Clinica Chimica Acta, 172 (1988) 199–210
Elsevier

CCA 04086

The presence and molecular forms of cardiodilatin immunoreactivity in the human and rat right atrium

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(Received 4 June 1987; revision received 29 October 1987; accepted after revision 1 November 1987)

Key words: Cardiodilatin; *N*-terminal pro-ANP; Radioimmunoassay; Chromatography; Atrium

Summary

A sensitive and specific radioimmunoassay has been developed for cardiodilatin, the N-terminal peptide sequence of the atrial natriuretic peptide (ANP) prohormone. Cardiodilatin-immunoreactivity (-IR) concentrations in the human right atrial appendage were found to correlate with ANP-IR concentrations, determined by an established radioimmunoassay, (cardiodilatin-IR = 13.2 ± 1.2 nmol/g, ANP-IR = 19.8 ± 2.0 nmol/g, $r = 0.80$, $p < 0.001$). Characterisation of the cardiodilatin-IR in the human and rat right atrium by gel permeation and fast protein liquid chromatography revealed only two cardiodilatin-IR molecular forms. The larger more hydrophobic form, the majority of the cardiodilatin-IR, contained in addition ANP-IR and therefore represents the prohormone. The smaller, less hydrophobic form, lacked ANP-IR and thus represents the cleaved N-terminal peptide sequence of the prohormone. These findings indicate that the prohormone is the major molecular form in the human and rat atrium. Furthermore, they demonstrate that a single large N-terminal peptide, cardiodilatin, derived from the prohormone, may exist as a distinct molecular form in the atrium of these species.

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Introduction

The atrial specific peptides have been isolated, and fully characterised, from the human and rat atrium in three molecular forms [1–6]. The 126 amino acid gamma atrial natriuretic peptide, or pro-ANP, alpha-ANP consisting of the 28 amino acid sequence of the C-terminal of pro-ANP and beta-ANP which is an antiparallel dimer of alpha-ANP in the human and an N-terminally extended alpha ANP in the rat [7]. A number of reports have appeared in the literature describing the presence of N-terminally extended and C- and N-terminally truncated forms of alpha-ANP in atrial tissues [8–14], which are probably extraction and purification artefacts [5,15] but the presence of the N-terminal peptide of pro-ANP, lacking alpha-ANP in its sequence, has not been reported to date.

The porcine atrial peptide cardiodilatin has been isolated in two molecular forms [16]. Cardiodilatin-126, the porcine equivalent of human pro-ANP [17] (> 80% homology) [18] and cardiodilatin-88 a N-terminally truncated form. The original use of the term cardiodilatin was to describe a partial N-terminal amino-acid sequence of the porcine atrial peptide [19] and we therefore employ this term now to describe the N-terminal peptide fragment of pro-ANP in the human and rat.

In order to investigate the presence of cardiodilatin-like immunoreactivity and its existence as a distinct product of pro-ANP in these two species we have developed a specific and sensitive radioimmunoassay employing a pure synthetic human sixteen amino-acid N-terminal pro-ANP fragment (Asn^{1}-Lys^{16} pro-ANP). Using this radioimmunoassay coupled with chromatographic fractionation we have shown pro-ANP to be the source of a second cardiac peptide, cardiodilatin, derived from its N-terminal as a single molecular form in the atrial tissues of the human and the rat.

Materials and methods

Development of antisera

Pure synthetic human Asn^{1}-Lys^{16} pro-ANP N-terminal fragment (cardiodilatin 1–16 (CDN 1–16); Peninsula Laboratories), either conjugated to bovine serum albumin with glutaraldehyde or carbodiimide, or unconjugated, was employed to raise antisera. Rabbits received a primary immunisation with 85 μg (45 nmol) of peptide in 2 ml of complete Freund's adjuvant injected in four subcutaneous sites, followed 3 months later with booster injections of 40 μg (21 nmol) of peptide in 2 ml of incomplete Freund's adjuvant. Subsequent booster injections were given at 2 monthly intervals and the animals were bled from a marginal ear vein 7–10 days after each booster injection.

All antisera were assessed for titre, avidity of binding to ^{125}I-CDN 1–16 and the degree of displacement of antibody bound to ^{125}I-CDN 1–16 by a standard concentration of pure synthetic human CDN 1–16. The ability of each antiserum to cross-react with the complete endogenous pro-ANP molecule was assessed by the degree of displacement of antibody bound to ^{125}I-CDN 1–16 by human and rat atrial extracts. The specificity of each antiserum was determined by testing the ability of up to 2 nmol additions of alpha-human-ANP to displace the binding between the antiserum and ^{125}I-CDN 1–16.

Iodination

Synthetic human CDN 1–16 (8 nmol) was iodinated using a modification of the chloramine T oxidation method [20] with 0.4 nmol $Na^{125}I$ (Amersham IMS 30) and 130 nmol chloramine T. The reaction was terminated after 10 s by the addition of 636 nmol sodium metabisulphite. Radiolabelled CDN 1–16 was purified on a HPLC Bondapak C-18 reverse phase column (3.9 × 300 mm) pre-equilibrated with 10% (v/v) acetonitrile in water containing 0.05% (v/v) trifluoroacetic acid. Initial elution with this solvent mixture for 20 min was followed by further elution with 30% (v/v) acetonitrile in water containing 0.05% (v/v) trifluoroacetic acid. The flow rate was 1 ml/min and 1 ml fractions were collected. The radioactivity of each fraction was assessed and the fractions of peak radioactivity were tested for immunoreactivity. The specific activity of the radiolabel was determined by self-displacement. The immunoreactive fraction selected for use in the assay was diluted, divided into aliquots, lyophilised, sealed in vacuo and stored at −20 °C in the dark.

Assay conditions

The standard used for the assay was the pure synthetic human CDN 1–16. Assays were set up in duplicate tubes each in a final volume of 700 μl. The buffer used was 0.06 mol/l sodium potassium hydrogen phosphate (pH 7.4) containing bovine serum albumin (30 g/l), EDTA (0.01 mol/l) and 0.05% (w/v) sodium azide. After incubation for 4 days at 4 °C the antibody bound and free cardiodilatin-IR were separated by the addition of 4 mg charcoal (Norit GSX, BDH Chemical Co.) with 400 μg dextran (Sigma Chemical Co., St. Louis, MO, USA) in 250 μl assay buffer containing 0.25% (w/v) gelatine per tube. Following centrifugation at 800 × *g* at 4 °C for 20 min, the supernatant (antibody bound peptide) was separated from the charcoal pellet (free peptide) and both bound and free fractions were counted on a gamma counter. The maximum sensitivity of the assay was calculated by determining the smallest standard addition of CDN 1–16 that produced a change of the percentage bound radioactivity that was greater than two standard deviations of the variation of the binding in the absence of cold peptide (zero standard).

ANP-immunoreactivity was measured by a well established radioimmunoassay developed in our laboratory [21].

Tissue extraction

Human right atrial appendage samples ($n = 20$) were obtained at the time of cardiac surgery for coronary artery by pass grafting and transported fresh to the laboratory. Samples were obtained with minimal tissue trauma before cannulation of the right atrium for cardiopulmonary bypass. The patients were in stable haemodynamic conditions and at normothermia at the time of tissue sampling.

Wistar rats ($n = 13$) fed on standard laboratory food and water for at least 5 days were killed by stunning and cardiac tissues obtained by immediate dissection. Tissues were weighed when fresh and extracted by boiling in 0.5 mol/l acetic acid (10% w/v) for 10 min. The extracts were stored at −20 °C until assay.

Chromatography

Fractionation of cardiodilatin and ANP-IR in tissue extracts was undertaken on

a 0.9 cm × 90 cm Pharmacia gel permeation column containing 60 ml of G-100 Sephadex. The column was eluted at a flow rate of 4.8 ml/h with assay buffer containing in addition 0.2 mol/l NaCl. Twelve-minute fractions (0.96 ml) were collected and an aliquot of each fraction was assayed in duplicate. Dextran blue, horse heart cytochrome C and K ^{125}I were included as markers of the void volume, 12 500 Da molecular size and the total volume, with each run. Cardiodilatin 1–67 N-terminal pro-ANP fragment (CDN 1–67; Peninsula), and alpha-human or rat-ANP were chromatographed separately and their elution positions determined by radioimmunoassay.

Fast protein liquid chromatography (FPLC, Pharmacia) was performed with a PEPRPC HR5/5 reverse phase C18 high resolution column. The column was eluted at a flow rate of 1 ml per minute and equilibrated with 20% (v/v) acetonitrile in water containing 0.05% (v/v) trifluoroacetic acid. Following the addition of a sample the column was eluted by a linear gradient of 20% to 30% (v/v) acetonitrile in water containing 0.05% (v/v) trifluoroacetic acid over 10 min, followed by further elution by a linear gradient of 30% to 40% (v/v) acetonitrile in water containing 0.05% (v/v) trifluoroacetic acid over 50 min. One-millilitre fractions were collected and aliquots of each fraction were assayed in duplicate. Synthetic human CDN 1–67, and alpha human or rat ANP were used as standard markers in runs before and after the samples.

Results

Antisera

A number of rabbits produced antisera to cardiodilatin but only one of these, antiserum XA3 produced by a rabbit immunised with the CDN 1–16 glutaraldehyde conjugate, was of sufficiently high titre and avidity for assay purposes. It not only exhibited the best displacement curve of antibody bound to ^{125}I CDN 1–16 by unlabelled CDN 1–16 but also demonstrated the highest cross-reactivity with endogenous pro-ANP in both human and rat. As the complete endogenous molecule (pro-ANP 1–126) was not available synthetically formal cross-reactivity studies could not be performed, but this antiserum could detect the highest concentrations of cardiodilatin-IR and exhibited the best correlation with ANP concentrations, determined by the ANP radioimmunoassay, in the same human atrial extracts. The cross-reactivity of the antiserum with human synthetic CDN 1–16 was 100% and with CDN 1–67 44%. The antiserum showed no cross-reactivity with alpha-human-ANP. The avidity of antibody binding to antigen (CDN 1–16), as determined by Scatchard analysis [20] was 2.5×10^{10} 1/mol. The antiserum was used in a final dilution of 1 : 3 500.

Iodination

The iodinated CDN 1–16 was eluted from the HPLC column by a concentration of 30% (v/v) acetonitrile in water containing 0.05% (v/v) trifluoroacetic acid, in a single radioactivity peak (specific activity of 60–70 Bq/fmol). In the antibody-free tube, non-specific binding was $< 3\%$, zero standard binding was 45–50% and excess antibody binding was 85–90%.

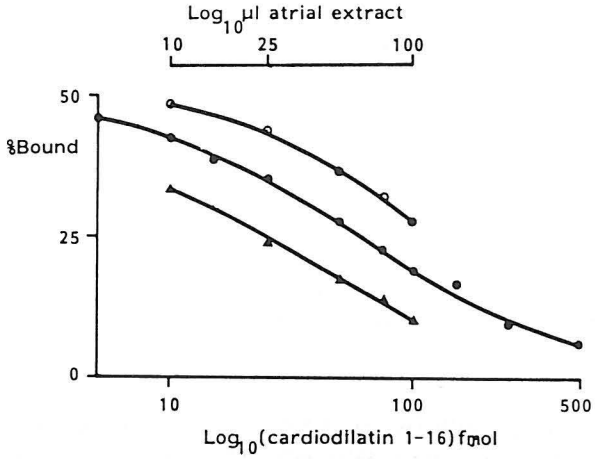

Fig. 1. Standard curve of synthetic human cardiodilatin 1–16 (●) showing parallelism with serial dilution curves of human (▲) and rat (○) right atrial extracts. Vertical axis, percentage ^{125}I CDN 1–16 bound; horizontal axis, $\log_{10}$ scale of CDN 1–16 concentration (bottom) and volume of human and rat atrial extracts (top).

Assay conditions

The least quantity of cardiodilatin-IR reliably detected by the assay at 95% confidence was 4 fmol/tube. The amount of added pure synthetic CDN 1–16 required to reduce to half the initial percentage binding was 70 fmol. A representative standard curve is shown in Fig. 1.

Tissue concentrations

Serial dilutions of human and rat right atrial extracts inhibited the binding of the radiolabel to the antibody in parallel with the standard curve (Fig. 1).

The concentrations of cardiodilatin-IR (expressed as CDN 1–16 IR-equivalents) and of ANP in samples of the human right atrial appendages and rat cardiac tissues are shown in Table I.

TABLE I

Cardiodilatin and ANP concentrations in human and rat cardiac tissues

Species	Tissue	CDN-IR [a]	ANP-IR [b]
Human	Rt Atrium	13.2 (1.2)	19.8 (2.0)
Rat	Rt Atrium	17.4 (1.8)	102.6 (10.5)
Rat	Lt Atrium	15.4 (2.0)	86.8 (5.4)
Rat	Rt Ventricle	0.03 (0.007)	0.32 (0.07)
Rat	Lt Ventricle	0.01 (0.004)	0.23 (0.04)
Rat	Interventricular septum	0.01 (0.003)	0.14 (0.02)

[a] Cardiodilatin-immunoreactivity.

[b] alpha-Human ANP-immunoreactivity.

All values are mean (SEM) nmol/g wet tissue.

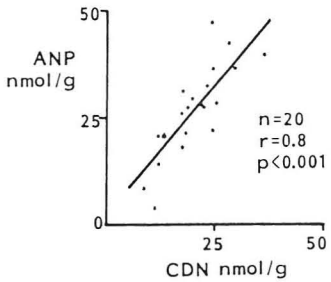

Fig. 2. Relationship between cardiodilatin and ANP concentrations in the human right atrial appendage ($n = 20$) by linear regression analysis. ANP, alpha-human ANP-IR; CDN, cardiodilatin-IR; r, correlation coefficient.

The mean of the ratios of cardiodilatin-IR to ANP in human right atrial appendage extracts was 0.71 ± 0.04 ($p < 0.01$). The relationship between cardiodilatin-IR and ANP concentrations in human right atrial appendage extracts (linear regression analysis) is shown in Fig. 2. with a correlation coefficient of 0.80 ($p < 0.001$).

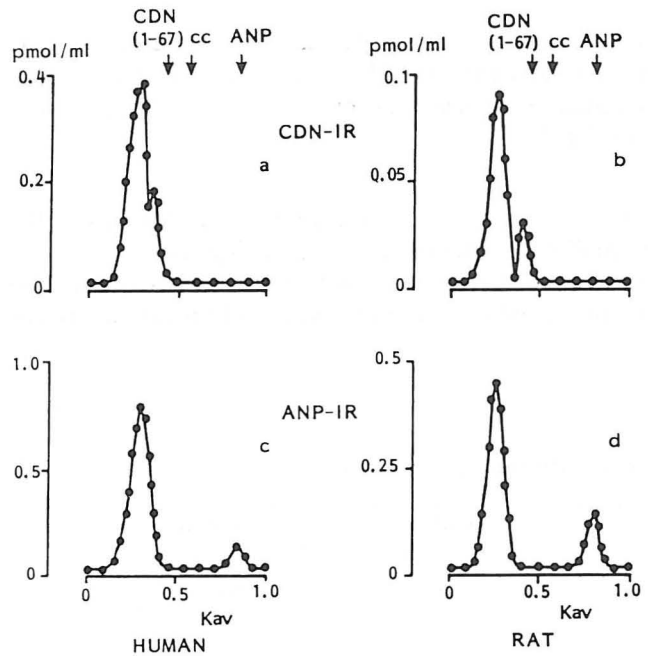

Fig. 3. Cardiodilatin (a and b) and ANP (c and d) radioimmunoassay of gel permeation chromatographic fractions of human (a and c) and rat (b and d) right atria. Two cardiodilatin-IR peaks are demonstrated. The major peak coelutes with the major ANP-IR peak. The smaller peak lacks ANP-IR. CC, horse heart cytochrome C; CDN 1–67, synthetic human cardiodilatin asn^{1}-arg^{67}; ANP, alpha-human-ANP; K_{av}, elution coefficient (horizontal axis); Vertical axis, pmol –IR/ml fraction.

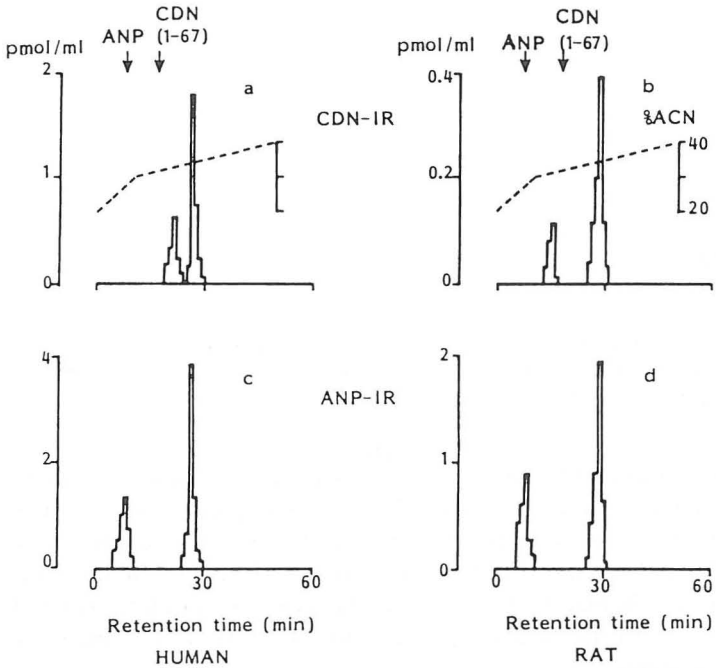

Fig. 4. Fast protein liquid chromatography of human (a and b) and rat (c and d) atria coupled with cardiodilatin (a and b) and ANP (c and d) radioimmunoassay. The major cardiodilatin peak also contains ANP-IR. The small, less hydrophobic peak lacks ANP-IR. The column was eluted with an increasing gradient of acetonitrile (ACN), from 20% to 40%.

Chromatography

Human right atrial appendage extracts fractionated by gel permeation chromatography and by FPLC, and assayed for cardiodilatin and ANP, revealed two immunoreactive peaks of each. The larger molecular size cardiodilatin-IR peak (K_{av} 0.30) on gel chromatography (Fig. 3a) and the cardiodilatin-IR peak of greater hydrophobicity on FPLC (Fig. 4a) contained in addition ANP-IR (Figs. 3c and 4c). The smaller molecular form cardiodilatin-IR peak (K_{av} 0.36) on gel chromatography (Fig. 3a) and the less hydrophobic molecular form cardiodilatin-IR peak on FPLC (Fig. 4a) lacked ANP-IR. The synthetic human CDN 1–67 marker eluted later (K_{av} 0.44) on gel chromatography and earlier on FPLC. A solely ANP-IR peak was also present co-eluting with synthetic alpha-human ANP in both chromatographic systems. The fractions constituting the less hydrophobic molecular form FPLC cardiodilatin-IR peak (lacking ANP-IR) when pooled and chromatographed by gel permeation (after evaporation of the acetonitrile in a Savant Vacuum centrifuge) revealed a single cardiodilatin-IR peak (Fig. 5) co-eluting with the smaller molecular size cardiodilatin-IR peak (K_{av} 0.36) of fractionated human right atrial extracts.

Gel permeation chromatography (Fig. 3b and d) and FPLC (Fig. 4b and d) of rat right atrial extracts coupled with cardiodilatin and ANP radioimmunoassay, re-

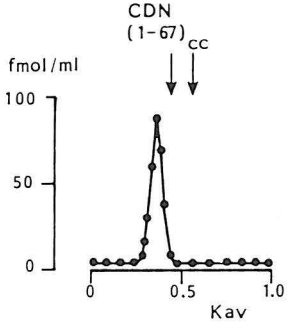

Fig. 5. Gel permeation chromatography of the less hydrophobic human atrial FPLC cardiodilatin peak. A single peak of cardiodilatin-IR eluted at the same position as the smaller cardiodilatin-IR peak obtained by gel chromatography of extracts of human atria.

vealed profiles similar to those obtained with the human tissue. Thus, two cardiodilatin-IR peaks were demonstrated. The first gel chromatographic peak (K_{av} 0.26) and the second FPLC peak also contained ANP-IR. The second gel chromatographic peak (K_{av} 0.40) and the first FPLC peak lacked ANP-IR and neither peak co-eluted with the synthetic human CDN 1–67 standard (no rat standard available synthetically). The solely ANP-IR peak co-eluted with rat alpha ANP standard in both chromatographic systems.

In both species the cardiodilatin-IR peaks that possessed ANP-IR always constituted > 65% of the total cardiodilatin-IR recovered, which was between 85–100%.

Discussion

A specific radioimmunoassay for cardiodilatin, the N-terminal peptide sequence of pro-ANP has been described. The antiserum used was raised in a rabbit immunised with a peptide fragment consisting of the first sixteen amino acid sequence of the N-terminal of pro-ANP. This sequence exhibits no homology with alpha-ANP and hence the antiserum showed no cross-reactivity with it. As the antiserum was raised to a pro-ANP fragment it would be expected to cross react with pro-ANP itself. This was demonstrated by the presence of nearly 50% cross-reactivity of the antiserum with the synthetic 67 amino acid N-terminal pro-ANP sequence. In addition, the cross-reactivity of the antiserum with the complete pro-ANP molecule (although this could not be determined precisely because synthetic pro-ANP was not available) was demonstrated by the parallelism obtained between serial dilutions of human and of rat right atrial extracts and the standard curve of synthetic CDN 1–16. This specific cross-reactivity is further substantiated by the highly significant correlation found in human right atrial extracts between cardiodilatin-IR concentrations, measured by the antiserum to the N-terminal of pro-ANP, and ANP-IR concentrations, measured by an established radioimmunoassay [21] with an antiserum to the C-terminal of pro-ANP. Therefore, this newly developed radioimmunoassay offers an alternative to the ANP radioimmunoassay

for the estimation of pro-ANP concentrations in human atrial tissues. Approximately 85% homology exists between human and rat pro-ANP [22] with three of the seventeen amino acid substitutions occurring in the N-terminal sixteen amino acid sequence. Therefore, the antiserum raised against synthetic human CDN 1–16 would be expected to cross-react less well with rat pro-ANP. This offers the most likely explanation for the observed discrepancy between cardiodilatin-IR and ANP-IR concentrations in rat cardiac tissues.

The chromatographic data demonstrate the presence of only two molecular forms of cardiodilatin-IR in the human and rat right atrium. Tissues were extracted under conditions which inhibit proteolysis [20] thus minimising the possible generation of artefactual forms. The atrial peptides are stored mainly in the pro-hormone form as has been demonstrated in rat atrial tissues [3,23,24] and rat atrial cell cultures [25]. In the present study we have demonstrated that the large molecular size (on gel chromatography) and more hydrophobic (on FPLC) ANP-IR peak, which represents the prohormone, is identical to the large molecular size and more hydrophobic cardiodilatin-IR peak. Thus the two radioimmunoassays, employing antisera raised to the extreme C- and N-terminals of pro-ANP, detect the same large molecular form in human and rat right atria. Therefore, the large molecular size and more hydrophobic cardiodilatin-IR peak is pro-ANP itself. This peak contained most of the -IR recovered thus indicating that the prohormone is the major molecular form in the rat right atrium confirming previous reports. In addition, we have shown that pro-ANP is the major molecular form in the human right atrium.

Antisera raised to N-terminal fragments of pro-ANP have been used in radioimmunoassays and immunocytochemical studies to investigate pro-ANP immunoreactivity in the heart and brain of several species [16,25–27]. The N-terminal antiserum employed in this study has not been previously employed by other groups. The use of this antiserum has enabled us to identify and characterise chromatographically a second cardiodilatin-IR molecular form in extracts of human and rat right atrium. This form is distinct from pro-ANP in that it lacks ANP-IR. The presence of alpha-ANP in human and rat atria has been previously described [1,2,5]. There have been no reports, though, of the presence of the N-terminal sequence of pro-ANP as a separate molecular form in atrial tissue extracts as demonstrated by the present study. The results presented here suggest that although pro-ANP is the major molecular form in the human and rat right atrium, a proportion of pro-ANP may undergo processing within atrial cells into its two constituent peptide fragments alpha-ANP (the 28 amino-acid sequence of the C-terminal of pro-ANP) and cardiodilatin (derived from the N-terminal sequence). The amino acid sequence and molecular size of cardiodilatin have not yet been determined. Further work involving purification of the peptide from animal atria which can then be subjected to sequence analysis as well as SDS PAGE of the tissues themselves will clearly be required. The data presented here in conjunction with the known sequence of pro-ANP allow us to postulate that cardiodilatin contains at least the first 67 amino acid N-terminal sequence of pro-ANP. It has been suggested that cleavage of pro-ANP at the Arg^{67}-Asp^{68} bond could yield an N-terminal peptide of pro-ANP [17]. The presence of such a 67 amino acid residue peptide has never been

demonstrated. In the present study the endogenous cardiodilatin-IR was shown to be of greater molecular size and hydrophobicity than synthetic CDN 1–67. Pro-ANP processing at the Arg^{98}-Ser^{99} bond yields alpha-ANP. Such processing can, in addition yield cardiodilatin, containing the complete N-terminal sequence of pro-ANP, ie a 98 amino acid peptide. Confirmation of this will be required by further work, as indicated above, and by the use of C-terminally directed antisera to cardiodilatin. This will establish whether cardiodilatin contains the complete N-terminal 98 amino acid sequence of pro-ANP or whether further processing occurs at any of the three arginine residues between Arg^{67} and Arg^{98}.

Acknowledgement

This study was supported by the Wynand M. Pon Foundation, Leusden, The Netherlands.

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