (Fig. 1b). The pattern of products was similar to that obtained with endopeptidase-24.11 (Fig. 1a). Experiments were also performed in the presence of selective inhibitors, the results of which are summarized in Table 3. The hydrolysis of α-hANP by microvillar membranes was almost completely inhibited by phosphoramidon (Fig. 1c) whereas captopril and amastatin had little or no effect.

DISCUSSION

Not all regulatory peptides are hydrolysed by the peptidases of the kidney brush border membrane. Vasopressin and insulin are essentially resistant to attack; oxytocin is slowly hydrolysed in comparison with substance P, bradykinin and the angiotensins (Stephenson & Kenny, 1987). α-hANP resembles the last group. In the present comparison, using a lower peptide concentration than previously, the half-life of α-hANP was about the same as that of angiotensin III. Both

Table 2. Identification of peptides released by incubation for 24 h with endopeptidase-24.11

Peaks (similar to those shown in Fig. 2) from several incubations were pooled and subjected to amino acid analysis. The yields were determined from the single incubation shown in Fig. 2, which contained 10 nmol of α-hANP. Peak 1 contained no peptide product. Resolution of the peptides in peaks 2, 3, 4, 5 was inadequate for interpretation of the analyses of the material in the collected fraction. Peak 8 contained insufficient peptide for analysis.

Peak no.	Retention time (min)	Yield (nmol)	Amino acid composition (molar ratio in parentheses)	Identity of fragment of α-hANP
6	4.94	3.6	Ala (1.0), Asp (1.8), Ser (4.0), Glu (1.4), Gly (2.1), Leu (1.2), Arg (1.2)	(1–7)–(17–25)
7	5.72	7.4	Phe (1.0), Asp (1.7), Gly (2.0), Arg (2.1)	8–14
9	8.32	7.1	Tyr (1.0), Phe (1.1), Arg (1.1)	26–28

Table 3. Effect of inhibitors on the hydrolyses of α-hANP by microvillar membranes

The incubation time was 15 min and contained 5 nmol of α-hANP. The peaks are numbered as in Fig. 1(a). The products were, where possible, identified by amino acid analysis. Peak 5 had an amino acid analysis identical with that of the substrate α-hANP (retention time 11.52 min) and is designated α-hANP', indicating that an unidentified peptide bond within the loop had been hydrolysed. The amino acid compositions for α-hANP and α-hANP' (the latter in parentheses) were Ile, 1.0 (1.0); Asp+Ser, 7.1 (7.3); Glu 1.6 (1.4); Gly 5.7 (5.5); Ala, 1.4 (1.1); Leu, 2.0 (2.0); Tyr 1.1 (1.0); Phe 2.1 (2.2); Arg 5.2 (4.8). Met and Cys were not quantified. Abbreviation: n.d., not determined.

Peak no.	Retention time (min)	Peptide	Yield (nmol)	Peptide remaining (%) relative to that in absence of inhibitors		
				Phosphoramidon (1 μM)	Captopril (1 μM)	Amastatin (1 μM)
1	3.80	F	0.19	0	82	93
2	5.92	FGGRMDR	0.13	0	100	100
3	8.52	FRY	0.27	0	100	117
4	9.52	n.d.	n.d.	0	86	81
5	10.76	α-hANP'	1.1	20	97	98

oxytocin and α-hANP contain disulphide linked loops of 6 and 17 amino acid residues respectively. The attack of endopeptidase-24.11 within the ring of oxytocin (at Tyr^2–Ile^3) was extremely slow, even in relation to the hydrolysis of the susceptible Pro^7–Leu^8 bond of the tail (Stephenson & Kenny, 1987). The larger ring of α-hANP permitted more rapid attack by endopeptidase-24.11. Peak 5 (Table 3, Fig. 1) referred to as α-hANP', had an amino acid composition identical with the substrate, but was fully resolved from it by h.p.l.c. The only interpretation is that one of the peptide bonds within the loop has been split. Since this was the major product (yield 22%) at 15 min with either endopeptidase-24.11 or microvillar membranes, the initial attack was at a site within the loop. The possibility that α-hANP' was an oxidized form can be excluded by the strong inhibition observed by 1 μM-phophoramidon. Hydrolysis of the Ser^{25}–Phe^{26} bond yielding the C-terminal fragment Phe-Arg-Tyr (yield 5% in 15 min) took place more slowly. Of the three points of hydrolysis shown to occur within the loop (Cys^7–Phe^8, Arg^{14}–Ile^{15} and Gly^{16}–Ala^{17}) we cannot say which is the preferred site. A marker for the dipeptide Ile^{15}–Gly^{16} eluted with a retention time of 2.5 min and could not be identified among the other fragments eluting in this region of the gradient. It is noteworthy that endopeptidase-24.11 cleaved adjacent to an alanine residue (Ala-17). This specificity had not been revealed by the other neuropeptides studied previously (Matsas et al., 1983, 1984a,b) but was confirmed by testing Ala-Ala-Ala-Ala as a substrate. This tetrapeptide (1 mM) was efficiently hydrolysed to Ala-Ala (30 μmol/min per mg of protein). Trialanine was not attacked. The sites of cleavage by endopeptidase-24.11 that we have identified in these studies are shown in Fig. 3. All the predicted bonds were found to be hydrolysed, with the exception of Gly^{20}–Leu^{21}; it is possible that fragments from such an attack were among the unidentified peaks in Fig. 2.

A surprising result was the failure of peptidyl dipeptidase A to hydrolyse α-hANP. The expected dipeptide product, Arg-Tyr (retention time, 2.8 min), was not formed even on prolonged incubation, nor was any evidence of an endopeptidase attack by this enzyme observed.

The pattern of products released by microvillar membranes resembled that produced by endopeptidase-24.11 (Figs. 1a and 1b). The formation of α-hANP' and the release of the C-terminal tripeptide Phe-Arg-Tyr were detected after 15 min incubation in both experiments. Free phenylalanine was released by the membranes, the result of secondary attack by aminopeptidase action, not observed with the enzyme incubation. The crucial contribution of endopeptidase-24.11 was confirmed by the effect of phosphoramidon on the formation of the products by microvillar membranes. All the products were strongly suppressed by 1 μM-phosphoramidon, most to undetectable levels and one to 20% of the uninhibited value (Table 3). Inhibitors of peptidyl dipeptidase A and aminopeptidases (captopriil and amastatin) had no significant effect on the hydrolysis of α-hANP.

The degradation of α-hANP by the brush border peptidases resembles that of substance P, bradykinin, the angiotensins and oxytocin in being predominantly initiated by endopeptidase-24.11. The points of attack revealed in this study seem likely to inactivate the peptide. The removal of the C-terminal tripeptide greatly reduces activity (Sugiyama et al., 1984) and, since the integrity of the disulphide bridge is essential (Chartier et al., 1984a), one may surmise that hydrolysis of peptide bonds within the loop would also inactivate. It seems likely therefore that passage of α-hANP through the proximal renal tubule in vivo will suffice to inactivate the peptide. Receptors for α-hANP and sites of action on Na$^+$ transport have been demonstrated in the collecting ducts and tubules (De Léan et al., 1985; Koseki et al., 1986; Briggs et al., 1982; Sonnenberg et al., 1986), but if these receptors in the distal nephron are physiologically important in the action of α-hANP they should be accessible from the contraluminal side. α-hANP has other postulated sites of action, e.g. in renal glomerular membranes (Lynch et al., 1986) where ANP may induce an increase in glomerular filtration rate (Maack et al., 1984), in the adrenal cortex causing inhibition of the release of aldosterone from the glomerulosa cells (Chartier et al., 1984b) as well as in the vascular smooth muscle where it is a vasorelaxant (Currie et al., 1983; O'Donnell et al., 1985) and in the brain (Quirion et al.,

1984, 1986). Since endopeptidase-24.11 has a widespread distribution on plasma membranes in many tissues and organs (Matsas *et al.*, 1983; Gee *et al.*, 1985; Bowes & Kenny, 1986), the inactivation of α-hANP by this enzyme may well occur at many other sites. It is particularly interesting that in the adrenal cortex, endopeptidase-24.11 is discretely localized to the glomerulosa cells (M. A. Bowes & A. J. Kenny, unpublished work) where it may serve to inactivate this and other peptide signals.

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