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Abstract

In vitro release of atrial natriuretic peptide (ANP) from atria was examined by ANP radioimmunoassay. Isolated right rat atria were incubated in Krebs-Ringer bicarbonate buffer, and test substances were added to the incubation medium. The fluid was assayed for rat ANP by a radioimmunoassay method recently developed in our laboratory. We produced an antiserum to human ANP(99-216) (alpha-hANP(1-28)) which showed a good cross-reactivity of 63% with rat ANP(99-126) (alpha-rANP(1-28)) and was useful for measuring rat ANP concentrations of the medium. Application of the medium to a reverse phase high performance liquid chromatography (HPLC) system resulted in a single peak of immunoreactive rat ANP corresponding to a small molecular weight synthetic rat ANP of 28 amino acid residues. Catecholamines (epinephrine, norepinephrine and isoproterenol) reduced the basal secretion of ANP, whereas acetylcholine stimulated the release of ANP. Forskolin and dibutyryl cyclic AMP did not affect the release of ANP. These results suggest the possibility that the regulation of ANP release may be partially associated with adrenergic and cholinergic mechanisms.

KEYWORDS: atrial natriuretic peptide, catecholamine, acetylcholine, radioimmunoassay

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In Vitro Release of Immunoreactive Atrial Natriuretic Peptide from the Rat Atria

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In vitro release of atrial natriuretic peptide (ANP) from atria was examined by ANP radioimmunoassay. Isolated right rat atria were incubated in Krebs-Ringer bicarbonate buffer, and test substances were added to the incubation medium. The fluid was assayed for rat ANP by a radioimmunoassay method recently developed in our laboratory. We produced an antiserum to human ANP(99-126) (α-hANP(1-28)) which showed a good cross-reactivity of 63% with rat ANP(99-126) (α-rANP(1-28)) and was useful for measuring rat ANP concentrations of the medium. Application of the medium to a reverse phase high performance liquid chromatography (HPLC) system resulted in a single peak of immunoreactive rat ANP corresponding to a small molecular weight synthetic rat ANP of 28 amino acid residues. Catecholamines (epinephrine, norepinephrine and isoproterenol) reduced the basal secretion of ANP, whereas acetylcholine stimulated the release of ANP. Forskolin and dibutyryl cyclic AMP did not affect the release of ANP. These results suggest the possibllity that the regulation of ANP release may be partially associated with adrenergic and cholinergic mechanisms.

Key words: atrial natriuretic peptide, catecholamine, acetylcholine, radioimmunoassay

Early ultrastructural studies (1,2) revealed that mammalian heart atria possess specific granules presumably formed in the Golgi complex, but their role remained unknown for years. The observation (3) that the granularity of atrial cardiocytes varied with changes in the electrolytes and body fluid balance implied that the granules could play some role in the control of electrolytes and fluid homeostasis. Bioassay studies (4, 5) have revealed that atrial extracts contain potent natriuretic, diuretic and vasorelaxant substances. Recently, a variety of atrial natriuretic peptides (ANPs) have been isolated from rat atrial tissue, sequenced and synthesized (6-11). The sequences determined to date are identical. These peptides contain the same sequence, but differ only in the number of amino acids.

Radioimmunoassay and immunohistochemical studies using specific antibody revealed the cardiac origin of the plasma ANP (12,13). Mechanical distension of the atria and volume expansion resulted in an increase in the plasma ANP concentration in the dog and rat (14-16). In addition,

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some biologically active substances given *in vivo* resulted in an increase in circulating ANP release (17). However, the cellular mechanism of ANP release from atrial cardiocytes remains controversial (18-20). In the present study, the effect of biologically active substances on the *in vitro* release of ANP from rat atria was examined using a newly developed ANP radioimmunoassay.

Materials and Methods

Experimental protocol. Male Wistar rats weighing 100-200 g were decapitated, and the right atria were immediately collected. Each atrium was cut into quarters, and two pieces were placed in a polyethylene tube with 1 ml of Krebs-Ringer bicarbonate buffer (KRBG, pH 7.4) containing 0.2% glucose, 0.25% bovine serum albumin, 100 μg/ml bacitracin and 1 mM ascorbic acid. All tubes were placed in a water bath (37°C) and gently shaken(90 cycles per min) in an atmosphere of 95% O_2-5% CO_2. A series of two incubations were performed at 30-min intervals after preincubation. The medium was changed with each incubation and stored at −70°C until assay. Test substances were added to the medium in the second incubation. The net rate of ANP secretion into the medium was indicated by the ratio of the second to the first incubation release of ANP.

Radioimmunoassay of rat ANP. Medium ANP was radioimmunoassayed using anti α-hANP(1-28) serum developed in our laboratory according to the method of Gutkowska *et al.* (21). Synthetic α-hANP(1-28) (Peptide Institute, Inc., Osaka, Japan) was conjugated to bovine thyroglobulin using water soluble carbodiimide (22). The conjugate was emulsified in Freund's complete adjuvant and injected intradermally into the shaved backs of Japanese white rabbits once a month. Eight weeks later, the rabbits were anesthetized with an intravenous injection of sodium pentobarbital (40 mg per kg body weight), and the blood was withdrawn via a silicon cannula which was inserted into the right jugular vein.

Synthetic α-rANP(1-28) was radioiodinated with ^{125}I using the chloramine T method (23). The iodinated peptide was purified on a Sephadex G-50 column (fine, 0.9x 60 cm) eluted with RIA assay buffer described below and was repurified with Sep-Pak C_{18} (Waters Associates, Milford, Massachusetts, U.S.A.) just before use.

Synthetic α-rANP(1-28) was serially diluted with Krebs-Ringer buffer and was used for constructing standard curves. Medium samples (50 μl) or standard solution (50 μl) and 250 μl assay buffer (containing 0.01M phosphate buffer, 0.15M NaCl, 0.5% bovine serum albumin, 0.02% sodium azide, 0.01M disodium EDTA and 0.1% Triton X-100, pH 7.4) were incubated with either 100 μl antiserum at a final dilution of 1 : 17,500 and 100 μl of ^{125}I-ANP as the tracer. After a 48h incubation, 100 μl of goat anti-rabbit gammaglobulin (1 : 40 dilution) and 100 μl of carrier rabbit serum (1 : 200 dilution) (Eiken Chemical Co., Tokyo, Japan) were added. Following an additional 24h incubation, all tubes were centrifuged at 1,200×g at 4°C for 30 min, and the radioactivity in the precipitate was determined.

To characterize the ANP-like immunoreactive substance in the medium, the medium samples(200 μl) were injected into a Hitachi 638 HPLC system (Hitachi Seisakusho, Tokyo, Japan), and eluted from the octadecyl silica column (Hitachi-Gel #3053, 4.0x 250 mm) with a linear gradient of acetonitrile from 20% to 50% in 0.1% trifluoroacetic acid at a rate of 1.0 ml per min over 60 min, collecting fractions of 1 ml. Aliquots of fraction were lyophilized, reconstituted with RIA buffer, and assayed for immunoreactive rat ANP.

ANP related peptides and test substances. α-hANP (3-28) was a gift of Dr. Ruth F. Nutt, and α-rANP(5-27) and α-rANP(5-28) were given by Dr. Philip Needleman. Synthetic α-hANP(1-28), α-rANP(1-28), and other related peptides were purchased from Peptide Institute, Inc., Osaka, Japan. Biologically active substances examined in the present stuby were isoproterenol, epinephrine, norepinephrine, forskolin, dibutyryl cyclic AMP, and acetylcholine. Forskolin was purchased from Calbiochem-Behring, La Jolla, California, U.S.A. Dibutyryl cyclic AMP was kindly provided by Daiichi Pharmaceutical Co., Ltd., Tokyo, and other substances were obtained

from Sigma Chemical Co., St. Louis, Missouri, U.S.A.

Statistical analysis. Values are presented as means ± SEM. The significance of differences between the values was determined by Student's *t*-test.

Results

Validity of the radioimmunoassay. An antiserum to α-hANP(1-28) was developed in rabbits as was that to rat ANP (21). The cross-reactivity of the antiserum with α-rANP(1-28), α-rANP(3-28), α-hANP(5-28), α-hANP(5-27), α-rANP(5-28), α-rANP(5-27) and α-rANP(5-25) were 63, 40, 4.5, 4.5, 0.8, 0.8 and 0.1%, respectively (Fig. 1). However, there was no cross reactivity (<0.001%) with LH-RH, β-LPH, somatostatin, ACTH, CRH, vasopressin, β-endorphin, angiotensin II, oxytocin, bradykinin, met-enkephalin, rat GH, rat TSH, and rat LH. The antiserum was used to measure the rat ANP concentration of the incubation medium using synthetic rat ANP (1-28) as a standard. The assay sensitivity of the RIA was 10 pg/0.5 ml, and the 50% displacement was 117 pg/0.5 ml. The intraassay variation was 3.91%. The recovery rate was 118.0±6.1% (mean± SD) for 100 pg of ANP. The dilution curve of the incubation medium paralled the standard curve.

Application of the medium to the HPLC system resulted in a single peak of ANP-like immunoreactivity at the position of α-rANP (1-28) (Fig. 2).

The in vitro release of ANP. Isolated rat atria showed a significant decrease in ANP release when exposed to norepinephrine (10^{-7}M and 10^{-5}M), epinephrine (10^{-5} M) and isoproterenol (10^{-7} M and 10^{-5} M), while ANP release was not statistically different from the values of the control in the presence of 10^{-7} M epinephrine or 10^{-9} M isoproterenol (Figs. 3,4). The addition of forskolin or dibutyryl cyclic AMP showed no significant effect on the release of ANP (Fig. 5). Acetylcholine (Fig. 6), however, caused a marked stimulation of ANP release from the atria at a concentration of 10^{-5}M ($p < 0.01$), whereas it did not significantly alter ANP release at lower concentrations (10^{-9}M or 10^{-7}M).

Discussion

In 1984, Sonnenberg and Veress reported the effect of biologically active sub-

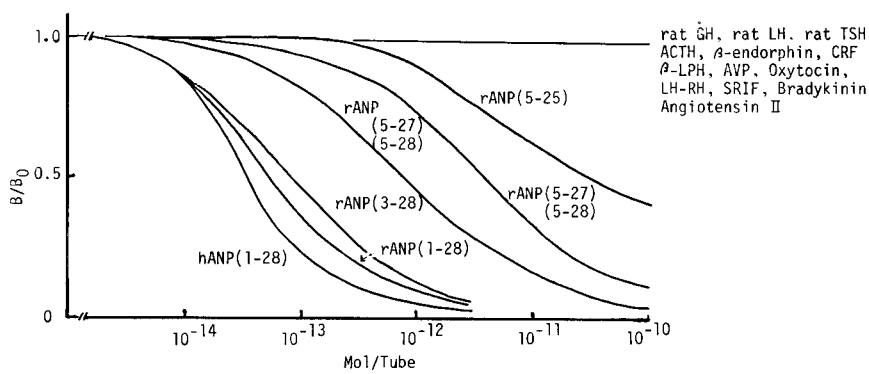

Fig. 1 Cross-reactivities of anti-α-hANP serum with α-ANP related peptides and other peptides.

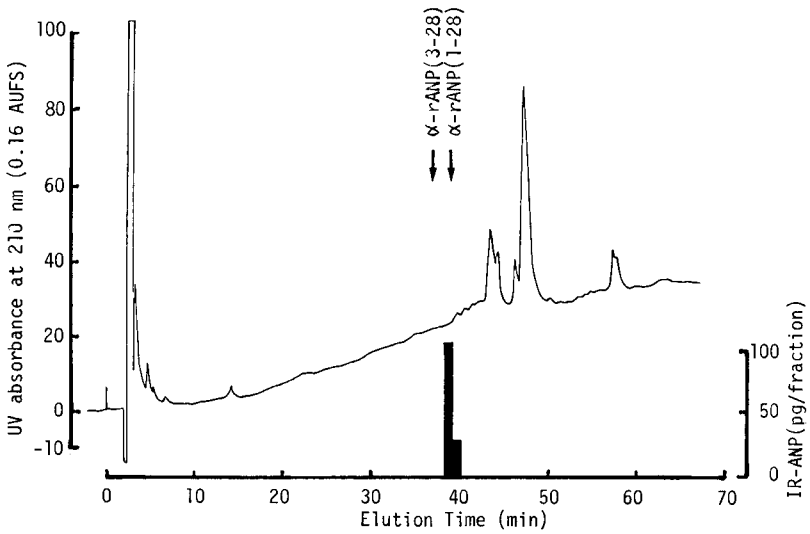

Fig. 2 Analysis of immunoreactive ANP in the incubation medium by reverse phase HPLC. The medium samples (200 μl) were injected into the HPLC system and eluted from the column (Hitachi-Gel #3053, 4.0x 250 mm) at 1.0 ml/min, using a gradient of acetonitrile from 20 to 50%. The arrows show the eluting positions of α-rANP(3-28) and α-rANP(1-28).

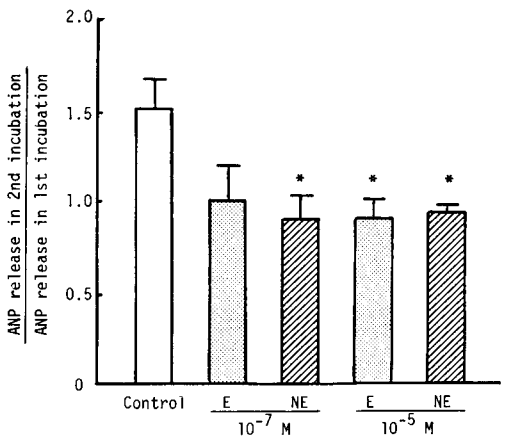

Fig. 3 Effect of norepinephrine (NE) and epinephrine (E) on the *in vitro* release of ANP from the isolated atria. The net rate of ANP secretion into the medium is indicated by the ratio of the second to the first incubation. Vertical lines represent the SEM. The number in each group is five ($n = 5$). Single asterisks indicate significant difference ($p < 0.05$) compared with control values.

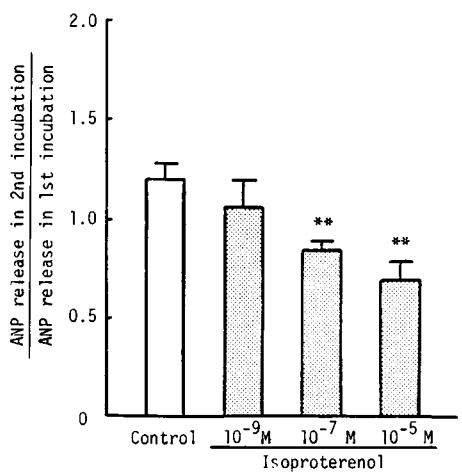

Fig. 4 Effect of isoproterenol on the *in vitro* release of ANP from the isolated atria. The net rate of ANP secretion is indicated by the ratio of the second to the first incubation. Vertical lines represent the SEM. The number of each group is five ($n = 5$). Double asterisks indicate significant difference ($p < 0.01$) compared with control values.

stances on the *in vitro* release of ANP from rat atria (18). They measured the rat ANP levels of incubation media by bioassay and found that epinephrine, vasopressin and acetylcholine stimulated the release of ANP. Other studies (19, 20), however,

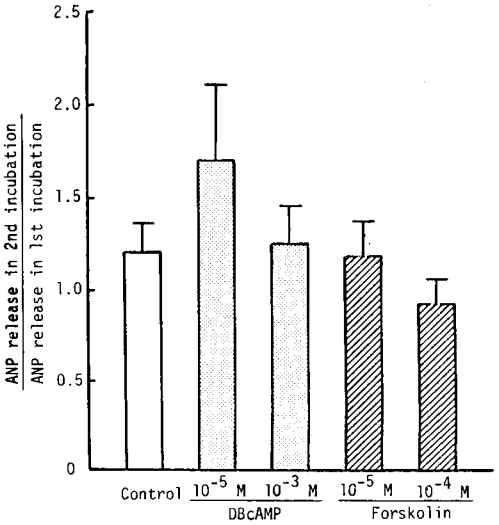

Fig. 5 Effect of dibutyryl cyclic AMP and forskolin on the *in vitro* release of ANP from the isolated atria. The net rate of ANP secretion is indicated by the ratio of second to the first incubation. Vertical lines represent the SEM. The number of each group is five.

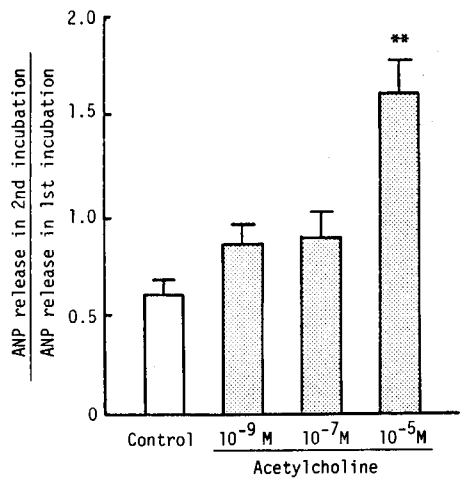

Fig. 6 Effect of acetylcholine on the *in vitro* release of ANP from the isolated atria. The net rate of ANP secretion is indicated by the ratio of the second to the first incubation. Vertical lines represent the SEM. The number of each group is five. Double asterisks indicate significant difference ($p < 0.01$) compared with control values.

have shown no effect of catecholamines and vasopressin. In the present study, adrenergic stimulation produced a significant decrease in ANP secretion. It is difficult to interpret these discrepancies. The inhibitory effect in our experiments might be a pharmacologic one since the effective doses of the substances were high, and cyclic AMP, a second messenger of the β-adrenergic receptor system showed no significant effect. In addition, forskolin which stimulates adenylate cyclase activity via a receptor-independent mechanism also showed no effect on the *in vitro* release of ANP. These results suggested that the cellular mechanism of ANP secretion is very complex. The cyclic AMP system is not involved, but phosphatidylinositol metabolism, which has been reported to be involved in the action of many hormones (24-26), might be involved in the release of ANP. We plan to examine the effects of adrenergic and cholinergic antagonists to confirm the present results.

According to the cross-reactivity experiment, our anti-α-human ANP serum recognizes the N-terminal portion of the amino acid sequence of α-ANP(1-28). α-Rat and human ANP(1-28) both consist of 28 amino acids, and their sequences are the same, except for one amino acid at the 12 th position. Therefore, the cross-reactivity of anti-human ANP serum with α-rANP (1-28) was fairly good, and we could measure α-rANP(1-28) with the antiserum.

Atrial tissue contains a large quantity of high molecular weight precursors (12,27). These precursors also possess biological activities similar to those of low molecular weight peptides (6-7). Gutkowska et al. (28) purified and sequenced a major ANP-like substance secreted from cultured rat atrial cardiocytes, and they found that the ANP-like substance had the same sequence as a 26-amino acid peptide which they previously reported (29). In contrast,

Glembotski and Gibson (30) demonstrated that the main immunoreactive ANP-like substance contained in the cultured cells and released into the medium was a large molecular weight polypeptide of about 15,000 daltons, presumably a precursor. It is difficult to explain the discrepancy between the results of their studies as both groups used similarly dispersed cultured cells. In the present study, when characterized by HPLC, ANP-like immunoreactive material in the medium exhibited a single peak at the position of a synthetic peptide of 28 amino acids, α-rANP(1-28). We cannot rule out the possibility that the high molecular weight form of ANP was converted to the low molecular weight form in the medium during the incubations, since we did not examine the proteolytic activity of the medium which we used in this study.

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