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Hydrolysis of human and pig brain natriuretic peptides, urodilatin, C-type natriuretic peptide and some C-receptor ligands by endopeptidase-24.11

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Endopeptidase-24.11 (E-24.11, EC 3.4.24.11) is widely believed to play a physiological role in metabolizing atrial natriuretic peptide (ANP). Since the discovery of ANP, new natriuretic peptides have been isolated and other peptides synthesized as receptor ligands. The hydrolysis *in vitro* of six related peptides by the endopeptidase has been studied, mainly by h.p.l.c. The initial attack on the 32-residue form of pig brain natriuretic peptide (pBNP-32) was shown to be at the Ser[20]–Leu[21] bond, as had been previously shown for the 26-residue form. In contrast, human brain natriuretic peptide-32 (hBNP-32), which differs in ten residues from pBNP-32, was attacked first at the Met[4]–Val[5] bond, releasing the N-terminal tetrapeptide, and only later at bonds within the ring: at Arg[17]–Ile[18] and subsequently at four other sites. Urodilatin, which has a four-residue extension at the N-terminus compared with α-human atrial natriuretic peptide-28 (α-hANP), was degraded at about half the rate of the latter, though the C-terminal Phe-Arg-Tyr was released at the same rate. The 22-residue C-type natriuretic peptide was hydrolysed more rapidly than α-hANP, as were two C-receptor ligands (peptides with deletions within the ring): C-ANP_{4-23} ($rANP_{4-23}$ des-Gln[18],Ser[19],Gly[20],Leu[21],Gly[22]) and SC 46542 ($hANP_{5-28}$ des-Phe[8],Gly[9],Ala[17],Gln[18]). Angiotensin-converting enzyme failed to hydrolyse pBNP-32, hBNP-32 or ^{125}I-rat (r) ANP, even after prolonged incubation. K_m and k_{cat} values were determined for the hydrolysis of α-hANP, porcine BNP-26, porcine BNP-32 and ^{125}I-rANP by E-24.11. K_i values were determined for six peptides, α-hANP, urodilatin, hBNP-32, C-type natriuretic peptide (CNP), SC 46542 and C-type natriuretic peptide (C-ANP_{4-23}), in radiometric assays of E-24.11 with either [^{125}I] insulin B chain or [^{125}I] rANP as substrate. The K_i values (2.5–13 μM) for CNP were the lowest of any of the group, whereas those for hBNP-32 (151–172 μM) were the highest. The physiological significance of these results is discussed, especially in regard to the relative resistance of hBNP-32 to attack and the ability of the C-receptor ligands to compete with natriuretic peptides for hydrolysis by E-24.11.

INTRODUCTION

A decade has now passed since atrial natriuretic peptide (ANP_{99-126}) was first isolated and characterized from cardiac atria. During this time, other structurally related peptides with natriuretic–diuretic and vasorelaxant activities have been found, including brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). In the human the main forms are: α-human (h) ANP (28 residues) (Kangawa and Matsuo, 1984), hBNP-32 (32 residues) (Sudoh et al., 1988, 1989), hCNP-22 (22 residues) (Sudoh et al., 1990; Komatsu et al., 1991) with another 53-residue form also in brain (Minamino et al., 1990) and urodilatin (32 residues). Urodilatin was first isolated from human urine and is identical with α-hANP, with four additional residues at the N-terminus (Schulz-Knappe et al., 1988). All have a disulphide-linked loop of 17 amino acids, the integrity of which is essential for biological activity. ANP is most abundant in cardiac atria, but is also found in several other tissues, including the brain, adrenal gland and testis. BNP is present in brain and heart, the highest concentration being in the atrium, though a greater quantity is present in the ventricles (Ogawa et al., 1991) and, in cardiac failure, BNP is the principal natriuretic peptide in plasma (Mukoyama et al., 1990, 1991a; Hosoda et al., 1991). CNP is essentially confined to the nervous system, with only small amounts in intestine and kidney (Komatsu et al., 1991; Ueda et al., 1991). The sequences of BNP from different species vary more widely than is the case for ANP.

Endopeptidase-24.11 (E-24.11) hydrolyses α-hANP, the initial attack opening the ring by cleavage at the Cys[7]–Phe[8] bond, with later points of attack at four more sites within the ring and others involving the head and tail sequences (Stephenson and Kenny, 1987a; Vanneste et al., 1988; Kenny and Stephenson, 1988). pBNP-26 is attacked differently, initially at Ser[14]–Leu[15] and subsequently at six other bonds, the hydrolysis of the Cys–Phe bond being a relatively late event (Vogt-Schaden et al., 1989; Bourne and Kenny, 1990). Urodilatin has been reported to be resistant to hydrolysis by dog kidney membranes (which contain E-24.11) (Gagelmann et al., 1988), but no information is available on the susceptibility of CNP to hydrolysis by E-24.11 and little on the susceptibility of pBNP-32 and hBNP-32, except for one report (Norman et al., 1991) indicating hydrolysis at Arg–Leu and Ser–Leu in pBNP, whereas hBNP was cleaved at Pro–Lys, Met–Val (in the N-terminal extension) and at Arg–Ile within the ring. Neither α-hANP or pBNP-26 is attacked by angiotensin converting enzyme, even after prolonged incubation (Stephenson and Kenny, 1987a; Bourne and Kenny, 1990).

A second mechanism for the clearance of these peptides *in vivo* has also been proposed, namely receptor-mediated endocytosis. ANP receptors have been identified in many tissues and are of two main types: one is a transmembrane protein capable of binding ligands at the extracytoplasmic domain and generating cyclic GMP at the cytoplasmic domain (Kuno et al., 1986; Chinkers et al., 1989). This class is therefore biologically active. Another type, the C-receptor, lacks the cyclase domain and is generally considered to be biologically 'silent' (Maack et al., 1987; Fuller et al., 1988). Whereas the first type of receptor displays rigorous criteria for ligand binding, C-receptors are much less demanding as regards structure, even tolerating D-

Abbreviations used: ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; α-hANP, human 28-residue peptide; p, porcine; r, rat; E-24.11, endopeptidase-24.11 (EC 3.4.24.11); ACE, peptidyl dipeptidase A (angiotensin converting enzyme, EC 3.4.15.1); SC 46542, des-Phe[8],Gly[9],Ala[17],Gln[18]-human atrial natriuretic peptide 5–28.

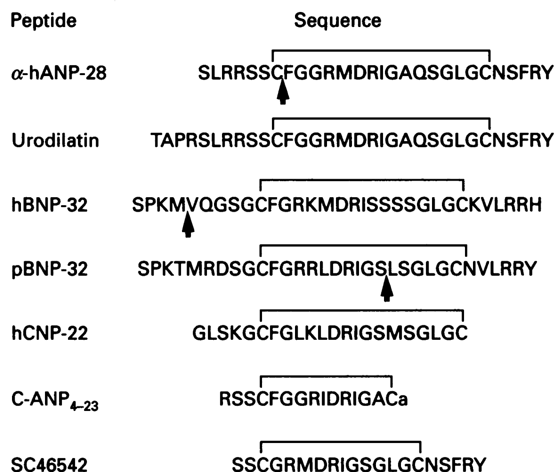

Figure 1 Sequences of the peptides studied

'a' Indicates an amidated C-terminus. Arrows indicate the initial points of attack, these data being from Kenny and Stephenson (1988), Bourne and Kenny (1990) and the present study.

amino acid substitutions (Scarborough et al., 1988). They outnumber the other types (Scarborough et al., 1986; Fuller et al., 1988) and have been shown to internalize ANP (Johnson et al., 1990; Rathinavelu and Isom, 1991), thus performing a clearance role. Analogues of ANP, with residues deleted from within the ring, bind selectively with high efficiency to C-receptors. Examples are C-ANP_{4-23} (C-ligand, des-Gln^{18},Ser^{19},Gly^{20},Leu^{21},Gly^{22}-$rANP_{4-23}$) and SC 46542 (des-Phe^{8},Gly^{9},Ala^{17},Gln^{18}-$hANP_{5-28}$). When C-ANP_{4-23} was administered *in vivo* an increase in plasma ANP levels was observed (Maack et al., 1987). However, C-receptor ligands may also be substrates for E-24.11 and might therefore have the potential to compete for hydrolysis with active natriuretic peptides. There is one report that purified E-24.11 degraded C-ANP_{4-23} at a rate of 0.7 μmol/min per mg of enzyme, initiating the attack at the Cys–Phe bond (Seymour et al., 1991), but otherwise little is known about their effectiveness as substrates compared with the natural natriuretic peptides.

In the present paper we have attempted a comprehensive study of the hydrolysis by E-24.11 of a group of natriuretic peptides (α-hANP, pBNP-32, hBNP-32, hCNP and urodilatin) and two analogues with C-receptor binding properties (SC 46542 and C-ANP_{4-23}). The sequences of the peptides are shown in Figure 1.

EXPERIMENTAL

Materials

Peptides

The natriuretic peptides and C-ligand were purchased from Sigma Chemical Co. ^{125}I-$rANP_{1-28}$ (3-[^{125}I]iodotyrosyl^{28} rat ANP) (Amersham Life Sciences) was kindly given by Dr. Michael Wurl (Solvay Pharma Deutschland) and SC 46542 was a gift from Dr. Martin Wilkins (Department of Clinical Pharmacology, Royal Postgraduate Medical School, London W12 0NN, U.K.)

Enzymes

E-24.11 and peptidyl dipeptidase A (angiotensin converting enzyme, ACE) were purified as previously described (Bourne and Kenny, 1990). The incubations of peptides with the peptidases, the sources and usage of the inhibitors, the h.p.l.c. analyses and identification of the peptide products were identical to those previously described (Bourne and Kenny, 1990).

Methods

Determination of kinetic constants

Two approaches were used. In the conventional method, yielding K_m and $k_{cat.}$ values, initial rates of hydrolysis were determined over a range of substrate concentrations. The times of incubation, amounts of enzyme and the range of concentrations of the substrates were: α-hANP, 12 min, 75 ng, 6–270 μM; pBNP-26, 12 min, 30 ng, 8.5–285 μM; pBNP-32, 15 min, 75 ng, 6–335 μM. The extent of hydrolysis of each peptide (mean of all concentrations) was: α-hANP, 26 %; pBNP-26, 20 %; pBNP-32, 15 %; however, at the lowest concentrations of the first two, hydrolysis exceeded 25 %.

In the second approach radioiodinated substrates were used and K_i values of the peptides determined as competitive inhibitors, rates of hydrolysis being determined by the release of trichloroacetic acid-soluble radioactive fragments. In one series ^{125}I-insulin B chain (0.1 mCi/nmol; concentration range 8–40 μM) was the substrate (Fulcher and Kenny, 1983), incubated for 15 min with 40–80 ng of E-24.11, with or without peptide as inhibitor. In the second series ^{125}I-$rANP_{1-28}$ was the substrate. The assay was set up as follows: 5 μCi of radioactive rANP was mixed with unlabelled rANP to give a stock solution, in assay buffer, of 600 μM rANP; assays were set up containing 8–150 μM rANP, with 5–40 ng of E-24.11 (so as to limit hydrolysis to less than 20 %), incubated for 30 min with and without peptide as inhibitor. The unlabelled peptides were present at appropriate concentrations from 25 μM to 200 μM; two concentrations were usually selected for each experiment. The kinetic constants were computed using a program (written by Dr. D. G. Herries of this Department) run on an Amdahl 5860 computer, such that greater weights were assigned to the higher substrate concentrations.

RESULTS

Mode of hydrolysis of pig and human BNP

Hydrolysis of pBNP-32

This peptide has a six-residue extension at the N-terminus compared with pBNP-26. The initial cleavage was defined in a time course, part of which is shown in Figure 2(a), being the h.p.l.c. profile after 25 min incubation of 5 nmol of pBNP-32 with 100 ng of E-24.11. Peak 1 indicates the initial product; the fraction containing this peptide was collected and the amino acid sequence determined. Two N-terminal residues (Ser, Leu) were detected, and pairs of amino acids were identified in each of the first 12 cycles with single residues in cycles 13–32. The results are therefore consistent with an initial attack within the ring at Ser^{20}–Leu^{21}.

Hydrolysis of hBNP-32

The sequence of this peptide differs in ten residues from that of the pig homologue, including the presence of Ser in place of Leu^{21}. The h.p.l.c. profiles at 60 min and 24 h, from a time-course experiment, are shown in Figures 2(b) and 2(c). The first illustrates the mode of the initial attack: peaks 1 and 5 being the first to appear, followed by peaks 2 and 4. The corresponding fractions were collected and subjected to amino acid sequencing, the results of which are in Table 1. The initial attack was in the

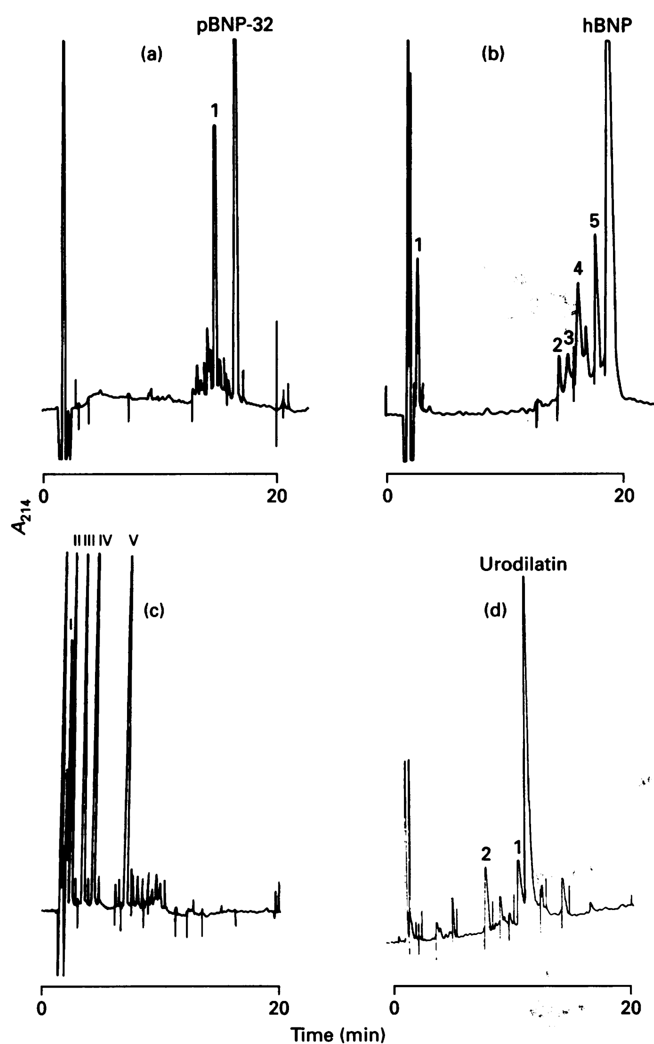

Figure 2 H.p.l.c. elution profiles of products of hydrolysis by E-24.11 of pBNP-32, hBNP-32 and urodilatin

See the Materials and methods section for details. (**a**) pBNP-32, after 25 min incubation; (**b, c**) hBNP-32 after 60 min and 24 h incubation; (**d**) urodilatin after 40 min incubation.

Table 1 Peptide products identified after incubation of hBNP-32 with E-24.11 for 60 min

An 8 nmol portion of hBNP32 was incubated with 160 ng of E-24.11 at 37 °C (vol. 0.16 ml). Yields were calculated from the peak areas (Stephenson and Kenny, 1987b). Abbreviation: n.d., not determined because more than one peptide present. Peaks are numbered as in Figure 2(b). Peak 3 was not identified. In this Table and Table 2 the one-letter amino acid code is used.

Peak	Retention time (min)	Identity of fragment	Bond(s) hydrolysed	Yield (nmol)
1	2.7	1–4 (trace of 29–32)	M^4–V^5; (V^{28}–L^{29})	n.d.
2	14.7	5–17; 18–32	M^4–V^5; R^{17}–I^{18}	0.7
4	16.4	1–17; 18–32	R^{17}–I^{18}	1.1
5	17.9	5–32	M^4–V^5	0.7
hBNP-32	18.7	–	–	2.7

head region, at Met⁴–Val⁵, releasing the N-terminal fragment (peak 1, Ser-Pro-Lys-Met) from the rest of the peptide, with the ring intact (peak 5). Subsequently hydrolysis occurred at

Table 2 Peptide products identified after incubation of hBNP-32 with E-24.11 for 24 h

An 8 nmol portion of hBNP-32 was incubated with 160 ng of E-24.11 at 37 °C (vol. 0.16 ml). Yields were calculated from the peak areas (Stephenson and Kenny, 1987b). Abbreviation: n.d., not determined because more than one peptide present. Peaks are numbered as in Figure 2(c).

Peak	Retention time (min)	Identity of fragment	Bonds hydrolysed	Yield (nmol)
I	2.4	15–17; 18–23	K^{14}–M^{15}, R^{17}–I^{18}; G^{23}–L^{24}	n.d.
II	2.7	15–17; 29–32	V^{28}–L^{29}; K^{14}–M^{15}; R^{17}–I^{18}	n.d.
III	3.8	11–14	C^{10}–F^{11}; K^{14}–M^{15}	4.7
IV	4.7	1–4	M^4–V^5	7.0
V	7.5	5–10; 24–28	M^4–V^5; C^{10}–F^{11} G^{23}–L^{24}; V^{28}–L^{29}	5.7

Arg¹⁷–Ile¹⁸, opening the ring and generating peaks 2 and 4. Some hydrolysis also occurred in the tail, releasing a very small quantity of the C-terminal peptide, Leu-Arg-Arg-His, which was co-eluted in peak 1.

The products at 24 h represent the final fragments generated by E-24.11 (Figure 2c), which were also identified by sequencing and are shown in Table 2. The bonds shown to be cleaved during this prolonged incubation were: Met^4–Val^5, Cys^{10}–Phe^{11}, Lys^{14}–Met^{15}, Arg^{17}–Ile^{18}, Gly^{23}–Leu^{24} and Val^{28}–Leu^{29}.

Hydrolysis of urodilatin

The hydrolysis of urodilatin was monitored over a period of 2 h. The initial product (retention time 10.9 min, compared with 11.5 min for the substrate) was just detectable at 10 min and reached a maximum at 60 min; it is peak 1 in Figure 2(d), which shows the profile after 40 min incubation. It was not analysed further, but, from its retention time relative to the substrate, it is probable that it corresponds to α-hANP′, the initial product of α-hANP hydrolysis, by cleavage at the Cys–Phe bond. The C-terminal tripeptide, Phe-Arg-Tyr, was identified by a synthetic marker (Stephenson and Kenny, 1987a) as peak 2 (retention time 8.1 min). The relative rates of hydrolysis of urodilatin and α-hANP are shown in Figure 3(a). In this study, 5 nmol of peptide was incubated at 37 °C with 100 ng of E-24.11 (vol. 100 μl) and the progress assessed by areas of the peaks corresponding to the substrate, primary product and C-terminal tripeptide. Under these conditions α-hANP and urodilatin were hydrolysed in pseudo-first-order manner, with $t_{0.5}$ values of 24 and 54 min respectively. The rise and fall of the primary products ANP′ and urodilatin′ are shown in Figure 3b, revealing that ANP′ accumulated at about twice the rate of urodilatin′. On the other hand, the generation of Phe-Arg-Tyr occurred at similar rates from the two peptides (Figure 3c).

Comparison of rates of hydrolysis by E-24.11 of α-hANP, hBNP, CNP, C-ANP$_{4-23}$ and SC 46542

Hydrolysis of each peptide was studied under similar conditions (50 μM peptide), and the curves are shown in Figure 4. It is notable that hBNP-32 is hydrolysed relatively slowly ($t_{0.5}$, 2 h, Figure 4a), compared with the other four peptides, which were rapidly degraded. The $t_{0.5}$ value for α-hANP in this experiment was 8 min (from the logarithmic plots in Figure 4b), whereas the other three peptides were hydrolysed even more rapidly, the $t_{0.5}$

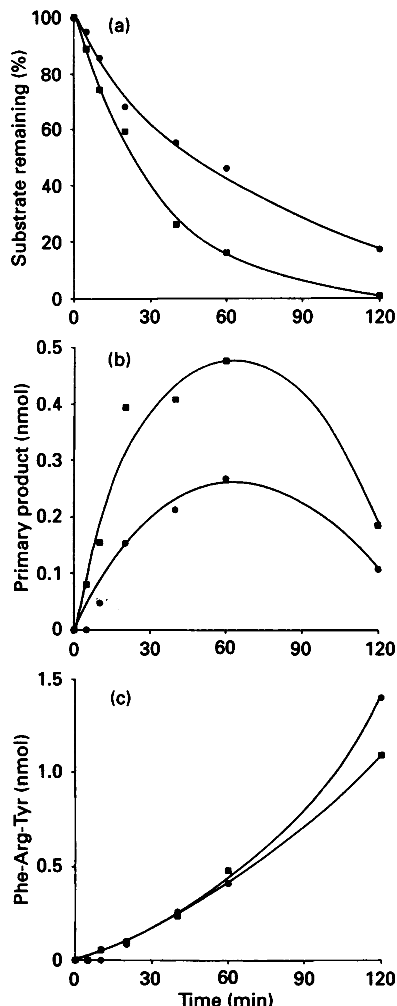

Figure 3 Time course of hydrolysis of α-hANP and urodilatin by E-24.11

See the Materials and methods section for details. ●, urodilatin; ■, α-hANP. (a) Substrate remaining; (b) primary products (α-hANP′ and urodilatin′); (c) C-terminal tripeptide (Phe-Arg-Tyr).

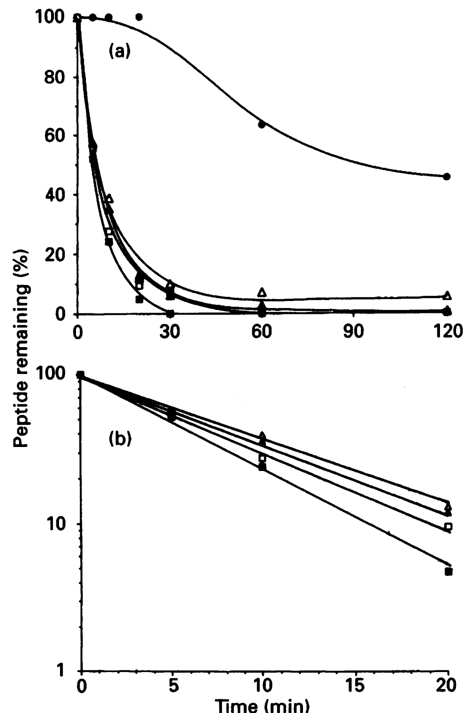

Figure 4 Time courses of hydrolysis of α-hANP, hBNP-32, CNP, C-ANP$_{4-23}$ and SC 46542 by E-24.11

All peptides were at 50 μM. See the Materials and methods section for details. (a) hydrolysis of peptides over 2 h period; (b) same data for four rapidly hydrolysed peptides over a 20 min period (logarithmic scale). Symbols: ●, hBNP-32; ▽, α-hANP; ■, CNP; □, C-ANP$_{4-23}$ and ▲, SC 46542.

values (relative to α-hANP, = 1.0) were: CNP, 0.6; C-ligand, 0.75 and SC 46542, 0.9.

Kinetic constants for the hydrolysis of natriuretic peptides and C-receptor ligands

Since these peptides can be hydrolysed at multiple sites, there is more than one way of assessing the initial rate from the h.p.l.c. analyses: either as disappearance of substrate or appearance of the initial or other product. All have drawbacks: if hydrolysis is limited to, say, 20 %, the peak area of the substrate decreases by a rather small amount and may be difficult to quantify accurately; the initial product has a transient existence, being degraded to other products, whereas an end product, e.g. the C-terminal tripeptide of α-hANP, can be generated directly from the substrate or indirectly from one or more fragments. The kinetic constants, K_m and $k_{cat.}$ assessed by this approach, for α-hANP, pBNP-26 and pBNP-32 are shown in Table 3. The K_m values for α-hANP and pBNP-32 were approx. 100 μM and that for pBNP-26 was < 20 μM, whereas α-hANP had the highest $k_{cat.}$ of the group.

Table 3 Kinetic constants of αhANP, pBNP-26 and pBNP-32

The values were calculated from disappearance of the substrate, assessed by peak area after h.p.l.c. When calculated from the areas of the initial product peaks, K_m values were slightly different (shown in parentheses). The conditions for each peptide are described in the Experimental section. Rates for the enzyme sample used in this experiment ($k_{cat.}$ 2162 min^{-1} for the hydrolysis of [D-Ala^2,Leu^5]enkephalin) were normalized to the value (5645 min^{-1}) reported by Matsas et al. (1984). $k_{cat.}$ values assumed one active site per subunit of M_r 90000. Values are means ± S.E.M.

Peptide	K_m (μM)	$V_{max.}$ (μmol/min per mg)	$k_{cat.}$ (min^{-1})	$k_{cat.}/K_m$ (min^{-1}·μM^{-1})
α-hANP	121 ± 14 (86 ± 9)	18.8 ± 1.0	1691	14.1
pBNP-26	15.3 ± 3.7 (17.7 ± 1.7)	4.2 ± 0.09	379	24.7
pBNP-32	111 ± 11 (99 ± 12)	9.4 ± 0.5	611	7.6

K_i values were determined for peptides as competitive inhibitors in radiometric assays of E-24.11 with either ^{125}I-insulin B chain or ^{125}I-rANP as substrates. The K_m values for the two labelled substrates (Table 4) were similar: insulin B chain (n = 11), 15.0 ± 2.3 μM and rANP (n = 7), 11.4 ± 3 μM, but the $k_{cat.}$ values were 10-fold different, rANP being the better substrate. The K_i values for six peptides (Table 5) indicate that CNP was the most potent (2.5–18 μM) followed by SC 46542, α-

Table 4 Kinetic constants for the hydrolysis of ^{125}I-insulin B-chain and ^{125}I-rANP.

For $k_{cat.}$ values, one active site per subunit of M_r 90000 was assumed. Values are means ± S.E.M.

Parameter	^{125}I-insulin B-chain ($n = 11$)	^{125}I-rANP ($n = 7$)
K_m (μM)	15.0 ± 2.3	11.4 ± 3.0
$V_{max.}$ (μmol/min per mg)	0.58 ± 0.036	5.58 ± 0.70
$k_{cat.}$ (min^{-1})	52	502
$k_{cat.}/K_m$ (min^{-1} · μM^{-1})	3.5	44.1

Table 5 K_i values of some natriuretic peptides and C-receptor ligands, using ^{125}I-insulin B-chain and ^{125}I-rANP as the assay substrates

See the Materials and methods section for details. Data were obtained from single experiments, usually with two concentrations of the inhibitor peptide, except for those marked *, which are means for two experiments. Values are means ± S.E.M.

	K_i (μM)	
Peptide Assay substrate...	^{125}I-insulin B-chain	^{125}I-rANP
α-hANP	26.4 ± 8.0*	33.6 ± 13
Urodilatin	31.1 ± 9	61.6 ± 33
hBNP-32	172 ± 48*	151 ± 80
CNP	13.1 ± 2.0*	2.47 ± 0.93
SC 46542	25.2 ± 4.8	2.7 ± 0.63
C-ANP$_{4-23}$	82.1 ± 19*	30.9 ± 13*

hANP, urodilatin, C-ANP$_{4-23}$ and hBNP-32, the last named being a very poor inhibitor (K_i > 150 μM) in both assays.

Effect of ACE on pBNP-26, pBNP-32 and ^{125}I-rANP

Incubation with 100 ng of purified ACE for 24 h at 37 °C in the presence of inhibitors of other peptidases (1 μM phosphoramidon, 100 μM amastatin, 100 μM di-isopropyl fluorophosphate) yielded no product peaks on analysis by h.p.l.c. and caused no loss of substrate peak area of pBNP-26 and pBNP-32. When ^{125}I-rANP was incubated under the same conditions, no radioactive product was detected in the supernatant fraction after precipitation with trichloroacetic acid.

DISCUSSION

Mode of hydrolysis of pBNP-32 and hBNP-32

The initial attack on pBNP-32 was at the Ser^{20}–Leu^{21} bond within the ring, a point of cleavage similar to that observed for pBNP-26. This is hardly surprising, since the sequences within the ring are identical for the two peptides, the only differences being in the N-terminal extension. We did not define the bonds which might have been cleaved later, but it is likely that the pattern of attack would resemble that defined for pBNP-26, namely at Cys–Phe, Arg–Leu, Arg–Ile, Ile–Gly, Ser–Leu and Gly–Leu (within the ring) and at Val–Leu in the tail (Bourne and Kenny, 1990).

Human BNP-32 was studied in some detail. The most significant finding was that the initial cleavage is outside the ring at Met^4–Val^5. It remains to be seen whether this has any effect on activity, but given the wide variation in the sequences of the N-terminal peptides of natriuretic peptides, it is likely that inactivation of hBNP-32 requires hydrolysis at bonds within the ring, perhaps at Arg^{17}–Ile^{18}, which was detected within the first hour. Analysis of the peptides remaining after incubation for 24 h revealed all the points of hydrolysis: Met–Val (in the head) Cys–Phe, Lys–Met, Arg–Ile and Gly–Leu (within the ring) and Val–Leu in the tail. The pattern of attack on bonds within the disulphide linked ring of hBNP differed substantially from that of pBNP-26. Where identical pairs of residues occur, similar bonds were split (Cys–Phe, Arg–Ile, Gly–Leu), but sequence differences elsewhere would seem to account for the reduced number of available sites for hydrolysis in hBNP-32. However, it is highly probable that conformational differences can play an important role in determining the availability of susceptible bonds and also have an effect on overall rates of hydrolysis. These considerations may explain the differences in rates observed for CNP, the C-receptor ligand peptides and urodilatin compared with α-hANP.

Comparing the site of initial attack on α-hANP with the three types of BNP (Figure 1) we propose that, where it is encountered, Ser–Leu is the preferred bond (as in pBNP-26 and pBNP-32), but where absent, other sites, such as Cys–Phe (in α-hANP) or Met–Val (in hBNP-32) become the points of initial attack. Not only is hBNP-32 unusual in respect of an initial cleavage point outside the ring, but it also stood out as being hydrolysed at a rate that was an order of magnitude lower than α-hANP or CNP and as being a poor inhibitor of the hydrolysis of labelled insulin B chain or labelled rANP. This poses interesting questions in regard to the role of E-24.11 in metabolizing hBNP compared with α-hANP and CNP. These questions may be especially relevant in pathological states, such as congestive heart failure (Mukoyama et al., 1990) and acute myocardial infarction (Mukoyama et al., 1991b), where hBNP is the main circulating natriuretic peptide, achieving levels 100-fold higher than in normal subjects.

Hydrolysis of CNP, urodilatin and C-receptor ligands

If hBNP-32 was unusually resistant to hydrolysis by E-24.11, CNP was, by contrast, the most rapidly attacked of the peptides studied. The two synthetic analogues, designed to bind specifically to C-receptors, C-ANP$_{4-23}$ and SC 46542 were also degraded rapidly, all three peptides being hydrolysed at rates comparable with that of α-hANP. Urodilatin is hydrolysed by E-24.11 at about half the rate of α-hANP, as judged by disappearance of the substrate and by the peak levels of the initial products, but the C-terminal fragments were released at identical rates. These results do not fit with the conclusion of Gagelmann et al. (1988) (based on experiments with dog kidney cortex membranes), namely that the N-terminal extension in the urodilatin sequence "prevents proteolytic attack". However, the time course in their paper shows degradation of urodilatin to have occurred at about 30% of the rate for ANP, slower than our finding, but not implying a totally resistant peptide.

Kinetic constants

The determination of kinetic constants for a substrate that yields multiple products, as is the case with the natriuretic peptides, raises practical and theoretical problems, since there will be several different enzyme–substrate complexes and a K_m assessed

by observation of the depletion of the intact peptide will be an average value for several reactions. With this approach, pBNP-26 had a lower K_m and higher $k_{cat.}/K_m$ value (specificity constant) than α-hANP or pBNP-32. The specificity constants for all three peptides, ranging from 8 to 25 $min^{-1} \cdot \mu M^{-1}$, can be compared with values of 44 for [Leu]enkephalin, 69 for bradykinin and 159 for substance P (Matsas et al., 1984). The K_m for the hydrolysis of ^{125}I-rANP was lower, 11 μM, and the specificity constant, 44 $min^{-1} \cdot \mu M^{-1}$, was comparable with that for [Leu]enkephalin. However it is important to bear in mind that the radioactive label is confined to a single residue, Tyr^{28}; hence the radiometric assay depends on the release of the C-terminal tripeptide.

K_i values, determined for the peptides as competitive inhibitors in the two radiometric assays of the enzyme, should approximate to K_m values for the peptides. Caution is necessary in comparing the values obtained by the two approaches (generally lower when computed as K_i values) for the reasons outlined above. Nonetheless, the ranking orders obtained with the two labelled substrates showed that CNP had the lowest K_i values, followed by SC 46542, whereas hBNP-32 had the highest value, at least an order of magnitude greater than that for CNP. There were some minor differences in the ranking order of the other three peptides, but urodilatin was comparable with α-hANP, and SC 46542 exhibited lower K_i values than C-ANP_{4-23}.

The high efficiency with which E-24.11 hydrolysed both C-receptor ligands is important in considering their mechanism of action *in vivo* or in studies on cells in culture, since their ability to protect endogenous natriuretic peptides will be a function of two actions: occupancy of C-receptors and competition with the peptides for hydrolysis by E-24.11.

ACE was ineffective *in vitro* in hydrolysing α-hANP (Stephenson and Kenny, 1987a), pBNP-26 and pBNP-32. This is at odds with the reports that captopril reduced the plasma clearance of ^{125}I-pBNP-26 in rats and that incubation for 1 h of this peptide with 20 μg of a commercial preparation of ACE yielded two radioactive products, including the C-terminal Arg-Tyr (Vanneste et al., 1990). It may be that iodination of the C-terminal residue, or other chemical changes to the peptide, may have rendered it susceptible to attack, but we found no evidence that ^{125}I-rANP was hydrolysed, even after prolonged incubation. Differences in the purity of the enzymes may possibly explain the discrepancy.

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REFERENCES

Bourne, A. and Kenny, A. J. (1990) Biochem. J. **271**, 381–385

Chinkers, M., Garbers, D. L., Chang, M.-S., Lowe, D. G., Chin, H., Goeddel, D. V. and Schulz, S. (1989) Nature (London) **338**, 78–83

Fulcher, I. S. & Kenny, A. J. (1983) Biochem. J. **211**, 743–753

Fuller, F., Porter, J. G., Arfsten, A. E., Miller, J., Schilling, J. W., Scarborough, R. M., Lewicki, J. A. and Schenk, D. B. (1988) J. Biol. Chem. **263**, 9395–9401

Gagelmann, M., Hock, D. and Forssmann, W. G. (1988) FEBS Lett. **233**, 249–254

Hosoda, K., Nakao, K., Mukoyama, M., Saito, Y., Jougasaki, M., Shirakami, G., Suga, S., Ogawa, Y. and Yasue, H. I. H. (1991) Hypertension **17**, 1152–1155

Johnson, G. R., Arik, L., Pitts, B. J. R. and Forster, C. J. (1990) Biochem. J. **268**, 771–776

Kangawa, K. and Matsuo, H. (1984) Biochem. Biophys. Res. Commun. **118**, 131–139

Kenny, A. J. and Stephenson, S. L. (1988) FEBS Lett. **232**, 1–8

Komatsu, Y., Nakao, K., Suga, S., Ogawa, Y., Mukoyama, M., Arai, H., Shirakami, G., Hosoda, K., Nakagawa, O., Hama, N., Kishimoto, I. and Imura, H. (1991) Endocrinology (Baltimore) **129**, 1104–1106

Kuno, T., Andresen, J. W., Kamisaki, Y., Waldman, S. A., Chang, L. Y., Saheki, S., Leitman, D. C., Naane, M. and Murad, F. (1986) J. Biol. Chem. **260**, 5817–5823

Maack, T., Suzuki, M., Almeida, F. A., Nussenzveig, D., Scarborough, R. M., McEnroe, G. A. and Lewicki, J. A. (1987) Science **238**, 675–678

Matsas, R., Kenny, A. J. and Turner, A. J. (1984) Biochem. J. **223**, 433–440

Minamino, N., Kangawa, K. and Matsuo, H. (1990) Biochem. Biophys. Res. Commun. **170**, 973–979

Mukoyama, M., Nakao, K., Saito, Y., Ogawa, Y., Hosoda, K., Suga, S., Shirakami, G., Jougasaki, M. and Imura, H. (1990) New Engl. J. Med. **323**, 757–758

Mukoyama, M., Nakao, K., Hosoda, K., Suga, S., Saito, Y., Ogawa, Y., Shirakami, G., Jougasaki, M., Obata, K., Yasue, H. et al. (1991a) J. Clin. Invest. **87**, 1402–1412

Mukoyama, M., Nakao, K., Obata, K., Jougasaki, M., Yoshimura, M., Morita, E., Hosoda, K., Suga, S., Ogawa, Y., Yasue, H. et al. (1991b) Biochem. Biophys. Res. Commun. **180**, 431–436

Norman, J. A., Little, D., Bolgar, M., and Donato, G. (1991) Biochem. Biophys. Res. Commun. **175**, 22–30

Ogawa, Y., Nakao, K., Mukoyama, M., Hosoda, K., Shirakami, G., Arai, H., Saito, Y., Suga, S., Jougasaki, M. and Imura, H. (1991) Circ. Res. **69**, 491–500

Rathinavelu, A. and Isom, G. E. (1991) Biochem. J. **276**, 493–497

Scarborough, R. M., Schenk, D. B., McEnroe, G. A., Arfsten, A., Kang, L.-L., Schwartz, K. and Lewecki, J. A. (1986) J. Biol. Chem. **261**, 12960–12964

Scarborough, R. M., McEnroe, G. A., Arfsten, A., Kang, L. L., Schwartz, K. and Lewicki, J. A. (1988) J. Biol. Chem. **263**, 16818–16822

Schulz-Knappe, P., Forssmann, K., Herbst, F., Hock, D., Pipkorn, R. and Forssmann, W. G. (1988) Klin. Wochenschr. **66**, 752–759

Seymour, A. A., Norman, J. A., Asaad, M. M., Fennell, S. A., Abboa-Offei, B., Little, D. K., Kratunis, V. J., Delaney, N. G., Hunt, J. T. and DiDonato, G. (1991) J. Pharmacol. Exp. Ther. **256**, 1002–1009

Stephenson, S. L. and Kenny, A. J. (1987a) Biochem. J. **243**, 183–187

Stephenson, S. L. and Kenny, A. J. (1987b) Biochem. J. **241**, 237–247

Sudoh, T., Kangawa, K, Minamino, N. and Matsuo, H. (1988) Nature (London) **332**, 78–81

Sudoh, T., Maekawa, K., Kojima, M., Minamino, N., Kangawa, K. and Matsuo, H. (1989) Biochem. Biophys. Res. Commun. **159**, 1427–1434

Sudoh, T., Minamino, N., Kangawa, K. and Matsuo, H. (1990) Biochem. Biophys. Res. Commun. **168**, 863–870

Ueda, S., Minamino, N., Aburaya, M., Kangawa, K., Matsukura, S. and Matsuo, H. (1991) Biochem. Biophys. Res. Commun. **175**, 759–767

Vanneste, Y., Michel, A., Dimaline, R., Najdovski, T. and Deschodt-Lanckman, M. (1988) Biochem. J. **254**, 531–537

Vanneste, Y., Pauwels, S., Lambotte, L. and Deschodt-Lanckmann, M. (1990) Biochem. Biophys. Res. Commun. **173**, 265–271

Vogt-Schaden, M., Gagelmann, M., Hock, D., Herbst, F. and Forsmann, W. G. (1989) Biochem. Biophys. Res. Commun. **161**, 1177–1183

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