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ATRIAL NATRIURETIC FACTOR IN MAMMALIAN TESTIS: IMMUNOLOGICAL DETECTION IN SPERMATOZOA

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SUMMARY: Immunoreactive atrial nutriuretic factor (ANF) was localized by immunochemistry and radioimmunoassay in mouse and rat testes. The analyses of acid extracts of testes by gel filtration and reverse phase high pressure liquid chromatography (HPLC) revealed the presence of a processed 31-residues peptide and the precursor form of 126-residues pro-ANF molecule corresponding to a molecular weights (M_r) of 3,300 and 18,000, respectively. The concentration of ANF in mice testis averaged 12 $\pm$ 3 ng and in rat testis 8 $\pm$ 2 ng per g of tissue. Specific immunochemical staining was localized in the spermatids and elongating spermatozoa of mammalian testis. The demonstration of immunoreactive ANF in testis and specific localization in spermatids reveals a new site at which ANF may be actively synthesized and regulate paracrine and/or autocrine function(s) during spermiogenesis, suggesting a broader spectrum of ANF action in addition to its known regulatory role in the control of blood pressure homeostasis.
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Atrial natriuretic factor (ANF), a peptide hormone synthesized in the specific granules of mammalian atrial cardiocytes and secreted into circulation elicits multiple physiological responses largely directed to the reduction of blood pressure and fluid volume (1). In addition to its effects on renal excretion of sodium and water (2-4) and relaxation of vascular smooth muscle cells (5-7), ANF is known to exert its effects on endocrine glands, such as the inhibition of aldosterone synthesis and release from adrenal cortical cells (8-10), vasopressin from posterior pituitary (11,12) and renin from renal cortical cells (13-15). The physiologic responses of ANF

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The Abbreviations used are: ANF, atrial natriuretic factor; HPLC, high pressure liquid chromotography; M_r, molecular weight; PBS, phosphate buffered saline.

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are associated with an increase in intracellular cGMP and a decrease in cAMP (16,17). Among various tissues examined the most remarkable ANF-induced activation of guanylate cyclase activity was observed in the membrane preparations of rat testis (18). ANF increased intracellular cGMP formation more than 1500-fold in cultured murine Leydig tumor cells (19). Recently, ANF was shown to stimulate androgen synthesis and release from normal Leydig cells (20-22) and progesterone from ovarian granulosa cells (23). These observations raised the possibility that ANF might be involved in the autocrine and/or paracrine regulation of testicular function, since it seemed unlikely that circulating concentrations of ANF were sufficient to elicit such responses. Accordingly we sought to determine whether ANF is present and localized within the testis itself. In the present study we report the first observation of the detection of ANF in the sperm precursors of mouse and rat testis. We have used radioimmunoassay and immunocytochemistry to measure the amount of ANF and to localize the intracellular distribution of this substance within the specific cell types of mammalian testis.

MATERIALS AND METHODS

Materials: $ANF_{(96-126)}$, $ANF_{(99-126)}$, $ANF_{(102-126)}$, $ANF_{(103-126)}$, and $ANF_{105-121)}$ were obtained from the peninsula Laboratories (Belmont, CA). SepPak C-18 cartridges were from Waters Associates (Milford, Mass.) and molecular weight markers were from Sigma Chemical Co. (St. Louis, MO). The perfix was obtained from Fisher Scientific (Pittsburgh,PA).

Production and Detection of Antibodies: Specific antibodies to $ANF_{(99-126)}$ were raised in male New Zealand rabbits by the methods described previously (24). $ANF\text{-}IV_{(102-126)}$ (25) was conjugated to bovine thyroglobulin, (Sigma Chemical Co.) by mixing 2.5 mg ANF-IV and 10 mg thyroglobulin in 1 ml 0.1M sodium phosphate buffer, pH 7.0. To this solution, 30 μl of 0.8 M glutaraldehyde was added in dropwise. The mixture was stirred for 4 h at room temperature and dialyzed against 10 mM sodium phosphate buffer, pH 7.0 for 24 h at 4°C. Conjugate containing 600 μg of ANF-IV was emulsified with an equal volume of Freund's complete adjuant and was used to immunize rabbits by multiple intradermal injections on the back. Animals were boosted at 4 week intervals and bled 2 weeks after the last immunization. Antiserum against synthetic rat $ANF\text{-}IV_{(102-126)}$ cross reacted 100% with $ANF_{(102-126)}$, $ANF_{(99-126)}$, and $ANF_{96-126)}$, 42% with α-human $ANF_{(102-126)}$ and 28% with atriopeptin I but did not cross react with arginine vasopressin, angiotensin I and II (26).

Tissues and peptide extraction: Normal male Sprague-Dawley rats weighing approximately 200g and normal male Swiss mice weighing approximately 30g were used. Tissues from rats were excised rapidly following decapitation, and from mice after cervical dislocation and immediately frozen in liquid nitrogen and stored at -80°C. Frozen testes were boiled for 10 min in 1 N acetic acid (1:10, weight/volume) and homogenized with a polytron (at setting 4, three times 30 seconds each). The homogenate was centrifuged at 2,000 rpm for 5 min and supernatant was recentrifuged at 25,000 rpm for 60 min at 4°C. Supernatant was collected and pellet was

reextracted and centrifuged as above. The combined supernatants were applied to an octadecyl-silica (ODS-silica) cartridge (SepPak C-18) prewashed as described previously (22). ANF was eluted by 80% methanol. The eluates were brought to dryness by vacuum centrifugation.

Determination of ANF by the Combination of High Pressure Liquid Chromatography (HPLC) and Radioimmunoassay: ANF in the tissue extracts was quantitated directly by specific radioimmunoassay as described previously (24). High performance gel filtration chromatography was performed on a BioRad TSK-125 column (7.5 x 60 mm, Richmond, CA) using a solvent system consisting of 30% acetonitrile, 0.1% trifluoroacetic acid and 0.2 M NaCl. To characterize ANF from testis extracts, a reverse phase HPLC column was used. Samples were applied to a Vydac C-18 column (0.46 x 25 cm, Altex Associates, Inc., Deerfield, IL) and eluted with an isocratic solvent system containing 22% acetonitrile and 0.1% trifluoroacetic acid as described previously (27). The HPLC eluate was collected in 0.25 ml fractions which were evaporated by vacuum centrifugation and ANF content was determined by radioimmunoassay by the published method (24). The amount of ANF was computed in reference to a standard curve obtained using the 28 residue peptide $ANF_{(99-126)}$ as standard. The high molecular weight ANF with 126 residue gives a value approximately 20% of the equivalent amount of the 28 residue peptide (27).

Immunolocalization of ANF: For immunocytochemical staining, six normal male mice and six normal male rats were used. After animals were sacrificed, each testis was removed and fixed immediately for 1 hour in Perfix. Each testis was then cut in half and fixation was continued for 2 additional hours followed by dehydration in graded alcohol and xylene, paraffin embedding and sectioning. ANF was immunolocalized by the peroxidase anti-peroxidase method of Sternberger et. al (28). For controls, the primary antiserum was replaced with (i) normal rabbit serum (ii) primary antiserum absorbed with excess ANF for 24-48 h or (iii) Tris-buffered saline. Tissue sections mounted on glass slides were treated with 1:100 30% hydrogen peroxide in methanol to quench endogenous peroxidase, rehydrated and treated with 10% normal swine serum in phosphate buffered saline (PBS) for 10 min. They were incubated sequentially with (i) rabbit antiserum against ANF (1:100 to 1:2,500) or with normal rabbit serum (both diluted in PBS with 0.4% Triton X-100), (ii) Swine anti-rabbit IgG (1:20) for 30 min. (iii) peroxidase antiperoxidase complex (1:50) for 30 min then stained with 0.1% 3,3'-diaminobenzidine plus 0.01% hydrogen peroxide for 10 min. All incubations were carried out in a humid chamber at room temperature and the tissue sections were rinsed with PBS.

RESULTS

The concentration of ANF in mice and rat testes averaged 12 ± 3 ng and 8 ± 2 ng per gram of tissues, respectively. Fig. 1 shows the characterization of ANF from the extracts of mouse and rat testes using high performance gel filtration chromatography by HPLC. The partially purified ANF corresponding to molecular weights (M_r) of 18,000 and 3,300 were resolved from both mouse and rat testis. As shown in Fig. 2, further examination of the material corresponding to the (M_r) of 3,300 by reverse phase HPLC in reference to various synthetic ANF fragments; $ANF_{(103-123)}$, $ANF_{(105-121)}$, $ANF_{(102-126)}$, $ANF_{(99-126)}$ and $ANF_{(96-126)}$ revealed that the elution position of this peptide corresponded to the 31-residues peptide $ANF_{(96-}$

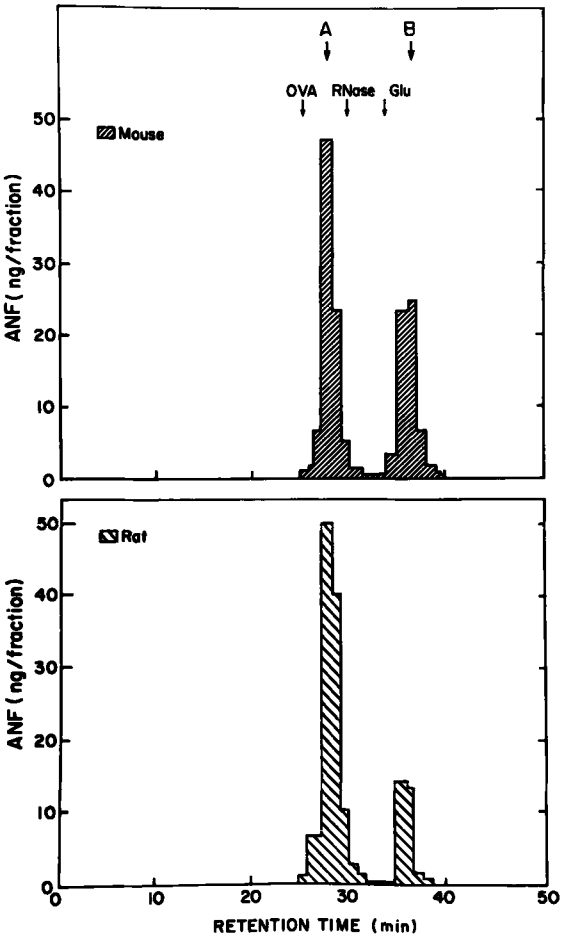

Fig. 1. Gel filtration chromatography of testis extracts. Samples were applied and high performance gel filtration chromatography was performed as described in the Materials and Methods. The amount of immunoreactive ANF in each fraction was determined by radioimmunoassay. Elution positions of the standard proteins indicated by arrows: ovalbumine (Ova;M_r=45,000), ribonuclease (RNase;M_r=12,000), and glucagon (Glu;M_r=3,500). Arrows A and B indicate the elution positions of pro-ANF and synthetic $ANF_{(96-126)}$, respectively. The upper and lower panels represent the chromatographic elution patterns of pro-ANF and $ANF_{(96-126)}$ from mouse and rat testis, respectively.

$_{126)}$. It is clearly a larger molecule than the circulating ANF with 28-amino acid residues. This observation suggests that ANF found in the testis is most likely the product of local synthesis and processing intrinsic to the testis.

In both rat and mouse testis, staining was observed only in round and elongated spermatids. In rat testis staining was first detected in the acrosomal caps of step 3 spermatids (29) persisted in the acrosome up to step 16 spermatids after which staining decreased (Fig. 3a).

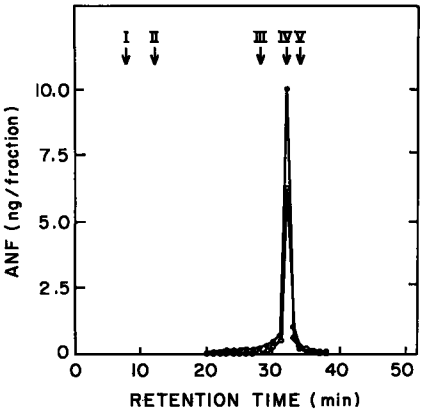

Fig. 2. Characterization of the testis extracts on octadecylcylanyl column (C-18, reverse phase HPLC): Samples were applied to a Vydac C-18 column and eluted with an isocratic solvent system as described in Materials and Methods. The authentic ANFs used for controls were: I: $ANF_{(103-123)}$, II: $ANF_{(105-121)}$, III: $ANF_{(102-126)}$, IV: $ANF_{(96-126)}$, V: $ANF_{(99-126)}$ The closed and open circles represent the elution pattern from mouse and rat testes extracts, respectively.

No staining could be detected in the spermatid cytoplasm or in any other cell types. In mouse testis, staining was first detected in the acrosomal cap of step 5 spermatids. Cytoplasm and flagella were also stained (Fig. 3b). Staining was completely abolished when normal rabbit serum was used and almost completely blocked when the antiserum was preincubated with ANF. (Fig. 3c).

DISCUSSION

The characterization of both pro-ANF and $ANF_{(96-126)}$ in testes homogenates and immunohistochemical demonstration of ANF in the spermatids and sperm cells suggest that the synthesis and processing of the prohormone to biologically active peptide hormone occurs in the male germ line. The target cells for such a locally produced ANF could be the Leydig cells since ANF has been shown to stimulate the testosterone synthesis and release (20-22), and the presence of ANF receptor has been demonstrated in both mouse and rat Leydig cells (30,31). Recently the guanylate cyclase-coupled ANF receptor from mouse Leydig tumor (MA-10) cells was cloned and sequence determined (32).

Quantitative analyses of ANF by radioimmunoassay showed that the testes of adult rats and mice contain on an average of 8 ng and 12 ng of ANF per g wet tissue, respectively. This

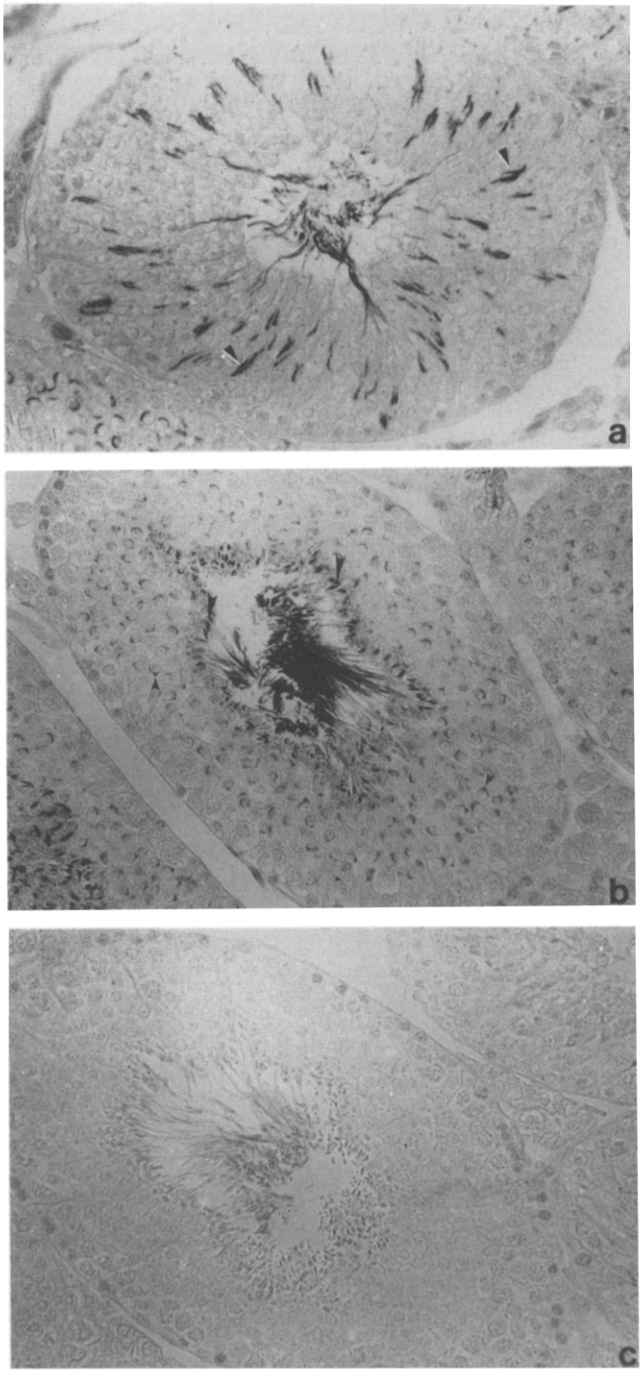

Fig. 3. Immunohistochemical localization of ANF:(a) Cross section of a stage I seminiferous tubule in rat testis. Note the unstained step I round spermatids and the staining of the heads of elongated spermatids in bundles (arrow) and of their flagella in the lumen. No counter stain, X350. (b) Cross section through a stage VII seminiferous tubule in mouse testis. Note the staining on the acrosomal cap of round spermatids (small arrow), the heads (large arrow), and the flagella of elongated spermatids. No counter stain, X350.(c) Cross section of a seminiferous tubule in mouse testis. Normal rabbit serum was used as a control. Note the absence of staining. No counter stain. X350.

concentration range is approximately 20-to 30-fold higher than plasma ANF concentration and 500-to 1000-fold higher than ANF in brain and pituitary but approximately 10-to 15-fold lower than in the cardiac atrium (33). Immunohistochemical staining of ANF has also been shown in the brain, pituitary, and heart (24,34-36). The specificity of antisera used in this study has been confirmed previously (22,24). It discriminates even truncated ANF peptides like atriopeptin I and cross reacts poorly with human ANF. Most importantly, it can be absorbed almost completely with synthetic ANF.

The immunolocalization of ANF-like material in both the acrosome and the flagellum of the spermatids was unexpected. The presence of other antigenic sites in the acrosome and the flagellum has also been reported (37,38). The exact function of ANF in the developing spermotozoa is not immediately clear, however, it is postulated to participate in capacitation, sperm motility and/or sperm development. The demonstration of ANF in the testis and its specific localization in the developing spermatozoa reveals a new site at which ANF may be actively synthesized, and suggests a broader spectrum of ANF action than originally postulated, and that it is not just limited to the control of fluid volume and blood pressure homeostasis.

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