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ATRIOPEPTINS: A FAMILY OF POTENT BIOLOGICALLY ACTIVE PEPTIDES DERIVED FROM MAMMALIAN ATRIA

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SUMMARY: Extracts of rat atria are potent stimulators of sodium and urine excretion, and relax vascular and intestinal smooth muscle preparations. The structures of six biologically active peptides obtained from atrial extracts are reported here. Ion exchange chromatography of a low molecular weight fraction obtained by gel filtration of atrial extracts produced two natriuretic fractions: the first induced relaxation of intestinal smooth muscle strips only, whereas the second also relaxed vascular strips as well. From the first fraction four pure biologically active peptides obtained by reverse phase HPLC have been sequenced: the 21 amino acid peptide, designated atriopeptin I, and three homologs (des-ser^{1}-, des-ser^{1}-ser^{2}-, and des-ser^{21}-atriopeptin I). From the second fraction two pure biologically active peptides were obtained, which had C-terminal extensions of atriopeptin I: atriopeptins II (23 amino acid residues) and III (24 residues), having respectively phe-arg and phe-arg-tyr C-termini. These results suggest that this family of six peptides, sharing the same 17 membered ring formed by an internal cystine disulfide, is derived from a common high molecular weight precursor.

Extracts of cardiac atria contain peptides which are candidates for the endocrine regulation of volume homeostasis by modulating the excretion of sodium, water, and vascular resistance (1-7). Gel filtration chromatography of rat atrial extracts produced a high molecular weight fraction which had natriuretic activity (*in vivo*) and a low molecular weight fraction which exhibited potent spasmolytic activity (*in vitro*) as well as natriuretic activity (*in vivo*) (4-7). Gentle proteolysis (*in vitro*) of the high molecular weight fraction markedly increased the smooth muscle relaxant activity of this fraction and resulted in the formation of product(s) that now chromatographically co-migrated with the low molecular weight fraction (7).

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We have resolved the low molecular weight fraction into at least six biologically active peptides. The sequence of the two major fractions indicated the presence of a 21 amino acid peptide (designated atriopeptin I) and a 23 amino acid peptide (atriopeptin II) (8). Atriopeptin I relaxes intestinal but not vascular smooth muscle and is natriuretic and diuretic *in vivo*. Atriopeptin II (having the atriopeptin I sequence with a phe-arg C-terminal extension) relaxes both vascular and intestinal smooth muscle as well as being a potent natriuretic-diuretic *in vivo* (8). In the current investigation we have isolated and characterized the remaining low molecular weight fractions and find that they are closely related substances apparently derived from a common precursor.

MATERIALS AND METHODS

Purification of Peptides - Fourteen hundred frozen rat atria (Biotrol, Indianapolis, IN) (153 gm wet wt) were homogenized in 10 volumes of phosphate buffered saline containing phenylmethylsulfonyl fluoride and pepstatin (Sigma) (each 1 μg/ml) and centrifuged (2500 x g 10 min). The supernatant (10 ml aliquots in 18 x 150 mm glass tubes) was immersed in a 100° water bath for 10 min, then chilled and centrifuged (10,000 x g 10 min). This supernatant was made 0.5 M in acetic acid and centrifuged again (27,000 x g 10 min). The acid supernatant (in 300 ml aliquots) then was applied to a Sephadex G-15 column (8 x 36 cm) and eluted with 0.5 M acetic acid (600 ml/hr). The protein fraction from this column was lyophilized, taken up in 0.5 M acetic acid, and 40 ml aliquots applied to a Sephadex G-75 column (5 x 90 cm) eluted with 0.5 M acetic acid (96 ml/hr). The low molecular weight fraction from the G-75 column (4) was lyophilized, then applied to a column of SP-Sephadex C-25 (20 g gel, 5 x 7 cm) in 25 mM ammonium acetate - 0.5 M acetic acid and eluted with a linear gradient of ammonium acetate (23.4 mM increment/hr at 96 ml/hr) in 0.5 M acetic acid. Two fractions which were biologically active were obtained: I (at 160 mM ammonium acetate) which relaxed intestinal smooth muscle strips only, and II (at 270 mM) which relaxed both intestinal and vascular smooth muscle. Fractions I and II were lyophilized and each subjected to high pressure liquid chromatography on a Brownlee RP-300 Aquapore column (4.6 mm x 25 cm) using a mixture of A (0.1% trifluoroacetic acid/acetonitrile) and B (0.1% trifluoroacetic acid/water) at 1 ml/min. Chromatography of I consisted of (a) 0 to 10%A in 3.8 min, (b) 10 to 14.8%A in 60 min, and (c) 14.8 to 16.4%A in 100 min; atriopeptin (AP) I appeared at 15.6%A, des-ser^{1} AP I and des-ser^{1}-ser^{2}- AP I (mixture) at 15.7%A, and des-ser^{21}-AP I at 15.8%A. Chromatography of II was done by (a) 0 to 16%A in 5.8 min, and (b) 16 to 22.4% in 80 min; AP II was obtained at 19.6% and AP III at 21.1%A. Each of these fractions was reapplied to a Vydac octadecasilyl column (300 A pore, 4.6 mm x 25 cm) at 1 ml/min using a mixture of A (0.05% trifluoroacetic acid/acetonitrile) and B (0.05% trifluoroacetic acid/water) employing a gradient of 0 to 30%A in 30 min. AP I appeared at 29.5%, des-ser^{1}-AP I and des-ser^{1}ser^{2}-AP I at 29.7% (these peptides could not be resolved by HPLC but were distinguishable during the N-terminal sequence analysis), des-ser^{21}-AP I at 29.9%. AP II appeared at 31.5% and AP III at 32.2%A in a gradient of 10 to 35% over 25 min.

Analysis of peptides. The atriopeptins were reduced and alkylated as follows. To each peptide (0.5-5 nmoles) in 90 μl 2% SDS in 0.4 M Tris acetate

pH 9.0, 10 µl 100 mM dithiothreitol was added, flushed with N_2, and incubated at 37° for 60 min. Then 20 µl of 120 mM iodoacetamide (3X recryst., freshly made) was added, flushed with N_2 and allowed to stand at room temperature for 10 min, when the mixture was dialyzed against 0.1% SDS for 2 hr, redialyzed overnight, then lyophilized. The peptides were sequentially degraded utilizing an Applied Biosystems Model 470 Gas Phase Sequencer (9). Thirty or more cycles were completed in each run with one degradation each for: the reduced and alkylated AP I (600 pmoles sequencer yield), des-ser^1-AP I (660 pmoles), des-ser^1-ser^2-AP I (650 pmoles), des-ser^{21}-AP I (520 pmoles), AP II (1200 pmoles), and AP III (850 pmoles). Phenylthiohyadantoin amino acids were identified using high performance liquid chromatography as a adapted from Hunkapiller and Hood (9,10). Average repetitive cycle yields were greater than 90% for each cycle whose signal allowed accurate quantitation. The protein concentration of the purified peptides was determined by a modification of the Lowry method (11).

Bioassays. Precontracted spiral strips of rabbit thoracic aorta and chick rectum were employed for the spasmolytic assay of the atrial peptides as previously described (4). The natriuretic-diuretic assay was performed as previously described (7).

RESULTS AND DISCUSSION

The purification protocol employed to produce peptides for sequence analysis is shown in Table 1. The low molecular weight fraction obtained by chromatography on Sephadex G-75 was separated into fractions I and II following purification by ion exchange. Final processing of fractions I and II by HPLC reverse phase yielded atriopeptin I (and three homolog peptides) from fraction I, and atriopeptins II and III from fraction II.

The results of sequence analyses of the six peptides are summarized in Fig. 1. All contain the same 17 member disulfide ring and differ only in their amino- and carboxy-terminal extensions. C-terminal analysis of atriopeptins I, II and III were carried out following incubations with carboxypeptidase-Y followed by amino acid analysis on HPLC. Atriopeptin I was a poor substrate for the carboxypeptidase only generating a weak serine signal. Short term incubation (10 sec) of atriopeptin II with the carboxypeptidase initially gave a stong arg signal and a weaker phe peak (i.e., 0.24 phe to 1.0 arg) while longer incubation (10 min) resulted in a strong arg and phe peak and a weak ser (i.e. a 1.00:1.24:.05 ratio of the 3 residues). Atriopeptin III initially (1 min) gave a strong tyr, arg, and a phe peak (i.e. 1.00:0.90:0.55 ratio) but on longer incubation all 3 residues were pronounced and a weak ser was present (i.e. 0.99:1.02:1.00:0.12 ratio).

Table 1. Purification of rat atrial peptides from 153 g tissue (atria from 1400 hearts). The biological activity was determined on intestinal smooth muscle strips (chick rectum). Quantitation was achieved by performing dose response curves with each peptide in comparison to the response to a standard curve achieved with isoproterenol (the intestinal relaxant). One unit of activity was set to be equivalent to 1 ng of isoproterenol.

	Total Protein mg	Specific Activity Units/mg		Total Activity Units	Recovery Percent
Crude	5764	7.7		44,000	
Homogenate					
Boiled Extract	728	154	(1X)	112,000	100%
Sephadex G-15	262	265	(1.7X)	69,400	62%
Sephadex G-75	17.0	2,890	(18.8X)	49,100	44%
SP Sephadex C-25:					
I	1.54	11,300	(73.4X)	17,400	16%
II	1.20	7,620	(49.5X)	9,140	8%
HPLC Fractions:					
Atriopeptin (AP) I	0.114	37,600	(244X)	4,280	3.8%
des-ser^1-API des-ser^1-ser^2-API	0.049	107,000	(695X)	5,220	4.6%
des-ser^{21}-API	0.073	62,500	(406X)	4,550	4.1%
Atriopeptin II	0.081	52,400	(340X)	4,260	3.8%
Atriopeptin III	0.061	62,600	(406X)	3,810	3.3%

A quantitative comparison of the biological activity of the various atrial peptides indicates that neither loss of serine residues on the N- or C-termini nor C-terminal extensions (as in atriopeptin II or III) markedly change the spasmolytic potency of the 6 atriopeptins on intestinal smooth

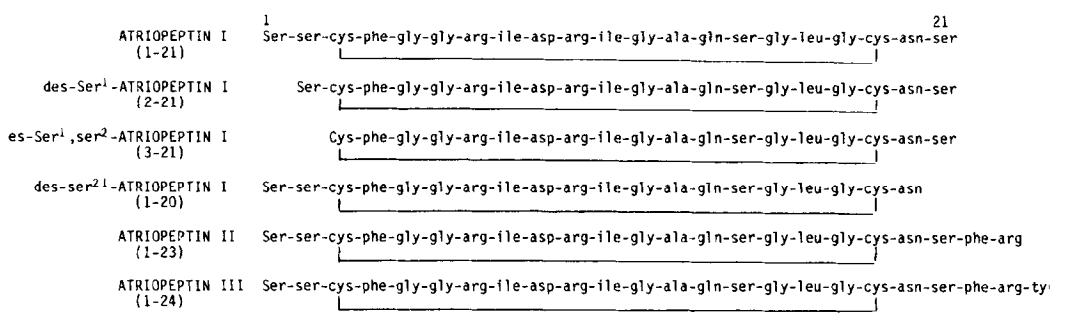

Figure 1. Structures of peptides derived from the low molecular weight fraction of atrial extracts.

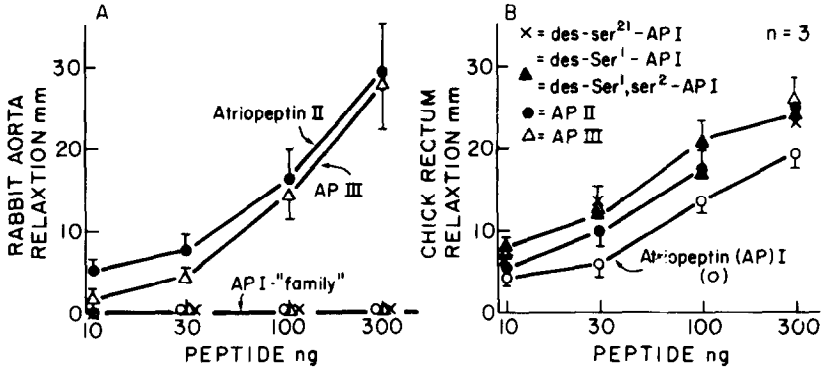

Figure 2. Comparative quantitative effects of atrial peptides as spasmolytic agents on smooth muscle strips. Left panel - The dose response curve for relaxation of rabbit thoracic aorta strips. API - "family" indicates atriopeptin I and its homologs (des-ser^{1}, des-ser^{1}-ser^{2}-, and des-ser^{21}). Right panel - The dose response curve for relaxation of chick rectum strips. The values are the means ±SEM for preparations made from 3 animals.

muscle strips (Fig. 2B). However, the lack of the phe-arg extension in the atriopeptin I and its homologs precludes vasorelaxant activity (Fig. 2A). Atriopeptin II and III are equipotent as blood vessel relaxants *in vitro* (Fig. 2A). Atroipeptin I, II, and III when injected intravenously as a 2 μg bolus exhibited a comparable natriuretic-diuretic response causing an 11-19 fold increase in $U_{Na}V$. The des-ser-atriopeptin I homologs (i.e., 1-21, 2-21 and 3-21, and 1-20) were less potent producing a 2 fold increase in $U_{Na}V$ with a 2 μg dose.

The common structure which comprises the bulk of each of the six peptides obtained from the low molecular weight fraction of atrial homogenates is the 17 member ring (Fig. 1) most likely derived from a single high molecular weight precursor. Gentle proteolysis of the high molecular weight fraction generated low molecular weight products which comigrate with fractions I and II derived from our low molecular weight fraction (7). Both Flynn *et al.* (12) and Kangawa and Matsuo (13) have very recently reported the structure of an atrial peptide with a ser-leu-arg-arg extension on the N-terminal of the peptide we designate as atriopeptin III in this paper. The human peptide described by Kangawa and Matsuo (13) contains a met for ileu^{12} substitution. The presence of the arg-arg in the N-terminal sequence predicts a logical *in vivo* site of enzymatic attack and suggests that atriopeptin III may be the

smallest likely candidate peptide to possess the full vascular and natriuretic-diuretic biological activity following N-terminal proteolysis.

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