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# Atrial Natriuretic Peptide Precursor Material in Rat Testis

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**Atrial natriuretic peptide (ANP) has recently been suggested to play a role in the regulation of male reproduction. Specifically, it has been shown to affect steroidogenesis in Leydig cells. The data presented here demonstrate that the rat testis also contains immunoreactive, ANP-like material (IR-ANP). Characterization of this material was conducted by high-pressure liquid chromatography (HPLC) analysis employing two different antisera to recognize either the C- or the N-terminals of the ANP prohormone (ANP 1-126). The IR-ANP corresponded to the 15 kd ANP precursor molecule (ANP 1-126), indicating the synthesis of ANP within the testis. This was supported by the detection of ANP messenger RNA transcripts (Northern blot hybridization) in the testis. These data indicate that ANP is expressed in the rat testis and support the notion that it may function in a paracrine and/or autocrine fashion in this organ.**

**Key words: ANP mRNA, RP-HPLC, northern blot hybridization, ANP (1-126)**

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Since the discovery of atrial natriuretic peptide (ANP) in the mammalian heart, most research has focused on its potent natriuretic, diuretic, and vasorelaxant effects (for review, see Cantin and Genest, 1985; Needleman et al, 1989). It is now well recognized that this peptide is involved in the humoral and central regulation of body fluid volume (for review, see Inagami et al, 1989; Baxter et al, 1988). However, there is increasing evidence that ANP peptide might serve additional functions. The peptide is present, and indeed is synthesized in various organs unrelated to cardiovascular physiology, some of these organs also have ANP binding sites (for review, see Gutkowska and Nemer, 1989; Stewart et al, 1988). With regard to the reproductive system, ANP seems to be associated with the corpus luteum (Vollmar et al, 1988b), the ovaries (Kim et al, 1989; Kim et al, 1987), and the anterior pituitary (Morel et al, 1989; Samson et al, 1988a,b). Moreover, there is evidence that ANP modulates steroidogenesis in Leydig cells through a guanylate cyclase-coupled ANP receptor (Pandey et al, 1985, 1986; Mukhopadhyay et al, 1986). However, the question of whether the testis itself expresses the ANP has remained unanswered. In this paper we report that the rat testis contains ANP-like immunoreactivity as well as the corresponding messenger RNA.

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## Materials and Methods

### Tissue Extraction

Male Sprague Dawley rats (300 to 400 g) were decapitated; their testes were removed and immediately transferred to hot (95°C) 0.1 N HCl (1 g tissue/10 ml) and were boiled for 2 minutes in a microwave oven. Subsequently, the tissue was homogenized (Polytron; setting 8, 15 seconds), and the testis homogenate was again kept at 95°C for 2 minutes. After centrifugation (20,000g, 20 minutes, 4°C) the clear supernatant (9 ml) was extracted by adsorption to activated Amberlite XAD-4 adsorbent resin (500 mg per column; Serva, Heidelberg, FRG). Elution was carried out with 2.5 ml of 80% acetonitrile in 0.1% trifluoroacetic acid; the material was lyophilized.

### Separation Techniques

*Gel filtration (GF).* Lyophilized extract was taken up in 0.1 N acetic acid (200 µl), centrifuged, and submitted to Sephadex G-50 chromatography (Pharmacia, Uppsala, Sweden; column 9 × 1000 mm 0.1 N acetic acid, flow rate 10 ml/hour). Calibration of the column was performed with bovine serum albumin ($V_0$), vitamin B 12($V_t$), rat pro-ANP (2-126), and rat ANP (99-126). Fractions (2.5 ml) were pooled, lyophilized, and detected for ANP by radioimmunoassay.

*Reverse phase high performance liquid chromatography (RP-HPLC).* The IR-ANP fractions from gel filtration were redissolved in 25 µl 0.1% trifluoroacetic acid (TFA), loaded onto a HPLC $C_{18}$ ODS Ultrasphere TM column (5 µm, 2 × 150 mm; Beckman Instruments), and eluted with a linear gradient of acetonitrile in 0.1% TFA (20–55%, 55 minutes). The flow rate was 0.2 ml/minute and the fraction size was 0.4 ml. Calibration was carried out with rat-ANP fragments (103-123), (103-126), (99-126) and the synthetic atrial natriuretic peptide precursor ANP (2-126).

### Antisera and Radioimmunoassay

Two different rabbit polyclonal antibodies directed to either the C- or N-terminal parts of ANP(1-126) were employed to detect ANP immunoreactivity. Antiserum "Loisl" (Vollmar et al, 1988a) was raised against the ring epitope of rat ANP (99-126). It does not detect any N-terminal fragments of ANP (1-126), but cross-reacts (40%) with synthetic rat pro-ANP (2-126). Antiserum "Loisl" showed negligible crossreactivity (<0.001%) with various neuropeptides such as β-endorphin, dynorphin B, dynorphin A, dynorphin $A_{1-8}$, α-neoendorphin, bovine-adrenal-medullary-22-peptide, N-acetyl-β-endorphin, Leu-enkephalin, ACTH, $arg^8$-vasopressin, oxytocin, angiotensin II, dopamine, and LHRH. Antiserum "GT-23" was generated against the N-terminal fragment ANP(11-37) of the precursor (Thibault et al, 1988). This antiserum does not recognize ANP(99-126), the major circulating form of ANP, but cross-reacts (100%) with the complete precursor ANP(1-126). The radioimmunoassay employing antiserum "Loisl" was performed as described by Arendt et al, 1985. The radioimmunoassay procedure for GT-23 followed the protocol of Thibault et al, 1988. Human ANP (1-30) was used as the standard, and the $^{125}$I-ANP (1-30) as tracer (both purchased from Peninsula Laboratories, UK).

### Recovery Experiment

Recovery of the synthetic ANP (2-126) (1/pmol) added to rat testis homogenate was performed as described by Vollmar et al (1988a), and amounted to 20 to 25%.

### Northern Blot Hybridization

For preparation of an ANP-specific cRNA hybridization probe, a 580 base Pst I-restricted ANP-cDNA fragment, obtained from K. Bloch and J. Seidman (Boston, MA), was subcloned in a pSP64 plasmid (Seidman et al, 1984). The Eco RI linearized plasmid served as a template for ANP-cRNA transcription using 100 µCi α-$^{32}$P-uridine triphosphate (400 Ci/mmol, Amersham, FRG) and the SP6-Polymerase-promotor system described by Melton et al, (1978). Total RNA was prepared by the LiCl-urea technique (Auffray and Rougeon, 1980). After quantification by absorption at 260 nm, 40 µg of mRNA was denatured in 1 mol/L glyoxal (pH 5), 50% dimethylsulfoxide, and 10 mmol/L Na-Phosphate buffer (pH 7) and was loaded onto a 1.2% agarose gel in bromophenol blue, Ficoll 400, and ethidium bromide for electrophoretic separation. The RNA was then blotted onto a Nytran 13 N membrane (Schleicher & Schuell, Dassel, FRG); the transfer was visualized as ethidium bromide-stained RNA using a UV source. Prehybridization was carried out in 50% formamide, 50 mmol/L Na-phosphate buffer (pH 7), 1 mol/L NaCl, 200 µg/ml salmon sperm DNA, 200 µg/ml yeast RNA, 5 × Denhardt's solution, 0.2% SDS, and 10 mmol for 3 hours at 70°C. Hybridization was then performed by adding 2 × $10^6$ cpm/ml of the ANP-probe followed by 18 hours of incubation. The membranes were then washed in 0.1 × SSC containing 0.1% sodium dodecyl sulfate at room temperature, and were subsequently exposed to an X-ray film with intensifying screens at −70°C.

## Results

Immunoreactive ANP (IR-ANP) was detected in hydrochloric extracts of rat testis purified on Amberlite XAD adsorbent resin and by Sephadex G-50 gel filtration. Figure 1A shows that the ANP immunoreactivity eluted as a single peak with a molecular weight corresponding to synthetic rat-pro-ANP (2-126). In some of the extracts, small amounts of low molecular weight ANP-like material were also detected. Correcting for the recovery rate of approximately 25% of exogenous ANP (2-126), the amount of IR-ANP extracted from rat testis was calculated to be approximately 2.5 pmol IR-ANP/g testis. In comparison, the atrium, hypothalamus, thymus of rats and the spleen of guinea pigs contain approximately 50 µmol, 6 nmol, 16

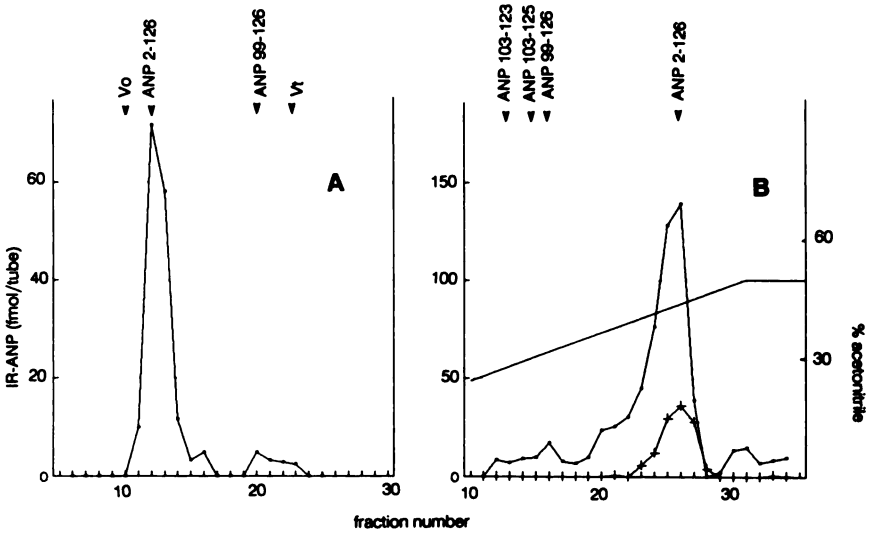


**Fig 1.**—Chromatographical analysis of IR-ANP extracted from rat testis. **A.** Sephadex G-50 gel filtration of acidic extract of testis tissue. Aliquots of immunoreactive fractions were detected by the antiserum "Loisl". Calibration of the column was performed as described in "Materials and Methods," and is indicated by arrows. **B.** RP-HPLC. Pooled material of the GF run was applied to the $C_{18}$ RP-HPLC column. The obtained fractions (0.4 ml) were assayed for IR-ANP using either antiserum GT-23 (●—●) or antiserum "Loisl" (+ — + —). Arrows indicate elution positions of rat-ANP (103-123), ANP (103-126), ANP (99-126), and ANP (2-126), respectively.

pmol, and 4.5 pmol IR-ANP/g wet tissue (Inagami et al, 1989; Vollmar and Schulz, 1988; Vollmar et al, 1989). ANP concentration in rat plasma has been reported to be between 20 and 50 fmol/ml (Thibault et al, 1988).

For further characterization and verification of the IR-ANP obtained by Sephadex gel filtration, pooled immunoreactive material was submitted to RP-HPLC analysis, and two antisera, capable of recognizing either the C- or the N-terminal sequence of the ANP prohormone molecule, were used for detection. As seen in Figure 1B, both antisera yielded an elution profile for testicular IR-ANP which again showed only a single peak coeluting with synthetic rat-ANP (2-126). The different amounts of IR-ANP detected by antiserum "Loisl" and "GT-23" reflect their different cross-reactivities for ANP(1-126).

Figure 2 demonstrates that the rat testis (lane 1) contains mRNA which hybridizes to a 580-base Pst I-restricted ANP cRNA probe (Seidman et al, 1984). The lower band clearly comigrates with the rat atrial ANP transcript employed as control tissue for detecting mRNA coding for ANP. Interestingly, we observed an additional hybridization band in the upper region. This material does not seem to be due to nonspecific hybridization, as it was visible even after higher stringency washing of the membranes. We assume, therefore, that this band may be tissue specific, ie, the transcriptional organization of the ANP gene might be different in the testis than the heart.

## Discussion

The data presented show that the rat testis contains ANP-like material. Chromatographic analysis, as well as employment of antisera capable of recognizing either the N- or the C-terminal part of the whole ANP precursor molecule, strongly suggests that the extracted IR-ANP represents the ANP prohormone (1-126). ANP(1-126) is considered to be the storage form of ANP (Cantin and Genest, 1985); since the prohormone is not present in the circulation of healthy rats (Thibault et al, 1988; unpublished observation), its synthesis within the testis is suggested. The low molecular weight ANP immunoreactivity identified with the C-terminal directed antibody may reflect degradation of the prohormone during extraction or origination from the circulation. Recently we have shown that the prohormone gets very rapidly degraded by tissue components (Vollmar et al, 1988a). Our low rate of recovery of ANP (1-126) may be due to rapid degradation of the peptide within the tissue.

Proof that a peptide is synthesized in a given tissue is provided by the presence of the corresponding mRNA therein. This essential requirement (Northern blot hybridization) has been demonstrated here for ANP and the rat testis. More detailed studies are required to determine whether the detected, second transcript of ANP indeed represents a characteristic for the rat testis.

Whether this locally produced ANP exerts any effects in vivo remains to be elucidated. In any case, there is clear evidence that the testis is a target organ for ANP. Firstly, Leydig cells have ANP binding sites partly coupled to guanylate cyclase (Pandey et al, 1988, Marala and Sharma, 1988; Vlasuk et al, 1988). Secondly, ANP has been reported

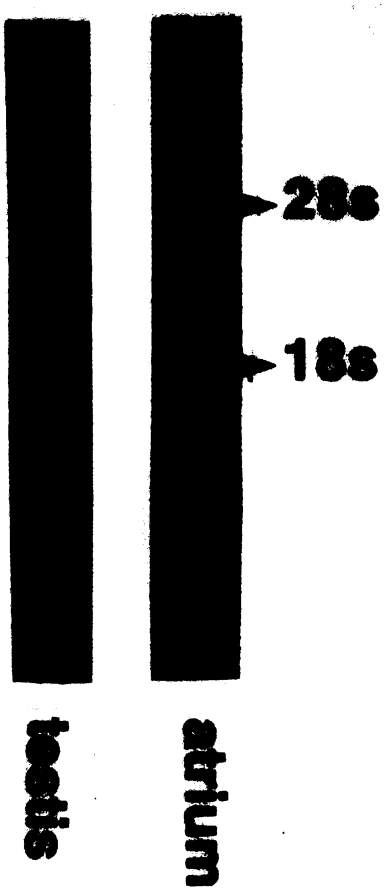


**Fig 2.**—Northern blot hybridization of rat testis mRNA. Lane 1: Total RNA (40 μg) of the rat testis was isolated, electrophoresed, and hybridized as described in "Materials and Methods." Lane 2: Total RNA (0.5 μg) from rat atria was employed as the control for ANP mRNA expression.

to alter steroidogenesis of isolated interstitial cells: ANP causes an inhibition of progesterone secretion in murine tumor Leydig cells (Pandey et al, 1985) and a stimulation of testosterone production in normal mouse Leydig cells (Pandey et al, 1986, Mukhopadhyay et al, 1986). Thus, a paracrine and/or an autocrine function for ANP in the testis is highly possible.

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## References

Arendt RM, Stangl E, Zähringer J, Liebisch DC, Herz A. Demonstration and characterization of α-human atrial natriuretic factor in human plasma. *FEBS Lett.* 1985; 189:57–61.

Auffray C, Rougeon F. Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur J Biochem.* 1980; 107:303–314.

Baxter JD, Lewicki JA, Gardner DG. Atrial natriuretic peptide. *Biotechnol.* 1988; 6:529–546.

Cantin M, Genest J. The heart and the atrial natriuretic factor. *Endocrinol Rev.* 1985; 6:107–127.

Gutkowska J, Nemer M. Structure, expression and function of atrial natriuretic factor in extraatrial tissues. *Endocrinol Rev.* 1989; 10:519–536.

Inagami T, Tanaka I, Mc Kenzie JC, Nakamaru M, Takayanagi R, Imada T, Pochet R, Resibois A, Naruse M, Naruse K, Shibasaki T. Discovery of atrial natriuretic factor in the brain: its characterization and cardiovascular implication. *Cell Mol Neurobiol.* 1989; 9:75–85.

Kim SH, Cho WK, Seul KH, Ryn H, Koh GY. Presence of immunoreactive atrial natriuretic peptide in follicular fluid, ovary and ovarian perfusates. *Life Sci.* 1989; 45:1581–1589.

Kim SJ, Shinjo M, Usuki S, Tada M, Miyazaki H, Murakami K. Binding sites for atrial natriuretic peptide in high concentrations in human ovary. *Biomed Res.* 1987; 8:415–420.

Marala RB, Sharma RK. Characterization of atrial natriuretic factor receptor coupled membrane guanylate cyclase from rat and mouse testes. *Biochem J.* 1988; 251:301–304.

Melton DA, Krieg PA, Rebagliati MR, Maniatis T, Zinn K and Green MR. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. *Nucleic Acids Res.* 1978; 12:7035–7056.

Morel G, Chabot JG, Gossard F, Heisler S. Is atrial natriuretic peptide synthesized and internalized by gonadotrophs? *Endocrinology.* 1989; 124:1703–1710.

Mukhopadhyay AK, Schumacher M, Leidenberger FA. Steroidogenic effect of atrial natriuretic factor in isolated mouse Leydig cells is mediated by cyclic GMP. *Biochem J.* 1986; 239:463–467.

Needleman P, Blaine EH, Greenwald JE, Michener ML, Saper CB, Stockmann PT, Tolunay HE. The biochemical pharmacology of atrial peptides. *Am Rev Pharm Tox.* 1989; 29:23–54.

Pandey KN, Kovacs WJ, Inagami T. The inhibition of progesterone secretion and the regulation of cyclic nucleotides by atrial natriuretic factor in gonadotropin responsive murine leydig tumor cells. *Biochem Biophys Res Comm.* 1985; 133:800–806.

Pandey KN, Pavlon SN, Kovacs WJ, Inagami T. Atrial natriuretic factor regulates steroidogenic responsiveness and cyclic nucleotide levels in mouse Leydig cells in vitro. *Biochem Biophys Res Comm.* 1986; 138:399–404.

Pandey KN, Pavlon SN, Inagami T. Identification and characterization of three distinct atrial natriuretic factor receptors. *J Biol Chem.* 1988; 263:13406–13413.

Samson WK, Aguila MC, Bianchi R. Atrial natriuretic factor inhibits luteinizing hormone secretion in the rat: evidence for a hypothalamic site of action. *Endocrinology.* 1988a; 122:1573–1582.

Samson WK, Bianchi R, Mogg R. Evidence for a dopaminergic mechanism for the prolactin inhibitory effect of atrial natriuretic factor. *Neuroendocrinology.* 1988b; 47:268–271.

Seidman C, Duby AD, Choi E, Graham RM, Haber E, Homcy

C, Smith JA and Seidman JG. The structure of rat preproatrial natriuretic factor as defined by complementary DNA clone. *Science*. 1984; 225:324–326.

Stewart RE, Swithers SE, Plunkett LN, McCarty R. ANF receptors: distribution and regulation in central and peripheral tissues. *Neurosci Biobehav Rev*. 1988; 12:151–168.

Thibault G, Murthy KK, Gutkowska J, Seidah NG, Lazure C, Chrétien M, Cantin M. $NH_2$-terminal fragment of rat proatrial natriuretic factor in the circulation: identification, radioimmunoassay and half-life. *Peptides*. 1988; 9:47–53.

Vollmar AM, Schulz R. Evidence for the presence of ANP-precursor material in the rat thymus. *Biochem Biophys Res Comm*. 1988; 155:700–708.

Vollmar AM, Friedrich A, Sinowatz F, Schulz R. Presence of atrial natriuretic peptide like material in guinea pig intestine. *Peptides*. 1988a; 9:965–971.

Vollmar AM, Myztka C, Arendt RM, Schulz R. Atrial natriuretic peptide in bovine corpus luteum. *Endocrinology*. 1988b; 123: 762–767.

Vollmar AM, Friedrich A, Schulz R. Immunoreactive atrial natriuretic peptide in the guinea pig spleen. *Life Sci*. 1989; 45: 1293–1297.

Vlasuk GP, Aracuri KE, Ciccarone TM, Nutt RF. Functional multiplicity of atrial natriuretic peptide receptors on cultured rat Leydig tumor cells. *FEBS Lett*. 1988; 228:290–294.

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