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Review Letter

Role of endopeptidase-24.11 in the inactivation of atrial natriuretic peptide

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The circulating form of atrial natriuretic factor is a 28-residue peptide containing a 17-residue disulphide-linked ring. It has important actions on the kidney, largely on its haemodynamics, and at other sites including the adrenal cortex and CNS. It has a short half-life in vivo and is rapidly inactivated when incubated with kidney microvillar membranes. Of the battery of peptidases present in that membrane, only one, endopeptidase-24.11, is responsible for initiating the attack, and this commences with hydrolysis of the Cys^7-Phe^8 bond within the ring. Hydrolysis at this and other points has been shown to inactivate the peptide and this information has pointed the way to the synthesis of resistant analogues.

Atrial natriuretic peptide; Membrane peptidase; Neuropeptide; Endopeptidase-24.11

1. DISCOVERY OF ATRIAL NATRIURETIC FACTOR

Atrial natriuretic factor (ANF) comprises a family of peptides with powerful natriuretic, diuretic and hypotensive activities which are present in storage granules of atrial muscle. The existence of these granules and their resemblance to secretory granules of other cells were reported more than 20 years ago [1,2] but the chemical nature of their contents was a mystery until quite recently. The first clues were the observation that the number of granules, assessed microscopically, could be affected by changes in the intake of water and sodium [3] and that injections of extracts of atria or granules promoted diuresis and natriuresis [4] and by 1983 it was becoming clear that these activities resided in a group of related peptides (for review and nomenclature see [5]). Several low-M_r peptides have been isolated from rat atria, e.g. atriopeptins I, II and III and α-rat atrial natriuretic peptide (α-rANP), all of which possess a disulphide-linked loop of 17 amino acid residues, with short N- and C-terminal extensions. However, the major circulating form of ANF in the rat appears to be the 28-residue peptide [6,7] which is derived from a high-M_r, 152-residue precursor, rat pre-pro ANP (see [5]). Human atria also release a 28-amino acid peptide (α-human atrial natriuretic peptide, α-hANP; [8]; fig.1) which differs from α-rANP by only one residue, Met^{12} for Ile^{12} [9].

Dedicated to Professor Giorgio Semenza on the occasion of his 60th birthday

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Abbreviations: ANF, atrial natriuretic factor; ANP, atrial natriuretic peptide (1–28) the prefixes h and r indicating human and rat; E-24.11, endopeptidase-24.11 (EC 3.4.24.11)

2. REGULATION OF RELEASE OF ANP

Several factors have been found to stimulate

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release of ANP. The infusion of isotonic saline into the rat, corresponding to a 30% expansion of volume, gave a 6-fold increase in plasma ANF-like material [10], the major component of the immunoreactive material being α-rANP(1–28) [11]. Administration of pharmacological doses of [Arg^8]vasopressin also stimulated release of ANP [12] and a negative feedback loop has been postulated whereby [Arg^8]vasopressin stimulates ANP release, which in turn suppresses [Arg^8]vasopressin secretion.

3. BIOLOGICAL EFFECTS AND MECHANISM OF ACTION OF ANP

Injection of atrial extract into rats causes natriuresis, diuresis and chloruresis [4]. The mechanism of the natriuretic effect of ANP has been the subject of considerable controversy but the findings obtained to date strongly support the theory that the ANP-induced natriuresis is largely due to its effects on renal haemodynamics, particularly the increase in glomerular filtration rate [13,14]. It seems that this is brought about by vasodilation of afferent arterioles and vasoconstriction of efferent vessels. Micropuncture studies have indicated that the natriuretic effect of ANP may be due, in part, to an inhibition of sodium transport along the collecting tubules and collecting ducts, but without affecting this process in proximal or distal tubules. It has been shown that α-rANP(Arg^3–Tyr^{28}) inhibits angiotensin II- and ACTH-stimulated aldosterone secretion in rat adrenal glomerulosa cells in vivo and in vitro [15,16]. The primary site of action of aldosterone is the cortical collecting tubule, where it slowly causes a decrease in sodium and an increase in potassium excretion [17]. However, since the ANP-induced natriuresis occurs within minutes [18], this rapid action cannot be related to reduced aldosterone output.

Apart from the renal effects mentioned above, ANP also reduces cardiac output, probably by indirectly reducing stroke volume and heart contractility [19], and causes relaxation of vascular smooth muscle [20,21] both of which could lead to the fall in blood pressure which is observed after ANP administration [18].

Some of the effects of ANP may be centrally mediated since ANP immunoreactivity has been found in various areas of the brain known to be involved in the regulation of fluid and electrolyte balance and cardiovascular homeostasis [22]. Several areas of the brain contain receptors for ANP, for example the subfornical organ and area postrema [23,24] and choroid plexus [25] and α-hANP has been demonstrated [26] in human cerebrospinal fluid. Furthermore, when α-rANP was injected intraventricularly it inhibited production of cerebrospinal fluid [27], suggesting that ANP is relevant to CNS water regulation under normal conditions and perhaps in the pathology of cerebral edema and hydrocephalus.

4. ANP STRUCTURE-ACTIVITY RELATIONSHIPS

The integrity of the disulphide bridge has been shown to be essential for activity. Reduction and carboxymethylation abolished natriuretic and diuretic actions [28] and no inhibitory effect on aldosterone secretion from adrenal glomerulosa cells was observed after performic acid oxidation [29]. The linear fragment α-rANP(Phe^8–Arg^{27}) had very little activity compared with α-rANP(Ser^5–Arg^{27}) [30] suggesting that hydrolysis of a peptide bond within the disulphide-linked loop would be sufficient to inactivate. Also, removal of the C-terminal tripeptide (Phe-Arg-Tyr) from α-rANP(Arg^4–Tyr^{28}) markedly decreased, but did not abolish, natriuretic and vasorelaxant actions [31].

5. INACTIVATION OF ANP BY TISSUES

Several groups have demonstrated a very rapid turnover of α-rANP in vivo, in which the kidneys play a major part [32–34], but the mode of attack and the nature of the peptidases involved has only recently become clear. An early report described an atrial enzyme activity which converted atriopeptin II [α-rANP(Ser^5–Arg^{27})] to atriopeptin I [α-rANP(Ser^5–Ser^{25})] by removal of the C-terminal dipeptide Phe-Arg [35]. Later it was observed that atriopeptin III [α-rANP(Ser^5–Tyr^{28})] was degraded by rabbit brush border membranes with hydrolysis at the Ser^5-Ser^6, Cys^7-Phe^8 and Ser^{25}-Phe^{26} peptide bonds [36]. More detailed studies on the mode of attack by renal brush

border membranes have now led to the identity of the peptidase responsible.

5.1. *Kidney microvillar peptidases*

No membrane is richer in peptidases than that of the renal brush border; they are listed and their specificity summarized in table 1. Many of the same enzymes are expressed on the surfaces of a variety of other cell types, not only in brush borders such as those of the intestine, placenta and choroid plexus, but on many other cells, including those of the nervous system (for recent review see [37]). The activity of this battery of peptidases, all active at neutral pH, is directed towards simple peptides, native proteins being wholly resistant to attack. Thus, substance P, bradykinin, angiotensins I, II and III, and insulin B chain, are all rapidly degraded in vitro by pig kidney microvillar membranes. In the same conditions, oxytocin was degraded an order of magnitude more slowly while vasopressin and insulin remained intact [39]. α-hANP is one of the group of very susceptible peptides, being attacked at rates comparable to angiotensins II and III [40].

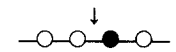
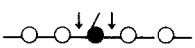
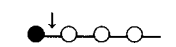
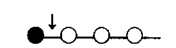
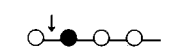
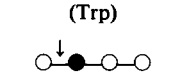
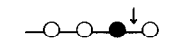
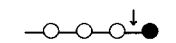
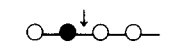
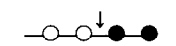
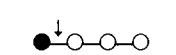
Microvillar membrane preparations provide a valuable model system for predicting the likely metabolism of a peptide in vivo, since most, though possibly not quite all, of the known cell surface peptidases are represented in high concentrations on this membrane. If conditions are selected to reveal the initial attack on a susceptible peptide, the products analysed and collected so that the bonds hydrolysed are defined, it is possible to predict the probable identity of the peptidases responsible. Fortunately, there are a number of specific inhibitors of the key peptidases (table 1) which can serve to confirm the identity as, too, can parallel experiments with isolated enzymes. This approach has shown that E-24.11 initiates the attack on bradykinin, substance P, angiotensins I, II, III, oxytocin and insulin B chain [39]. In some respects this was a surprising conclusion: peptidyl dipeptidase A (angiotensin converting enzyme) converts angiotensin I to II and inactivates bradykinin by removal of the C-terminal dipeptides, His-Leu and Phe-Arg, respectively. While this is a physiologically important process in the capillaries of the lung (where peptidyl dipeptidase A but no E-24.11 is present; see [37]), the relative activities of the two peptidases in kidney microvilli dictate a different fate whereby the principal attack is dependent on E-24.11.

With this background in mind and because the kidney is an important target organ for ANP, we have used the same approach to investigate the metabolism of this peptide [40]. In short incubations of α-hANP with pig E-24.11, the main product was a peptide with the same amino acid composition, but with a shorter retention time on HPLC. It was designated α-hANP′ and attributed to the hydrolysis of a single peptide bond within the S-S-bridged 17-residue ring. When the digestion was allowed to run to completion, α-hANP′ was further degraded and 9 peptides could be resolved on HPLC. Amino acid analysis of the four main peptide products indicated attacks at three points within the ring and one in the tail (solid arrows, fig.1). In contrast, incubation of α-hANP with peptidyl dipeptidase A revealed no evidence of any fragments even after 24 h. With microvillar membranes incubated for 15 min the main product (22% yield), was α-hANP′, together with the C-terminal fragments (Phe-Arg-Tyr and free phenylalanine, 9% yield) and traces of other fragments from hydrolysis within the ring. Of the inhibitors, only phosphoramidon had a clear-cut effect, suppressing the yield of the fragments by 80–100% (fig.2). The formation of identical peptide products by the membranes and purified E-24.11, and the inhibitory effect of phosphoramidon, confirm that the attack on α-hANP is initiated by E-24.11 in this membrane. The identity of the bond initially hydrolysed to generate α-hANP′ became clear when the products formed by pig E-24.11 on α-rANP (which differs only in the substitution of Met^{12} by Ile^{12}) were analysed by sequencing (kindly performed by Merrell Dow Research Institute). Two peptide sequences were revealed, α-rANP(Ser^{1}–Cys^{7}) and α-rANP(Phe^{8}–Tyr^{28}), thus showing that the first attack was at the Cys^{7}-Phe^{8} bond. This point of hydrolysis has also been shown for rat E-24.11 [41]. Two other groups had reported hydrolysis of the same bond after incubation of α-rANP with rabbit [36] or rat [42] brush border membranes, but in neither case was the identity of the peptidase established. Later, it was suggested that the rabbit enzyme might be E-24.11 [43] and its identity was firmly established in the case of the rat [44].

Rat kidney microvilli contain endopeptidase-2,

Table 1

Peptidases so far identified in kidney microvilli

Class	Enzyme	Active site	Specificity	Specific inhibitor
Endopeptidases	endopeptidase-24.11 (EC 3.4.24.11, ? all species)	Zn^{2+}	 (hydrophobic)	phosphoramidon
	endopeptidase-2 (rat kidney)	Zn^{2+}	 (hydrophobic)	–
	meprin (mouse kidney)	Zn^{2+}	 (hydrophobic)	–
Aminopeptidases	aminopeptidase N (EC 3.4.11.2)	Zn^{2+}	 (many)	amastatin
	aminopeptidase A (EC 3.4.11.7)	Ca^{2+}	 (Glu/Asp)	amastatin
	aminopeptidase W (EC 3.4.11.–)	Zn^{2+}	 (Trp)	amastatin
	aminopeptidase P (EC 3.4.11.9)	?	 (Pro)	–
Carboxypeptidases	carboxypeptidase P (EC 3.4.17.–)	Zn^{2+}	 (Pro,Ala,Gly)	–
	carboxypeptidase M (EC 3.4.17.–)	?	 (Lys,Arg)	GEMSA
Dipeptidyl peptidase	dipeptidyl peptidase IV (EC 3.4.14.5)	serine	 (Pro,Ala)	Dip-F
Peptidyl dipeptidase	peptidyl dipeptidase A (ACE) (EC 3.4.15.1)	Zn^{2+}	 (nonspecific)	captopril
Dipeptidase	microsomal dipeptidase (EC 3.4.13.11)	Zn^{2+}	 (nonspecific)	cilastatin
Transferase	γ-glutamyl transferase (EC 2.3.2.2)	–	 (γ-Glu)	AT-125

The data refer to pig except where otherwise stated. For reviews see [37,38]. GEMSA, guanidinoethylmercaptosuccinic acid; Dip-F, diisopropylfluorophosphate; AT-125, L-(*S*,5-*S*)-amino-3-chloro-4,5-dihydro-5- isoxazoleacetic acid

in addition to E-24.11 [45,46], and this raises the possibility that membranes from this species might metabolise ANP differently from those of pig, rabbit or human in which only E-24.11 is to be found. In fact the pattern of products released by brief exposure of α-hANP to rat microvillar membranes is

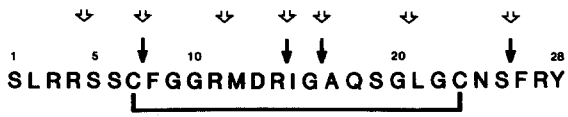


Fig.1. Sequence of α-hANP and points of hydrolysis by E-24.11. This human sequence differs from that of the rat (α-rANP) in containing Met^{12} in place of Ile^{12}. The solid arrows show the main points of hydrolyses which we reported [40] and the open arrows indicate bonds cleaved by E-24.11 purified from human kidney [48]. The initial attack takes place at Cys^7-Phe^8.

indistinguishable from that observed with pig membranes and the hydrolysis was inhibited by phosphoramidon. Purified endopeptidase-2 hydrolysed α-hANP very slowly (being incomplete after incubation for 6 h with 100 ng enzyme and 5 nmol peptide) and the products differed from those observed with rat or pig microvillar membranes [47]. Endopeptidase-2 therefore appears to play little or no role in the metabolism of ANP, in agreement with the observation that the activity in rat kidney membranes responsible for the hydrolysis of α-rANP at the Cys^7-Phe^8 bond is E-24.11 [44]. Another recent study [48] has used E-24.11 purified from human kidneys. Seven sites of attack were noted: Arg^4-Ser^5, Cys^7-Phe^8, Arg^{11}-Met^{12}, Arg^{14}-Ile^{15}, Gly^{16}-Ala^{17}, Gly^{20}-Leu^{21} and Ser^{25}-Phe^{26} (open arrows, fig.1). Of these, the cleavage of an Arg-Ser bond is atypical for an enzyme which normally attacks bonds involving the amino group of hydrophobic residues. Yet the effect of phosphoramidon on suppressing hydrolysis

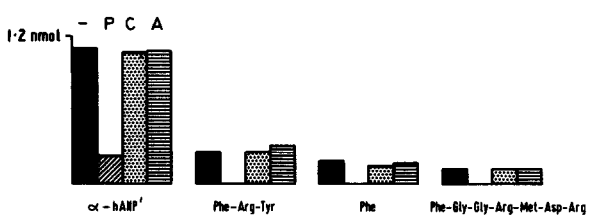


Fig.2. Effect of peptidase inhibitors on the formation of the main products when α-hANP was incubated with pig microvillar membranes. The bars show the yield of each product. Solid bar, no inhibitors; cross-hatched, 1 μM phosphoramidon (P); stippled, 1 μM captopril (C); horizontal hatched, 1 μM amastatin (A). Data redrawn from [40]. All bars are plotted on the same scale, the yield of the main product, α-hANP', being 1.1 nmol, corresponding to 22% hydrolysis at the Cys^7-Phe^8 bond.

at this and other sites is consistent with this attack being attributable to E-24.11 and not to a minor contaminating activity. The initial point of hydrolysis at Cys^7-Phe^8 was again confirmed.

5.2. *Effect of the E-24.11 attack on the biological activity of α-hANP*

The degradation of α-hANP by the brush border peptidases resembled that of substance P, bradykinin, the angiotensins and oxytocin in being predominantly initiated by E-24.11. The points of attack observed seem likely to inactivate ANP: the removal of the C-terminal tripeptide greatly reduces activity [31] and, since the integrity of the disulphide bridge is essential [28–30], it was predictable that hydrolysis of peptide bonds within the loop would also inactivate. Recently it has been confirmed that ring-opened α-rANP(Ser^5–Tyr^{28}), generated by incubation with E-24.11, had approx. 2% of the vasorelaxant activity of the parent peptide [49] and was at least 10-times less active than the parent peptide in causing hypotension, natriuresis and diuresis in spontaneously hypertensive rats [50]. The ring-opened metabolite of α-rANP(Ser^5–Ser^{25}) which also lacks the C-terminal tripeptide Phe-Arg-Tyr, was essentially inactive. It seems likely therefore that passage of ANP through the proximal renal tubule in vivo will suffice to inactivate the peptide. In assessing the physiological significance of these findings it should be recalled that the renal receptors for ANP and sites of action on Na^+ transport are reported to be in the collecting ducts and tubules [51–54] and would be physiologically relevant only if accessible to ANP at the basolateral surface, since ANP will be cleared from the luminal fluid. ANP has other postulated sites of action, e.g. in renal glomerular membranes [25] where ANP may induce an increase in glomerular filtration rate [18], in the adrenal cortex causing inhibition of the release of aldosterone from the glomerulosa cells [15] as well as in the vascular smooth muscle where it is a vasorelaxant [20,21] and in the brain [23,24]. Since E-24.11 has a widespread distribution on plasma membranes in many tissues and organs [55–59] the inactivation of ANP by E-24.11 may well occur at other sites in addition to the kidney. It is particularly interesting that in the adrenal cortex, endopeptidase-24.11 is discretely localized in the glomerulosa cells [37] where it may serve to in-

activate ANP and other peptide signals such as angiotensins II and III.

5.3. *Possible clinical importance*

α-hANP has been found to have therapeutic actions in patients with essential hypertension and congestive heart failure [60] indicating the potential of α-hANP as a new drug for such disorders. Much research is now being directed towards the synthesis of longer lasting, more potent analogues of this naturally occurring peptide. An analogue of α-rANP(Cys^7–Tyr^{28}), lacking the six, N-terminal, ('head') amino acid residues, with 3-mercaptopropionic acid replacing Cys^7, has been shown to have several-fold higher natriuretic and diuretic activities than α-rANP(Arg^3–Tyr^{28}) [61]. The resultant des-amino analogue would be resistant to attack by E-24.11 at Cys^7-Phe^8 (its specificity requires as a minimum the binding at S_1 and S_2 sites) and also to aminopeptidase attack. This resistance to enzymic degradation would suffice to explain the high potency of the analogue.

Recently two atrial natriuretic peptides have been identified in human coronary sinus plasma [62]. The amino acid sequence of one of the peptides indicated that it was identical with α-hANP. However, the amino acid sequence of the second peptide was consistent with α-hANP(Ser^1–Cys^7) linked to α-hANP(Phe^8–Tyr^{28}) by the disulphide bond, which is now known to be the structure of α-hANP'. The molar ratio of the peptides was 10:3, respectively. E-24.11 has been detected in heart tissue albeit in very low quantities [56], and we have shown that it hydrolyses the Cys^7-Phe^8 bond of α-hANP, but its role in generating this derivative in the coronary sinus in vivo has yet to be established.

6. WHAT IS THE PHYSIOLOGICAL ROLE OF THE RENAL BRUSH BORDER PEPTIDASES?

It will be apparent from what has been said that the powerful hydrolytic activity of the microvillar peptidases, especially E-24.11, can, in vitro, rapidly degrade many regulatory peptides, including ANP. Micropuncture and microperfusion studies have shown that the passage of injected labelled peptides (e.g. bradykinin, angiotensins I, II, though not oxytocin) through the proximal tubule is associated with their degradation [63–65], while injection of bradykinin into the distal tubule leads to complete recovery in the urine [63]. What is more difficult to comprehend is why such mechanisms exist to clear the glomerular filtrate of these peptides. Failure to do so would, in the absence of endocytic mechanisms for peptides (as distinct from proteins, e.g. insulin [66]), lead to their passage into the urine after some degree of concentration. The nutritional losses would appear to be trivial, amounting in the human to only a few milligrams of peptide. In regard to their biological actions the peptides will, in most cases, have been sequestered from their target organs, so that the choice of degradation versus excretion would not affect their biological turnover rate. There remains a possibility (one that we have argued before [37,67]) that the brush border peptidases exert a protective role by preventing certain peptides from reaching the distal part of the urinary system. ANP may be such a peptide. The concentrating power of the nephron would generate pharmacological rather than physiological concentrations of ANP, if not degraded in the proximal tubule. It has been shown that intraluminal injection of high concentrations of bradykinin (50 × physiological) into the late proximal tubule caused increased ^{22}Na excretion [68]. It may be that this peptide and ANP in abnormally high intraluminal concentrations could gain access to receptors on the contraluminal surfaces to produce this response. Blockade of E-24.11 by intravenously infused phosphoramidon into anaesthesised rats has been reported to cause natriuresis and diuresis [69]. We have, in preliminary experiments, confirmed this effect of phosphoramidon on renal function, but unlike the first report, which suggested that it resulted from the protection of kinins from inactivation, we would surmise that there is a strong argument for the involvement of ANP.

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