Purification and Sequence Analysis of Bioactive Atrial Peptides (Atriopeptins)

Abstract. *Mammalian cardiac atria have several biologically active peptides that exert profound effects on sodium excretion, urine volume, and smooth muscle tone. In the present study two such peptides of low molecular weight were purified and separated from each other on the basis of differences in charge, hydrophobicity, and biological profile. The first peptide, designated atriopeptin I, exhibits natriuretic and diuretic activity and selectivity relaxes intestinal smooth muscle but not vascular smooth muscle strips. The second peptide, atriopeptin II, is a potent natriuretic and diuretic that relaxes both intestinal and vascular strips. Sequence analysis of atriopeptin I indicates that it is composed of 21 amino acids, of which serine and glycine residues predominate. The amino terminal sequence of atriopeptin II up to residue 21 is the same as that of atriopeptin I, with the addition of the Phe-Arg extension at the carboxyl terminus. Both peptides appear to be derived from a common high molecular weight precursor (designated atriopeptigen); their biological selectivity and potency may be determined by the site of carboxyl terminal cleavage.*

Mammalian atria contain peptides that exert potent effects on kidney function and regional vascular resistance. Atrial extracts have natriuretic, diuretic, and smooth muscle relaxant activities (*1–7*). Initial characterization of the atrial extracts indicated that the active substances are heat-stable and sensitive to proteolytic inactivation (*1, 3, 4*). The atrial extract was purified by gel filtration column chromatography (Sephadex G-75), yielding two active fractions with apparent molecular weights of 20,000 to 30,000 and less than 10,000 (*4*). Mild proteolytic digestion of the high molecular weight fractions resulted in material that had increased biological activity and that comigrated with the active low molecular weight fraction (*7*). These results suggest that the high molecular weight material may be a precursor to the more active low molecular weight species. Subsequent purification of the low molecular weight fraction by ion-exchange chromotography (SP-Sephadex) resolved two major peaks of biological activity. The first peak possessed potent chick rectum relaxant activity and was natriuretic and diuretic. The second peak was vasorelaxant in addition to relaxing intestinal smooth muscle and being natriuretic and diuretic (*7*). In the present study we purified to homogeneity and characterized two of the low molecular weight atrial peptides.

We developed a processing scheme that results in pure peptides suitable for structural analysis (see legend to Fig. 1). The lyophilized chick rectum relaxant fraction obtained from the ion-exchange (SP Sephadex) column (*7*) was fractionated by reversed-phase (Brownlee) high-pressure liquid chromatography (HPLC). The major fraction was then purified to homogeneity by HPLC on a Vydac column. This material (purified 244 times from boiled atrial extract), designated atriopeptin I, caused a concentration-dependent relaxation of chick rectum strips at 10 to 100 ng but did not relax precontracted aorta strips at doses as high as 1000 ng (Table 1). The pure (340-fold purification from boiled atrial extract), low molecular weight rabbit aorta relaxant factor, designated atriopeptin II, is a potent vascular smooth muscle relaxant in vitro (threshold aorta relaxation achieved with 10 ng). Both peptides are equipotent as intestinal smooth muscle relaxants (10 to 100 ng). A relative determination of the effectiveness of the atriopeptins in vivo was precluded by the limited amount of material available. However, atriopeptin I was clearly natriuretic and diuretic, being about one-fourth as potent as atriopeptin II. Intravenous administration of 3 μg of atriopeptin I or 1 μg of atriopeptin II was required to induce a 10- to 15-fold increase in urinary sodium excretion (Table 1).

Results of the gas-phase sequence analyses of atriopeptins I and II are presented in Fig. 1. The sequences of the initial 21 amino acids of both atriopeptins

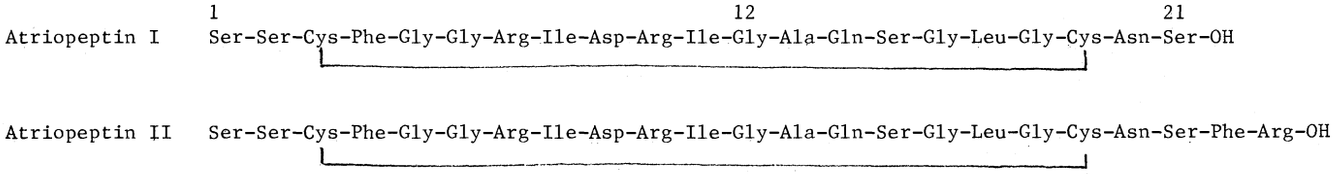

Fig. 1. Amino acid sequences of the two primary bioactive low molecular weight atrial peptides. Rat atria were homogenized in the presence of phenylmethylsulfonyl fluoride (1 μg/ml) and pepstatin (1 μg/ml) and centrifuged at 2500*g* for 10 minutes. The supernatant was heated (100°C) in phosphate-buffered saline (10 volumes per gram of tissue sample), centrifuged, and lyophilized (*4, 7*). The atrial extract was then applied to a G-15 Sephadex column (8 by 36 cm) and eluted with 0.5*M* acetic acid (600 ml per hour). This was followed by chromatography of the lyophilized residue on G-75 Sephadex column (5 by 90 cm) eluted with 0.5*M* acetic acid at 96 ml per hour. The lyophilized low molecular weight fraction from the G-75 column (derived from 1200 atria) was applied to SP-Sephadex C-25 (20 g of gel in a column 5 by 7 cm) in 25 m*M* ammonium acetate in 0.5*M* acetic acid and eluted with a linear gradient of ammonium acetate (23.4 m*M* per hour at 96 ml per hour) in 0.5*M* acetic acid. Active fractions eluted at 160 m*M* (atriopeptin I) and at 270 m*M* (atriopeptin II). After freeze-drying these were individually purified by reversed-phase HPLC on a Brownlee RP-300 Aquapore column (4.6 mm by 25 cm) with the following gradients at 1.0 ml/min. Atriopeptin I: 0 to 10 percent solvent A (see below) for 3.8 minutes, then 10 to 14.8 percent A for 60 minutes, then 14.8 to 16.4 percent for 100 minutes. The atriopeptin I (major peak) eluted at 15.6 percent A. Atriopeptin II: 0 to 16 percent A for 3.6 minutes and then 16 to 22.4 percent A for 80 minutes. The atriopeptin II eluted at 19.6 percent A. The solvent system on the Brownlee column was A = 0.1 percent trifluoroacetic acid in acetonitrile and B = 0.1 percent trifluoroacetic acid in water. The bioactive fractions were reapplied on a Vydac column (octadecasilyl reversed-phase; 300-Å pore size; 4.6 mm by 25 cm) and eluted at 1.0 ml/min with a gradient of 10 to 35 percent A over 25 minutes. The solvent system used on the Vydac column was A = 0.05 percent trifluoroacetic acid in acetonitrile and B = 0.05 percent trifluoroacetic acid in water. Atriopeptin I appeared at 29.7 percent and atriopeptin II appeared at 31.0 percent A. The polypeptides were sequentially degraded with an Applied Biosystems model 470A gas phase sequencer (*12*). Thirty or more cycles were completed in each run, with one degradation each for atriopeptin I (yield, 665 pmole), reduced and alkylated atriopeptin I (600 pmole), and atriopeptin II (1178 pmole). The atriopeptins were reduced and alkylated by dissolving the peptide (0.5 to 5 nmole) in 90 μl of 2 percent sodium dodecyl sulfate (SDS) in 0.4*M* tris acetate (*p*H 9.0). Then 10 μl of 100 m*M* dithiothreitol was added and the solution was flushed with N_2, capped, and incubated at 37°C for 60 minutes. Next, 20 μl of a fresh solution of 120 m*M* iodoacetamide (which had been recrystallized three times) was flushed with N_2, capped, incubated at room temperature for 10 minutes, transferred to boiled dialysis tubing, dialyzed against 0.1 percent SDS for 2 hours, redialyzed overnight, and lyophilized. Phenylthiohydantoin amino acids were identified with HPLC (*13*). Average repetitive cycle yields were greater than 90 percent for each cycle whose signal allowed accurate quantitation. The abbreviations for the amino acid residues are as follows: Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Cys, cysteine; Gln, glutamine; Gly, glycine; Ile, isoleucine; Leu, leucine; Phe, phenylalanine; and Ser, serine.

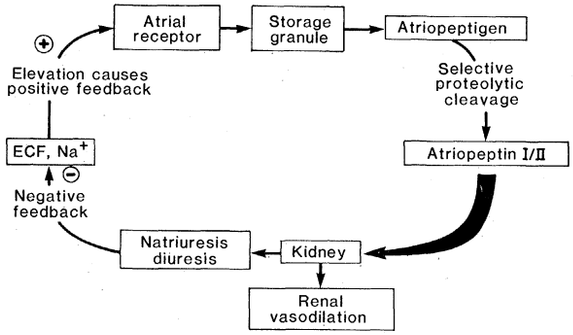

Fig. 2. Hypothetical model for the storage, release, and activation of the bioactive atrial peptides (*ECF*, extracellular fluid).

are exactly the same. The peptides exhibit numerous serine and glycine residues, with the difference consisting of the carboxyl terminal Phe-Arg of atriopeptin II. The amino acid analyses of the two peptides are consistent with the sequencing data and indicate that atriopeptin II contains a higher content of phenylalanine and arginine (Table 2). Two separate carboxyl terminal analyses (*8*) of atriopeptin II clearly indicate the presence of the additional Phe-Arg terminal sequence. The amino terminal sequence analysis of 2 to 3 nmole of atriopeptin II (purified from 5000 rat atria) provided a strong signal for the 23 amino acids that terminated as Phe-Arg. On the other hand, atriopeptin I was resistant to carboxyl terminal analysis. Others have had difficulty with carboxypeptidase digestion of amino acid residues adjacent to a cystine disulfide bridge (*9*). We found that reductive alkylation with dithiothreitol and iodoacetamide abolished the biological activity of atrial extracts; this is consistent with, but not proof of, the requirement for an internal disulfide bridge. In recent analyses of atrial extracts 36 or 47 amino acid residues rich in serine and glycine have been reported (*10, 11*). Obviously, we are studying a smaller peptide, and the differences may reflect modification of the peptides by proteolysis during the extraction procedure. However, the peptides we sequenced are biologically active and may approach the size of the minimum effective structure, as evidenced by the loss of the vascular relaxant activity of atriopeptin I.

An understanding of the source, receptor recognition, processing, and regulation of bioactive peptides requires elucidation of the amino acid composition and sequence. Atriopeptin I and II can readily be differentiated by charge (on ion-exchange chromatography) and mobility (on reversed-phase HPLC). The carboxyl terminal sequence of the peptides dictates their biological specificity. The shortened carboxyl terminus on atriopeptin I restricts its biological activity to relaxation of the intestinal smooth muscle and reduces its natriuretic and diuretic activity. Atriopeptin I does not relax isolated blood vessel strips but is effective in vivo as a natriuretic and diuretic. On the other hand, the extended carboxyl terminal Phe-Arg in atriopeptin II includes the structural features required for vascular receptor recognition. The presence of the identical sequence of amino terminal amino acids strongly suggests that atriopeptins I and II are derived from the same precursor peptide (designated atriopeptigen). Indeed, we previously demonstrated that gentle tryptic hydrolysis of the high molecular weight peptide fraction (atriopeptigen) derived from rat atria resulted in enhanced biological activity (*7*). This observation was associated with the generation of low molecular weight peptides exhibiting the chromatographic properties of the small peptides present in the original extract (*7*).

A hypothetical scheme of the regulation and function of these potent atrial peptides in the endocrine control of extracellular volume and sodium homeostasis is provided in Fig. 2 and is designed to serve as a focus for continued investigations. This model suggests that changes in extracellular volume and sodium serve as the triggers for peptide release. The homogeneity of the low molecular weight peptides (atriopeptins) suggests a limited sequential proteolytic cleavage of a common biosynthetic precursor. Further experimentation (utilizing selective immunoassays or gentic probes) are required to elucidate the function of atriopeptigen, which may be the primary storage form, and proteolysis with subsequent activation may occur during secretion or at a more distal site. The qualitative and quantitative correlation of blood levels of the atriopeptins must be demonstrated to establish their role in physiological or pathophysiological processes. Furthermore, an extensive analysis of the biological properties

Table 1. Relative biological activities of the atriopeptins. The in vitro intestinal (chick rectum) and vascular (rabbit aorta spiral strip) smooth muscle bioassay methods were as previously described (*4*). Strips were continuously perfused (10 ml/min) with Krebs-Henseleit solution (37°C). The chick rectal strips were precontracted with $2 \times 10^{-8} M$ carbachol and standardized such that 10 ng of isoproterenol gave a 40-mm relaxation of the intestinal smooth muscle. The rabbit thoracic aorta spiral strip was precontracted with $2 \times 10^{-8} M$ norepinephrine and standardized to give a 20-mm relaxation with 10 ng of nitroglycerin. The natriuretic-diuretic assay was performed in dialurethane-anesthetized Sprague-Dawley rats (*7*). A suprapubic silastic bladder was placed for urine collection and a tail vein catheter was used for infusion of 0.225 percent NaCl in 5 percent dextrose at 38 μl/min. After an equilibration period of 1 hour, two 10-minute urine collections (baseline) were followed by rapid intravenous injection of the test substance and three more 10-minute urine collections were completed. Sodium concentration was measured by flame photometry.

Protein (ng)	Relaxation in vitro (mm)		Urinary sodium excretion in vivo (percent of baseline)*
	Chick rectum	Rabbit aorta	
	Atriopeptin I		
10	0	0	
30	25	0	
100	37	0	
1000		0	102 ± 38 (4)
2000			548 ± 191 (5)
3000			1004 ± 498 (3)
	Atriopeptin II		
10	0	4	
30	20	10	
100	30	26	
300		44	
500			343 ± 146 (3)
1000		70	1895 ± 662 (3)
2000			2443 ± 342 (3)

*Values are means ± standard errors for the number of determinations shown in parentheses.

Table 2. Amino acid analysis of the two primary low molecular weight peptides purified from rat atrial extracts. Analyses were performed by hydrolyzing 1 nmole of peptide with 6*N* HCl for 48 hours. The hydrolyzates were lyophilized, redissolved, and applied to a Waters amino acid analysis system utilizing *o*-phthalaldehyde precolumn derivatization (*14*) followed by reversed-phase HPLC. N. D., not determined.

Amino acid	Atriopeptin I		Atriopeptin II	
	Amino acid analysis	Sequence	Amino acid analysis	Sequence
Asp	1.9	2	2.5	2
Glu	1.3	1	1.8	1
Ser	3.1	4	3.9	4
Gly	4.7	5	5.7	5
Arg	1.7	2	2.7	3
Ala	1.3	1	1.5	1
Phe	1.0	1	2.0	2
Ile	1.6	2	1.9	2
Leu	1.3	1	1.6	1
Cys	N.D.*	2	N.D.*	2

*The reduced, alkylated peptide permitted detection but not quantitation of the cysteine groups.

and receptors of the atrial peptides can be explored with the preparation of synthetic analogs. The continued investigation of this unique family of atrial peptides may provide insight into the properties of a major endocrine regulatory system.

Note added in proof: Synthetic atriopeptins I and II exhibit the same chromatographic and biological activity as the authentic biologically derived peptides. The synthetic peptides required oxidation to elicit their biological effect, thereby supporting the requirement for the cysteine disulfide ring.

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References and Notes

1. A. J. DeBold, H. B. Borenstein, A. T. Veress, H. Sonnenberg, *Life Sci.* **28**, 89 (1981).
2. H. Sonnenberg, W. A. Cupples, A. J. DeBold, A. T. Veress, *Can. J. Physiol. Pharmacol.* **60**, 1149 (1982).
3. N. C. Trippodo *et al.*, *Proc. Soc. Exp. Biol. Med.* **170**, 502 (1982).
4. M. G. Currie *et al.*, *Science* **221**, 71 (1983).
5. R. Keeler, *Can. J. Physiol. Pharmacol.* **60**, 1078 (1982).
6. G. Thibault, R. Garcia, M. Cantin, J. Genest, *Hypertension* **5**, 175 (1983).
7. M. G. Currie, D. M. Geller, B. R. Cole, P. Needleman, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
8. The carboxyl terminus was determined by digestion with carboxypeptidase [R. Hayashi, *Methods Enzymol.* **47**, 84 (1977)]. Atrial peptides (1000 to 2000 pmole) were dissolved in 280 μl of 0.05*M* sodium acetate buffer (*p*H 5.5). To this, 2 μg (20 μl) of carboxypeptidase Y (Pierce) was added. At intervals 50 μl was removed and mixed with 25 μl of 1 percent trifluoroacetic acid. The released amino acids (50-μl portions) were measured with a Waters amino acid analyzer.
9. J. A. Santome *et al.*, *Biochem. Biophys. Res. Commun.* **20**, 482 (1965).
10. A. J. DeBold and T. G. Flynn, *Life Sci.* **33**, 297 (1983).
11. R. T. Grammer *et al.*, *Biochem. Biophys. Res. Commun.* **116**, 696 (1983).
12. M. W. Hunkapiller, R. W. Hewick, W. J. Dreyer, L. E. Hood, *Methods Enzymol.* **91**, 399 (1983).
13. M. W. Hunkapiller and L. E. Hood, *ibid.*, p. 486.
14. D. W. Hill, F. H. Walters, T. D. Wilson, J. D. Stuart, *Anal. Chem.* **51**, 1338 (1979).
15. This work could not have been accomplished without the excellent technical assistance of S. W. Holmberg, A. Johnston, M. Kuhnline, and J. G. Boylan. Reported in part at the Annual Meeting of the American Heart Association, Anaheim, Calif., on 16 November 1983. M.G.C. is a Pharmacology-Morphology Fellow of the P.M.A. Supported by Monsanto.

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Activation of Antitumor Agent Gilvocarcins by Visible Light

Abstract. *Gilvocarcins that are antitumor agents are activated by low doses of visible light to induce bacteriophage lambda in* Escherichia coli. *This result is dependent on interaction with DNA. Gilvocarcin M, an analog without antitumor activity, failed to induce the prophage after light exposure, thus demonstrating a correlation between photosensitizing and antitumor activities. These results raise several possibilities regarding the mode of action of gilvocarcins as antitumor agents in vivo, involving light or enzymatic activating systems, which could be exploited in human cancer therapy.*

Current strategies for the development of new anticancer drugs involve the design or selection of agents that are tumor specific and less toxic than those used in the past. Several groups of investigators (*1–5*) have reported the isolation from Streptomycetes of antitumor active compounds with the gilvocarcin chromophore (*4–9*) which exhibit little toxicity in animals (*4, 9, 10*). A similar compound, chrysomycin A, was isolated 25 years ago (*11*), but was not completely characterized until recently (*9*). In view of the antimicrobial activity and mammalian cell cytotoxicity exhibited by gilvocarcins (*1–5, 10, 11*) the tolerance of animals for high doses (LD_{50} ~ 1000 mg/kg) (*4, 9, 10*) has been a mystery.

In our program, gilvocarcin-producing fermentation broths were detected as inducers of bacteriophage lambda, utilizing a colorimetric assay (*5*). However, tests of the purified active component (gilvocarcin V) here and elsewhere yielded conflicting results regarding its prophage-inducing activity (*4, 5*). These results, along with experimental observations on DNA strand break formation after irradiation of gilvocarcin V–DNA complexes (*12*), led us to examine the role of light in the prophage-inducing activity of this class of compounds. We now present data demonstrating the critical role of light in the activity of antitumor active gilvocarcins, a role previously unsuspected, we believe, because of their extreme sensitivity to normal fluorescent light. Comparison of the activity of gilvocarcins with that of related chemicals, including psoralens (Fig. 1), provides clues to the structural requirements for their activation and serves to emphasize the special characteristics of these compounds.

Prophage induction experiments were performed with *Escherichia coli* strain BR 513, a strain designed for screening of carcinogens and antitumor agents (*13*). These bacteria contain a chromosomally integrated lambda-lacZ fusion phage under control of the lambda repressor. Treatment with DNA-damaging agents leads to induction of the "SOS response," derepression of prophage, and synthesis of β-galactosidase, product of the *lacZ* gene. β-Galactosidase was detected by reaction with substrates giving colored products that could be quantitated or observed in spot tests on agar. In the latter case, comparisons could be made among many chemicals irradiated together on the same plate.

Figure 2a shows the dose response for prophage induction with chrysomycin A and gilvocarcin V in the presence and absence of light. Whereas both compounds showed strong prophage-inducing activity after a 15- or 20-minute exposure of solutions to fluorescent light, no activity was seen when the experiment was performed in the dark (or under yellow lights). These results accounted for the experimental variation seen in two laboratories at Frederick.

Other experiments indicated that prophage induction occurred only when the bacteria and chemical were present together during light exposure, a result reminiscent of the behavior of psoralens. Incubation of chemical alone in the light, followed by addition of bacteria, did not result in prophage-inducing activity (Fig. 2a). In a control experiment, irradiation of bacteria alone did not induce the pro-

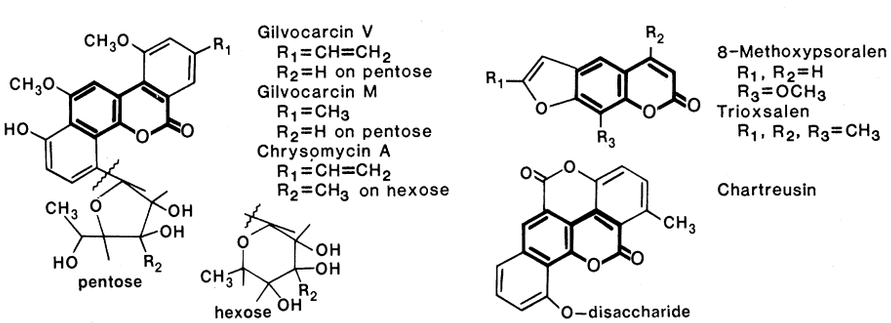

Fig. 1. Structures of gilvocarcins and related compounds. Bold-faced structure is coumarin.