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Urodilatin (CDD/ANP-95–126) is not biologically inactivated by a peptidase from dog kidney cortex membranes in contrast to atrial natriuretic peptide/cardiodilatin (α-hANP/CDD-99–126)

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Atrial natriuretic peptide (CDD/ANP-99–126) is rapidly inactivated by a membrane preparation from dog kidney cortex. Inactivation occurs by cleavage of the ring structure in the position between Cys-105 and Phe-106. A unique proteolytic product separated by HPLC on reverse-phase column appears as a single peak which elutes prior the intact peptide. In contrast, CDD/ANP-95–126 (urodilatin) which is released from the kidney is not destroyed by proteolysis using an identical membrane preparation.

Atrial natriuretic peptide; Cardiodilatin; Urodilatin; Proteolysis; Biological activity; (Vascular smooth muscle, Kidney membrane)

1. INTRODUCTION

In the atrial specific granules [1] of mammalian heart cardiodilatin/atrial natriuretic peptide (CDD/ANP) which was first functionally characterized by its diuretic activity [2] is stored as a prohormone of 126 amino acids in atrial myoendocrine cells [3–5]. In the blood plasma a circulating bioactive form of CDD/ANP-1–126 containing 28 residues of the C-terminus of the prohormone has been characterized [6,7]. This circulating peptide exhibits potent natriuretic, diuretic and vasorelaxant potency in vitro and in vivo [2,5,8–12]. Beside CDD/ANP-99–126, a variety of shorter forms have been isolated (reviews [3,13]). However, it is likely that these smaller peptides may be simply the result of hydrolytic artifacts during the purification procedure [3,13]. Upon cleavage of the prohormone at a single arginine peptide bond, the biologically active form is liberated and circulates as a single peptide chain with a disulfide loop formed by two cysteine residues [3]. The peptide exhibits a short half-life of 3–4 min [11]. Inactivation of the bioactive 28-residue peptide in the blood was reported to occur (i) by proteolysis of the loop between Cys-105 and Phe-106 [6] and (ii) by cAMP-dependent phosphorylation of a serine residue in position 104, originally described by Rittenhouse et al. [14]. The phosphorylation prevents receptor binding thus decreasing the vasorelaxant potency [15,16]. The humoral role of the heart peptide in electrolyte and body fluid volume homeostasis is indicated by the presence of high-affinity receptors for CDD/ANP in the kidney [17,18]. Studies implicate that the kidney is an important site for the clearance and subsequent degradation of CDD/ANP [19]. Olins et al. [20] and later Koehn et al. [21] reported that kidney cortex membranes contain CDD/ANP degrading activity. The authors found that inactivation of CDD/ANP occurs by a single cleavage in the disulfide loop region of Cys-105 and Phe-106.

Cardiac hormones are known to occur also in other organs as revealed by mRNA hybridisation [22], polypeptide extraction [23] and immuno-

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logical studies [24–26]. By use of the same isolation procedure as described for the isolation of the circulating form of CDD/ANP from human blood [7] a peptide of 32 amino acids was isolated from urine [27]. Amino acid sequence analysis revealed that the CDD/ANP peptide in the urine is a 32-amino-acid-residue containing molecule (CDD/ANP-95–126). The molecule is N-terminally extended compared to CDD/ANP-99–126. It may be concluded that the urine peptide is produced and processed in the kidney tubules. The urine peptide exhibits vasorelaxant potency [28] and was called urodilatin [27].

A recent finding by our group [29] obtained from in vitro perfusion of rabbit kidneys with CDD-99–126/α-hANP and urodilatin shows that urine outflow was enhanced with urodilatin. Therefore, in an effort to elucidate the fate of urodilatin interaction with the kidney we have investigated the metabolism of CDD-99–126/α-hANP and urodilatin (CDD/ANP-95–126) by a membrane preparation from the kidney cortex.

2. MATERIALS AND METHODS

Synthetic CDD-99–126/α-hANP and urodilatin (CDD/ANP-95–126) were obtained from Bissendorf Biochemicals (Hannover, FRG). All other reagents used were of analytical grade. Reagents for amino acid sequence analysis were ultra pure and purchased from Applied Biosystems. Preparation of membranes from kidney cortex followed basically the procedure originally described [21]. Membrane preparation was carried out at 0–4°C on ice. Kidneys were obtained from dogs and stored at −20°C until use. The cortex was dissected, minced and resuspended in 3 ml homogenization buffer [10 mM sodium phosphate (pH 7.4), 1 mM $MgCl_2$, 30 mM NaCl, 0.01% NaN_3 and 10 μg/l DNase]. Homogenization was carried out by use of an Ultra-Turrax (3 × 3-s bursts with 2-min intervals). The homogenate was centrifuged twice for 10 min at 500 × g and layered (6 ml) onto 5 ml sucrose (41%, w/v). Following centrifugation at 100 000 × g for 30 min the membranes were collected from the buffer/sucrose interfase and washed twice with 10 mM Tris (pH 7.4). Storage was with the same buffer at −70°C. Protein determination was done by the method of Lowry et al. [30] using bovine serum albumin as a standard.

For proteolysis (37°C) CDD/ANP was dissolved in phosphate-buffered saline (PBS: 0.05 M phosphate, 0.1 M NaCl, pH 7.4) at a final concentration of 100 μg/ml. The reaction was started by addition of membranes (80 μg/100 μg CDD/ANP) and terminated by addition of 0.1 ml of 0.1% trifluoroacetic acid (TFA) to 0.05 ml reaction volume. This was followed by centrifugation for 1 min in an Eppendorf centrifuge 3200 at maximum speed and rapid freezing with liquid N_2. The samples were separated by high-performance liquid chromatography (HPLC) on a reverse-phase column (Aquapore RP-300, 4.6 × 100 mm, Applied Biosystems). The column was developed by a linear gradient of acetonitrile (10–70%) containing 0.1% TFA using a device from Applied Biosystems. Absorption was determined at 220 nm. Fractions were collected manually.

Bioassays were carried out as in [16] using rabbit aorta strips in an organ bath.

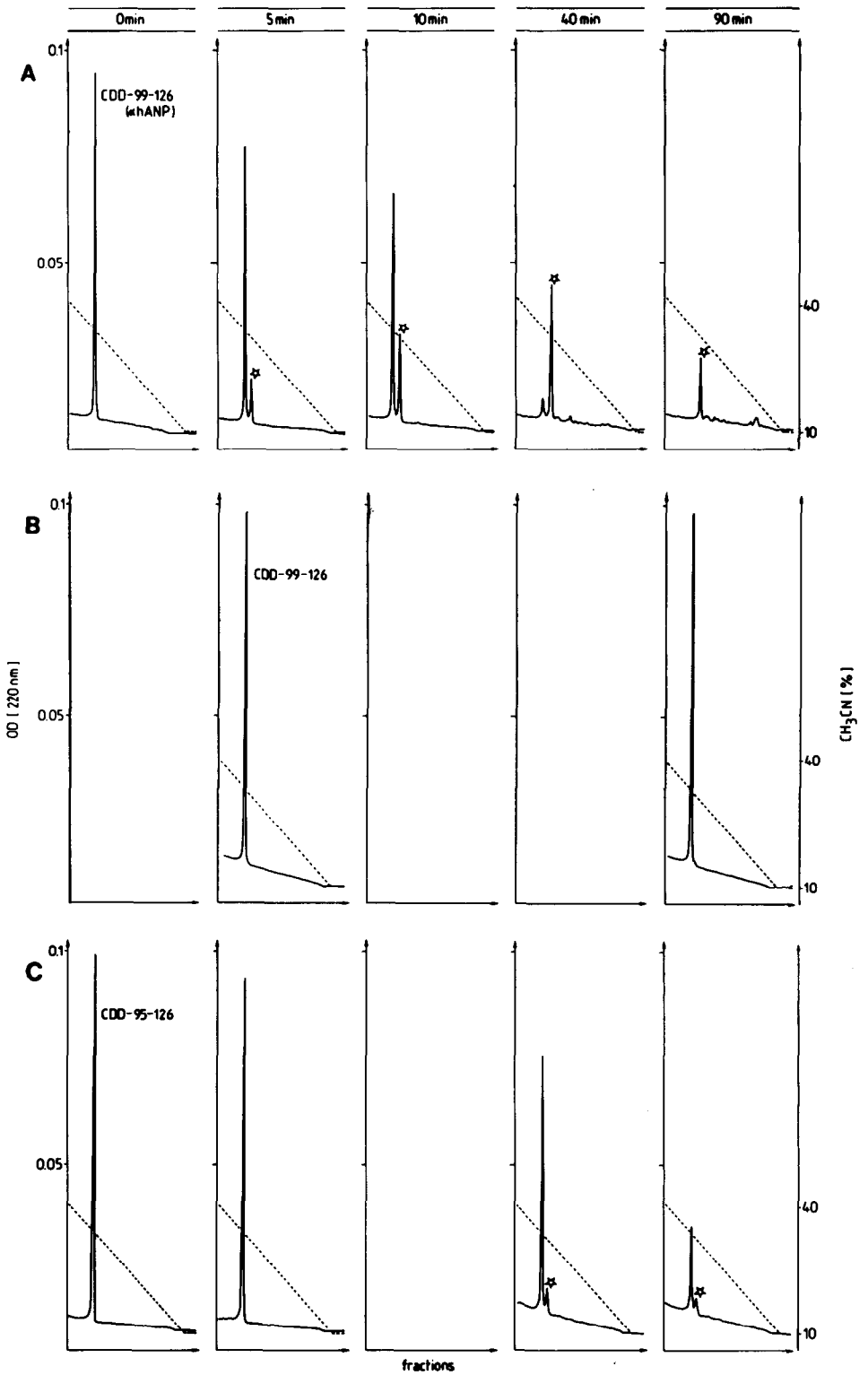
Sequence analysis was performed on a gas-phase sequencer model 477A from Applied Biosystems. Phenylthiohydantoin derivates were separated by reverse-phase HPLC according to [31].

3. RESULTS AND DISCUSSION

CDD-99–126/α-hANP (25 μg) was incubated with 20 μg kidney cortex membranes from dog. At various time intervals aliquots were removed from the reaction volume: immediately after the start of the enzymatic reaction (0 min), after 5, 10, 40 and 90 min of incubation. A typical experiment is demonstrated in fig.1A showing consecutive HPLC profiles of CDD/ANP-99–126 proteolysis. At time zero CDD/ANP-99–126 is eluted as a single peak with 38% acetonitrile. During incubation with the kidney membrane preparation the content of CDD/ANP-99–126 decreased at the cost of a minor peak (indicated by an asterisk in fig.1A) eluting with 36% acetonitrile. The conversion of substrate into product is summarized as a function of time in fig.2. After 90 min incubation the reaction reached the maximum level where all CDD/ANP-99–126 had been degraded completely. Only the product eluting at 36% acetonitrile was visible.

Biotests using rabbit aorta strips ensured that only the major peak (eluting at 38% acetonitrile)

Fig.1. Reverse-phase HPLC profiles of time-dependent (0, 5, 10, 40 and 90 min incubation) CDD/ANP-99–126 degradation by dog kidney cortex membranes (A) and heat-treated (5 and 90 min incubation) membrane preparations (B) and incubation of CDD/ANP-95–126 (urodilatin) with kidney cortex membranes (0, 5, 40 and 90 min). At various time intervals aliquots (5 μg CDD/ANP) were removed and separated by HPLC. Absorbance of the column effluent was measured at 220 nm (——). The column was developped by increasing the concentration of acetonitrile (– – –). The bioactive form of CDD/ANP-99–126 and CDD/ANP-95–126 elutes at 38% acetonitrile, while the degraded product appears with 36% acetonitrile. The cleaved product is indicated by the asterisk.


0min
5min
10min
40min
90min
A
0.1
0.05
CDD-99-126
(αhANP)
40
10
B
CDD-99-126
OD [220nm]
CH3CN [%]
C
CDD-95-126
fractions

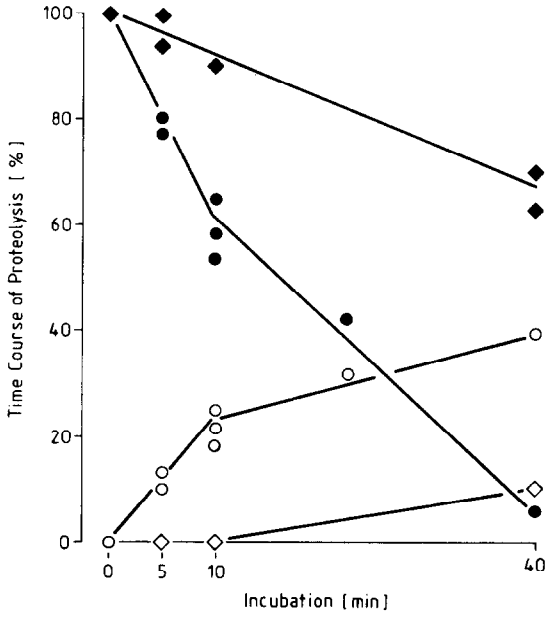


Fig.2. Rate of degradation of CDD/ANP-99-126 (●) and CDD/ANP-95-126 (urodilatin) (◆) during incubation with kidney membranes and appearance of its proteolytic products (○,◇). The maximal absorbance of the peaks of the reverse-phase HPLC column at the appropriate time intervals was plotted vs time (cf. fig.1).

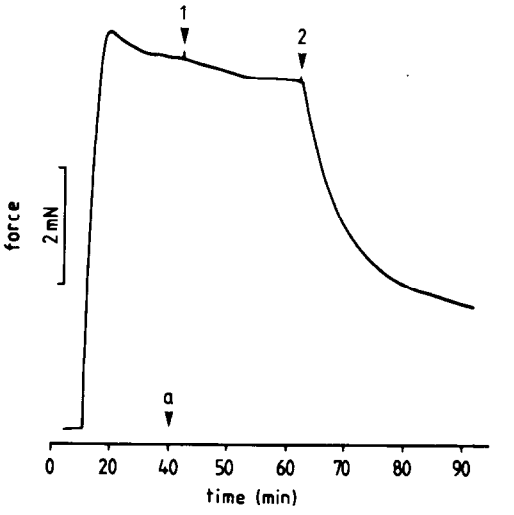


Fig.3. Effect of degraded CDD/ANP-99-126 after separation by reverse-phase HPLC (36% acetonitrile) of 10 min incubation with kidney cortex membranes on rabbit aorta. For control intact CDD/ANP-99-126 (eluted with 38% acetonitrile) from the same column run was used. The aorta was precontracted with 10^{-7} M norepinephrine. Upon a plateau force level being achieved the proteolytically cleaved CDD/ANP-99-126 (5 μl) was added, (1) followed by incubation with 2 μl of the bioactive peptide (2). The ratio of proteolytically degraded CDD/ANP-99-126 to intact peptide was 1:2.5 as estimated from the corresponding absorbance maxima (cf. also fig.1A, 10 min incubation). At position a (40 min incubation) the recorder speed was increased.

represents the bioactive form and corresponds to CDD/ANP-99-126. A typical experiment for analysis of bioactivity is given in fig.3. For the test the two peaks (eluting at 38 and 36% acetonitrile) were collected manually from HPLC runs. Both fractions were lyophilized and after resolubilization in organ bath solution directly applied to the precontracted aorta muscle preparation. The peak generated during incubation with the membrane preparation eluting at 36% acetonitrile exhibited no vasorelaxant potency indicating proteolytic processing as described by others [20,21].

The structure of the cleaved product was analysed by revealing the amino acid sequence. CDD/ANP-99-126 was incubated with kidney membranes for 90 min and the peak eluting at 36% acetonitrile was collected. Incubation for 90 min ensures complete degradation of the bioactive peak eluting at 38% acetonitrile which corresponds to intact CDD/ANP-99-126. Therefore, only the product of the enzymatic degradation was used for the sequence studies. The peptide was submitted to 17 cycles of amino-terminal sequence analysis. In the first cycle two distinct amino-terminal residues (serine and phenylalanine) were identified. Further sequence analysis revealed that the peak was similar to CDD/ANP-99-126 except that it contained a single cleavage between the Cys-105 and Phe-106 bond. Cys was not determined by the sequence analysis but assigned from the known sequence. Therefore, in our experiment using CDD/ANP-99-126 as a substrate proteolysis by the membrane preparation from dog kidney caused a single cleavage within the disulfide loop between the residues in position Cys-105 and Phe-106. Thus, the enzymatic processing that inactivates CDD/ANP-99-126 is similar to that demonstrated with membrane preparations from rabbit [20] and rat kidneys [21].

As a control the cortex membrane preparation was inactivated by boiling (15 min) prior to incubation with CDD/ANP-99-126. The experiment is shown in fig.1B. Heat treatment of the membrane preparation completely destroys the proteo-

lytic activity. CDD/ANP-99–126 is not degraded during incubation for at least 90 min.

Urodilatin (CDD/ANP-95–126) which belongs to the CDD family was submitted to the same experimental procedure as described above for CDD/ANP-99–126. The experiment is shown in fig.1C. Urodilatin was incubated with the kidney cortex membrane preparation by using the same incubation procedure and concentrations. After 0, 5, 40 and 90 min aliquots were removed from the test tube and separated by HPLC. In contrast to CDD/ANP-99–126 urodilatin (CDD/ANP-95–126) was not proteolytically degraded within 5 min incubation. Only minor proteolysis was observed within 40 min incubation without further increase in product. Note that under similar conditions CDD/ANP-99–126 was completely destroyed (fig.1A). As depicted in fig.2 the total amount of urodilatin (CDD/ANP-95–126) decreases during prolonged incubation as can also be seen with CDD/ANP-99–126. This is not surprising because a crude membrane preparation was used. Therefore, one has to expect a variety of proteolytic activities [20,21,32]. However, the decrease of urodilatin is much slower. Treatment of a sample containing CDD/ANP-99–126 with the cortex membrane preparation thus enables rapid detection of the according peptide by appropriate alteration of the elution profile.

The proteolytic enzyme in rat kidney cortex membrane preparations responsible for CDD/ANP-99–126 degradation has been characterized as a metalloendoprotease [21]. The enzyme cleaves atrial natriuretic peptide (CDD/ANP-99–126) at a single cleavage site between Cys-105 and Phe-106. A similar proteolytic enzyme preparation was prepared from rabbit kidney membranes [20]. This preparation degraded CDD/ANP-99–126 as well as a shorter form of CDD/ANP-103–126 (atriopeptin III) indicating that the presence of the tetrapeptide Ser-99–Leu-100–Arg-101–Arg-102 at the amino terminus does not protect against digestion by the proteolytic activity in the membrane preparation [20]. It was reported that CDD/ANP-103–126 was not cleaved exclusively between the Cys–Phe bond. At least 3 cleavage sites have been reported [20]. This observation was confirmed as Koehn et al. [21] also report appearance of multiple products by use of shorter peptides. Our results, however, achieved by the use of a more extended peptide (CDD/ANP-95–126), demonstrate that the proteolytic enzyme in dog kidney membrane preparations which cleaves CDD/ANP-99–126 at the ring structure is inactive against urodilatin (CDD/ANP-95–126). Therefore, the tetrapeptide Thr-95–Ala-96–Pro-97–Arg, 98 prevents proteolytic attack and metabolization by the kidney while CDD/ANP-99–126 undergoes hydrolysis and inactivation. The present data demonstrate for the first time a degradation-resistent peptide that belongs to the CDD/ANP family. From the fact that urodilatin (CDD/ANP-95–126) is excreted by the kidney [27] and since the kidney may be considered as the target tissue for the peptide action, it appears logical that the secreted peptide needs a design which prevents biological inactivation.

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