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Peptide Regulators of Steroid Production

ATRIAL AND BRAIN NATRIURETIC PEPTIDE IN ADRENAL STEROIDOGENESIS

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Summary—We elucidated the role of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) in human and bovine adrenocortical steroidogenesis. The urinary volume, sodium excretion and cyclic GMP (cGMP) excretion and plasma cGMP were markedly increased by the synthetic α-human ANP (α-hANP) infusion in healthy volunteers. Plasma arginine vasopressin (AVP) and aldosterone levels were significantly suppressed. Both ANP and BNP inhibited aldosterone, 19-OH-androstenedione, cortisol and DHEA secretion dose-dependently and increased the accumulation of intracellular cGMP in cultured human and bovine adrenal cells. α-hANP significantly suppressed *P*450scc-mRNA in cultured bovine adrenal cells stimulated by ACTH. Autoradiography and affinity labeling of [^{125}I]hANP, and Scatchard plot demonstrated a specific ANP receptor in bovine and human adrenal glands. Purified ANP receptor from bovine adrenal glands identified two distinct types of ANP receptors, one is biologically active, the other is silent. A specific BNP receptor was also identified on the human and bovine adrenocortical cell membranes. The binding sites were displaced by unlabelled ANP as well as BNP. BNP showed an effect possibly via a receptor which may be shared with ANP. The mean basal plasma α-hANP level was 25 ± 5 pg/ml in young men. We confirmed the presence of ANP and BNP in bovine and porcine adrenal medulla. Plasma or medullary ANP or BNP may directly modulate the adrenocortical steroidogenesis. We demonstrated that the lack of inhibitory effect of α-hANP on cultured aldosterone-producing adenoma (APA) cells was due to the decrease of ANP-specific receptor, which caused the loss of suppression of aldosterone and an increase in intracellular cGMP.

INTRODUCTION

α-Human atrial natriuretic peptide (α-hANP), a peptide composed of 28 amino acids containing a disulfide linkage between 7 and 23 cysteine [1, 2] and porcine brain natriuretic peptide (pBNP) composed of 26 amino acid residues (pBNP-26), were identified in the human or rat atria and brain and in the porcine brain, respectively [3, 4].

These peptides are homologous polypeptide hormones and produce vasodilatory, diuretic and natriuretic effects when injected into rats and humans during the regulation of fluid and electrolyte homeostasis [5]. Very recently a C-type natriuretic peptide composed of 22 residues (CNP-22) was identified in the porcine brain as a 3rd member of the mammalian natriuretic peptide family (Fig. 1) [6].

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Molecular cloning has defined 3 types of natriuretic peptide receptor, atrial natriuretic peptide (ANP) A and B receptors (120–140 K) which are coupled to cyclic GMP (cGMP) production [7, 8, 9] and ANP C receptor which is not coupled to cGMP production (clearance of ANP) (Fig. 2) [10, 11].

The precise mechanism of the biological effects of these peptides is unknown. Here we review our results about the mechanism of action of these peptides especially on adrenal steroidogenesis.

EXPERIMENTAL

α-hANP infusion test

Six healthy Japanese volunteers and five patients with primary aldosteronism were put on regular diets which contained 12 g NaCl per day for 1 week before initiation of the test. A synthetic α-hANP (Peptide Institute, Osaka, Japan) infusion test was done as follows [12].

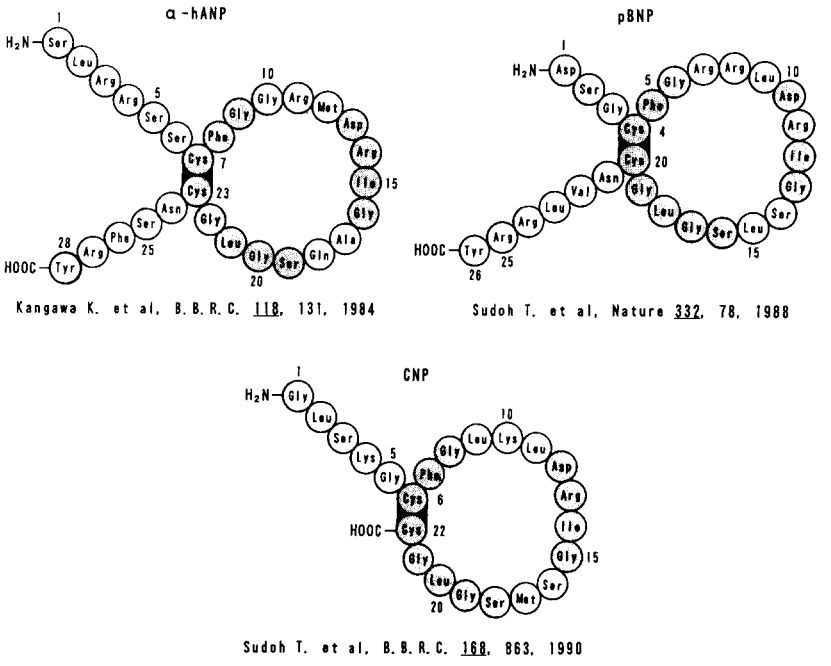


Fig. 1. Structure of natriuretic peptide family.

On the day of the test, the subjects were kept fasting and supine until the study was completed. The collection of urine and intake of 200 ml of water were carried out in all subjects every hour on 3 occasions. At 09.00 h, synthetic α-hANP was administered i.v. at 0.1 μg/kg per min for 20 min. As a control study, the same volume of saline was infused for 20 min.

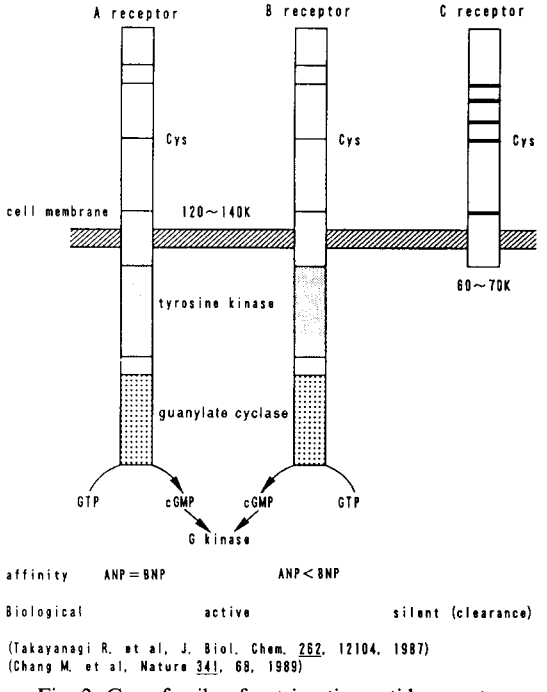


Fig. 2. Gene family of natriuretic peptide receptor.

Blood pressure and heart rates were monitored continuously during the study using an automatic sphygmomanometer, samples were drawn into a tube containing EDTA-2Na and Trasylol (500 KIU/ml), centrifuged at 5000 *g* for 10 min and the plasma sample was stored at −20°C for hormone assay. The urine sample collected during this interval of 60 min was also stored at −20°C. The urinary cGMP and cAMP concentrations were measured using cGMP and cAMP assay kits (Yamasa Shoyu Co., Chiba, Japan). Plasma arginine vasopressin (AVP) was determined by RIA using specific anti-AVP antisera (Mitsubishi Yuka Medical Science, Tokyo, Japan). Plasma aldosterone and cortisol were determined by specific RIA (CEA IRE-Sorin RIA, Paris, France and Daiichi RIA kit, respectively). Plasma DHEA and 19-OH-androstenedione were measured by specific RIA.

Primary monolayer culture of human and bovine adrenal cells

Bovine adrenal glands were collected at a local slaughter house immediately after the animal was killed. Human adrenal glands were obtained at the time of adrenalectomy from patients with advanced breast cancer, and aldosterone-producing adenoma (APA) and adjacent normal adrenal tissues were obtained immediately after adrenalectomy from patients with primary aldosteronism. Primary mono-

layer culture of adrenal cells was done as follows [13]: the adrenal glands were minced with scissors, put in Modified Eagle's Medium (MEM) containing 0.25% collagenase (type I), and incubated with agitation in enzyme solution at room temperature for 60 min. The dispersed adrenal cells were filtered through 3 layers of gauze and centrifuged at 100 *g* for 10 min. The cells were washed 3 times with MEM and put in 35 × 10 mm plastic culture dishes (Falcon 3001) at a density of 1×10^5 cells/dish. The cells were incubated in 1 ml MEM containing 10% fetal calf serum, 100 U penicillin, and 100 μg streptomycin at 37°C under 5% CO_2 in air. The culture medium was changed daily.

On day 4, the cell culture dishes were washed and preincubated for 10 min with serum-free MEM containing 40 μg/ml bacitracin. Five minutes after the addition of various amounts of hANP (10^{-9}–10^{-7} M), triplicate cell culture dishes were stimulated with ACTH or angiotensin II (AII) for 3 h. All media were stored at −20°C for steroid assay. The cultured adrenal cells were harvested using 0.05% trypsin and 0.02% EDTA, and cells precipitated by centrifugation were stored at −20°C to determine intracellular levels of cGMP.

In vitro receptor autoradiography

In vitro receptor autoradiographic studies were done using methods as described previously [14]. Briefly, bovine adrenal tissues, APA and normal adrenal tissues were rapidly isolated and frozen in O.C.T. compounds at −70°C. 25-μm-Thick sections were then cut in a cryostat at −20°C and collected on cold microscope slides. Some of these slides were used for staining with hematoxylin-eosin. Other slides were desiccated for at least 3 h under vacuum at −20°C. For binding assays, slide-mounted frozen sections were incubated in 50 mM Tris–HCl buffer (pH 7.4), containing 100 mM NaCl, 5 mM $MgCl_2$, 0.5 μg/ml phenylmethylsulfonylfluoride (PMSF), 40 μg/ml bacitracin, 4 μg/ml leupeptin, 2 μg/ml chymostatin and 0.5% BSA at room temperature for 3 h with 50 pM [^{125}I]α-hANP for total binding, and 50 pM [^{125}I]α-hANP plus 1 μM unlabeled α-hANP for nonspecific binding. At the end of the incubation time, the slides were rinsed 3 times in cold buffer and rapidly dried. These slides were tightly juxtaposed with Kodak XAR film (Eastman Kodak, Rochester, NY, U.S.A.) and stored at −70°C for 6 days. After exposure, the films were processed. Specific binding was evaluated by the difference in [^{125}I]α-hANP bound in the presence or absence of 1.0 μM α-hANP.

Binding of [^{125}I]α-hANP or [^{125}I]pBNP to human or bovine adrenal membrane fractions

The preparation of membrane fractions and binding assays were performed as described previously. The bovine or human adrenal tissues were minced finely and homogenized with Polytron in 10 vol of ice-cold 50 mM Tris–HCl buffer (pH 7.6), containing 1 mM EDTA, 1 mM dithiothreitol and 0.1 mM PMSF. The membrane fractions were prepared as 800–100,000 *g* pellets and stored at −80°C. α-hANP and pBNP were radioiodinated using a lactoperoxidase method and were purified by reverse-phase HPLC. The specific radioactivity of [^{125}I]hANP was 1000–1400 Ci/mmol. The specific radioactivity of [^{125}I]pBNP was 1400–2200 Ci/mmol. The binding of [^{125}I]α-hANP to human adrenal membrane fractions was determined as described previously [15]. The standard binding assay was conducted in 300 μl of 50 mM Tris–HCl (pH 7.6), 1 mM EDTA, 1 mM cystamine, 150 mM NaCl, 5 mM $MnCl_2$, 0.1 mM PMSF, 100–200 μg of human adrenal membrane fractions and 5–300 pM of [^{125}I]α-hANP, with or without α-hANP (10^{-6} M) or 50–100 μg of human adrenal membrane fractions and 10 fmol of [^{125}I]pBNP, with or without various amounts of pBNP. The incubation was carried out at 22°C for 45 min in a polystyrene culture test tube (12 × 75 mm). Bound [^{125}I]α-hANP and [^{125}I]pBNP were separated from the free ligand by filtration on a 934 AH glass fibre filter which had been pretreated with 0.3% (w/v) polyethylenimine. The filters were washed 3 times with phosphate-buffered saline and the radioactivity trapped in filters was counted.

Affinity labeling of [^{125}I]pBNP to human adrenal membrane fractions

Human adrenal membrane fractions (2 mg protein) were incubated with [^{125}I]pBNP (1.5 mM), 50 mM Tris–HCl, 1 mM EDTA, 1 mM cystamine, 150 mM NaCl, 5 mM $MnCl_2$, 0.1 mM PMSF, in the absence or presence of pBNP (10^{-7} M) or α-rANP (10^{-7} M) in a final volume of 1 ml for 45 min at 25°C. After incubation, DSS (in dimethylsulfoxide) was added to a final concentration of 0.5 mM, and the crosslinking reaction was allowed to complete for 15 min at 25°C. Reactions were terminated by the addition of 50 μl of 0.5 M glycine. The

reaction mixtures were centrifuged to collect the pellets. The resultant pellets were solubilized in 100 μl of 0.2 M Tris–HCl (pH 6.8), containing 15% (w/v) sodium dodecyl sulfate (SDS), 20% glycerol, 200 mM dithiothreitol, 10 mM mercaptoethanol and 0.15 mg/ml of bromophenol blue, and heated for 3 min at 100°C. Gel electrophoresis was performed with slab gels according to the method described previously [16] using 8% (w/v) polyacrylamide gel. After the gels had been stained with Coomassie brilliant blue, the dried materials were subjected to autoradiography using Kodak XAR-5 film.

RIA for α-hANP and BNP

Plasma hANP concentration was measured by RIA. Rabbit anti-α-hANP serum raised against synthetic α-hANP conjugated to bovine thyroglobulin was provided by Eiken Chemical Co. Ltd (Tokyo, Japan). This anti-α-hANP antiserum was directed mainly against the C terminal sequence in the α-hANP molecule. RIA for α-hANP was determined as described previously [17]. α-hANP was iodinated using a lactoperoxidase method and purified by reverse-phase HPLC. The assay mixture contained [^{125}I]α-hANP (10,000 cpm), rabbit anti-α-hANP anti-serum (final dilution, 1:80,000) and unextracted chilled plasma in 0.1 M phosphate buffer (pH 7.5), containing 50 mM NaCl, 0.1% BSA, 0.3% dextran, 0.1% Triton X-100 and 0.1% NaN_3. Antibody-bound and free α-hANP were separated by the addition of anti-rabbit immunoglobulin G serum. The minimum detectable quantity was 2 pg per tube. RIA for BNP was performed by the method reported previously [18]. The RIA buffer consisted of 0.1 M sodium phosphate (pH 7.4), 1% BSA, 0.1% Triton X-100, 0.025 M EDTA-2Na and 0.05% NaN_3. A sample (100 μl) was pre-incubated with 100 μl of anti-BNP-26 anti-serum for 24 h. Anti-serum recognizes mainly the ring portion of BNP-26 flanked by a disulfide linkage. Then 100 μl of [^{125}I]pBNP-26 (*ca* 20,000 cpm) was added, and incubation continued for an additional 24 h. Free and bound tracers were separated by the polyethyleneglycol (PEG) method. All procedures were carried out at 4°C.

Characterization of ANP and BNP in bovine adrenal medulla

Bovine adrenal medulla, obtained from freshly killed animals at a local slaughter house was separated from cortex portions on ice at 4°C. After weighing, tissues were diced and then boiled in water for 10 min to inactivate proteases. After cooling, glacial acetic acid was added (a final concentration of 1 M) and tissues were homogenized with a Polytron mixer for 4 min. The homogenates were centrifuged at 8000 ***g*** for 20 min, and the supernatants were then collected and loaded onto a Sep-Pak C_{18} cartridge column. The adsorbed materials were eluted with a solution of 40% CH_3CN containing 0.01% TFA, and the eluate was evaporated to dryness *in vacuo*. The residual material was dissolved in 1 M acetic acid and then used for gel filtration and reversed-phase HPLC.

The dried extract was dissolved in 1 M acetic acid at the concentration of 1 g wet wt/ml, loaded onto a Sephadex G-50 fine column (1.5 × 120 cm, Pharmacia) and eluted with 1 M acetic acid. Each fraction was lyophilized and redissolved in a small volume of RIA buffer, and then submitted to RIAs for BNP and ANP. The molecular forms in the extract were also analyzed by reversed-phase HPLC on a Shim-Pak CLC-ODS (6 × 150 mm) eluted with a linear gradient of CH_3CN from 10 to 40% in 0.01% TFA for 45 min at a flow rate 1.5 ml/tube. Aliquots of all fractions were assayed for ANP and BNP.

Dot blot analysis of P450scc-mRNA

Cultured bovine adrenal cells were treated with 10^{-8} M ACTH, 10^{-7} M rANP or 10^{-7} M pBNP. After 24 h incubation, cells were collected and total RNA collected by acid–guanidium, thiocyanate–phenol–chloroform extraction. The denature, blotting and hybridization with ^{32}P-labeled cDNA of *P*450 side chain cleavage (scc) were carried out as described previously [18].

Statistical methods

Statistical significance was determined by Student's *t*-test. Results are expressed as the mean ± SD.

RESULTS

Effect of α-hANP infusion

The i.v. administration of α-hANP led to a remarkable reduction in mean blood pressure (−10 mmHg, $P < 0.05$), and there was an increase in pulse rate (+10 bpm, $P < 0.05$), in each normal healthy volunteer. The urinary volume, sodium excretion and cGMP excretion

were increased to 3.5 ($P < 0.05$), 2.5 ($P < 0.05$) and 8-fold ($P < 0.01$), respectively, during the α-hANP infusion [12]. The mean plasma cGMP concentration was clearly elevated and attained 8-fold of the mean basal concentrations by the end of infusion. However, there was no apparent change on plasma or urinary cAMP during the infusion [19]. The result clearly demonstrated that synthetic α-hANP selectively induces the cGMP in concomitant with the marked diuretic, natriuretic and hypotensive effects in human subjects. The plasma AVP concentrations decreased from 0.39 ± 0.09 pg/ml to the undetectable levels (Fig. 3) [12]. There were no significant changes in plasma ACTH concentration and plasma renin activity following the infusion. The mean plasma cortisol and aldosterone concentrations were significantly reduced during the infusion (Fig. 3). Our data suggest that synthetic α-hANP inhibits adrenocortical steroidogenesis in man [20]. The effects of α-hANP in patients with primary aldosteronism were compared with those in normal volunteers. There were significant decreases in the mean blood pressure (−10 to −15 mmHg) in patients with primary aldosteronism [21]. The plasma cGMP concentrations and the rate of sodium excretion were increased following the infusion. However, the plasma cortisol and aldosterone concentrations did not significantly change during α-hANP infusion.

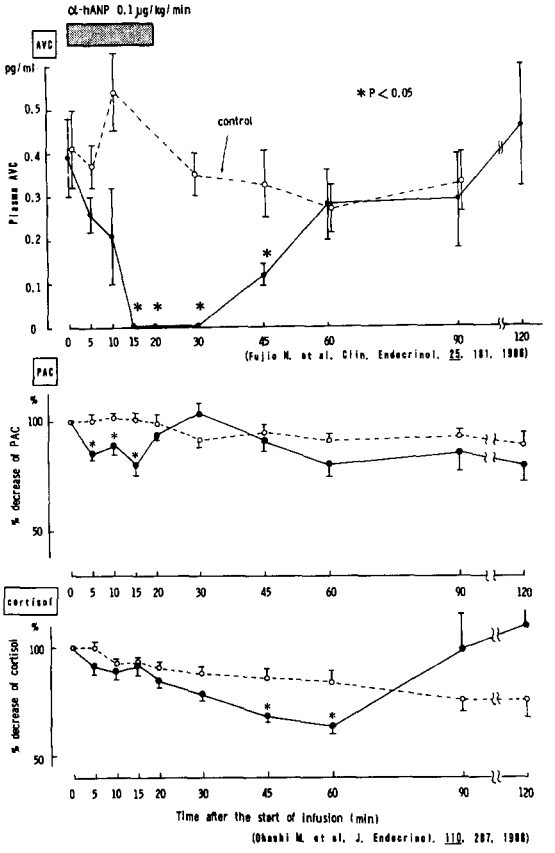


Fig. 3. Effect of α-hANP infusion on plasma AVP, aldosterone and cortisol in human healthy volunteers.

Effect of ANP and BNP on adrenal steroidogenesis

The effects of α-hANP on steroidogenesis in bovine adrenocortical cells in primary monolayer culture were investigated. α-hANP induced a significant dose-dependent inhibition of basal levels and ACTH (10^{-8} M)- and angiotensin II (10^{-7} M)-stimulated increases of aldosterone, cortisol and DHEA secretion [14]. Visualization of [^{125}I]α-hANP binding sites by *in vitro* autoradiography demonstrated that these sites were highly localized in the bovine adrenal cortex, especially the zona glomerulosa (Fig. 4) [14]. The ability of α-hANP to alter steroidogenesis by human adrenal glands was investigated in cultured human adrenal cells [22, 23]. α-hANP (10^{-9}–10^{-7} M) inhibited basal and ACTH (10^{-8} M)-stimulated aldosterone (Fig. 5), cortisol, DHEA and 19-OH-andros-

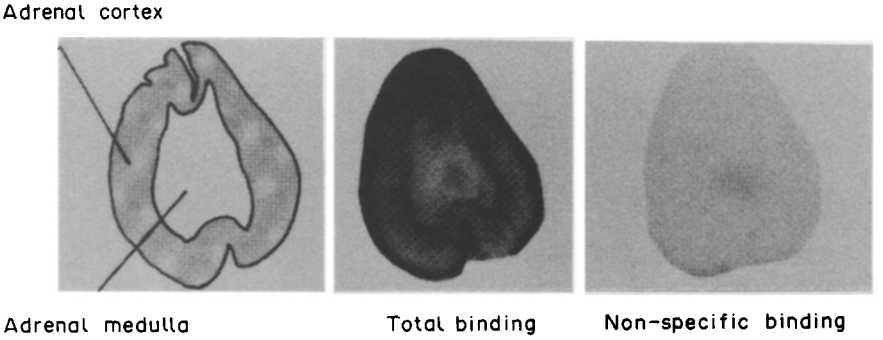
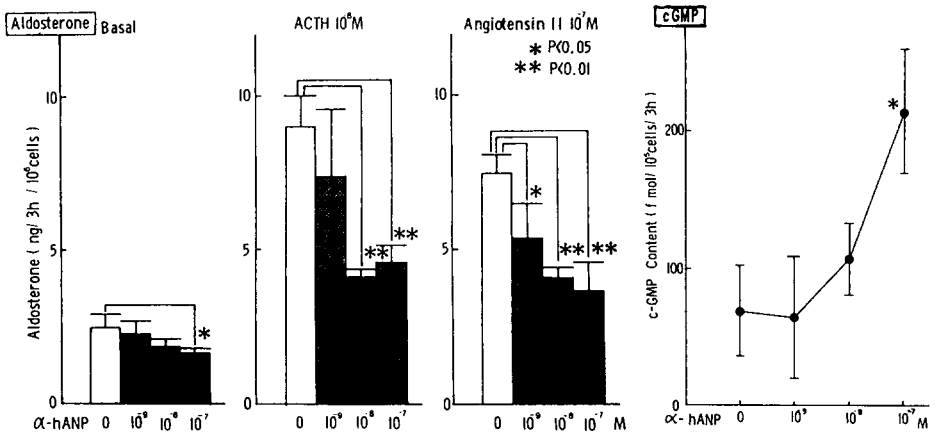


Fig. 4. *In vitro* receptor autoradiography of [^{125}I]α-hANP binding to bovine adrenal gland.



(Higuchi K. et al, J. Clin. Metab. Res. 62, 941, 1986)

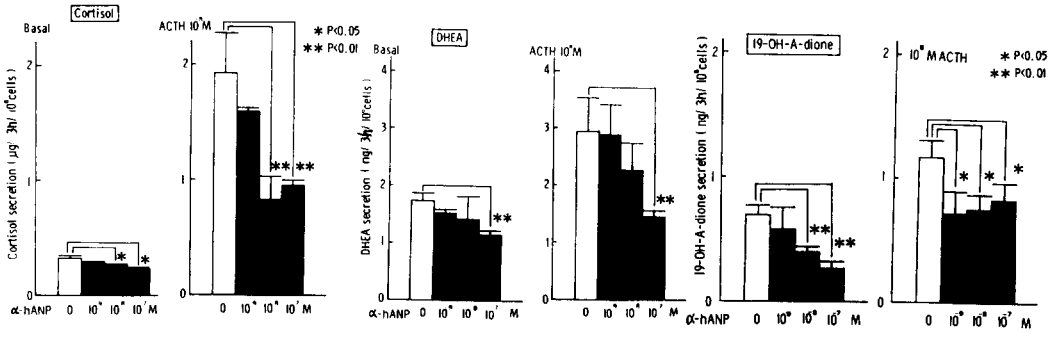
Fig. 5. Effect of α-hANP on aldosterone secretion and formation of intracellular cGMP in cultured human adrenal cells.

tenedione (aldosterone amplifier) secretion in a dose-dependent manner (Fig. 6) [23]. In addition, α-hANP enhanced the accumulation of intracellular cGMP in a dose-dependent manner [22].

As aldosterone, cortisol, and DHEA secretion from cultured human adrenal cells was inhibited by α-hANP, the site of inhibition of steroidogenesis by α-hANP is probably localized in the early pathway of steroidogenesis in human adrenal cells. We determined *P*450scc-mRNA in cultured bovine adrenal cells. α-hANP or pBNP significantly suppressed the *P*450scc-mRNA induced by 10^{-8} M ACTH (Fig. 7). The results also suggest a link between inhibitory effects of α-hANP and accumulation of intracellular cGMP. pBNP also had a significant suppressive effect on aldosterone and cortisol secretion in ACTH-treated cultured human adrenal cells [16] and cultured bovine adrenocortical cells. Concomitantly, the intracellular cGMP formation was enhanced by pBNP treatment (Fig. 8).

α-hANP and pBNP binding sites in human and bovine adrenal membrane fraction

Using crude membrane fractions prepared from human adrenal tissues, we noted the existence and molecular weight of specific binding sites for [^{125}I]α-hANP. The mean maximal binding capacity (B_{max}) and dissociation constant (K_d) of 4 human adrenal membrane fractions were 8.0 ± 1.6 fmol/mg/protein and 25.7 ± 7.4 pM, respectively, as calculated by Scatchard plot analysis (Fig. 9) [15]. When the membrane fractions were incubated with [^{125}I]α-hANP and then cross-linked with disuccinimidyl suberate (5 mM), the 135 and 67 kDa proteins were specifically radiolabeled (Fig. 14) [15]. The very high affinity of [^{125}I]α-hANP binding sites suggests that human adrenal steroidogenesis may be influenced by



(Higuchi K. et al, J. Clin. Metab. Res. 62, 941, 1986) (Higuchi K. et al, Horm. Metab. Res. 21, 92, 1989)

Fig. 6. Effect of α-hANP on cortisol, DHEA, 19-OH-androstenedione (19-OH-A-dione) in cultured human adrenal cells.

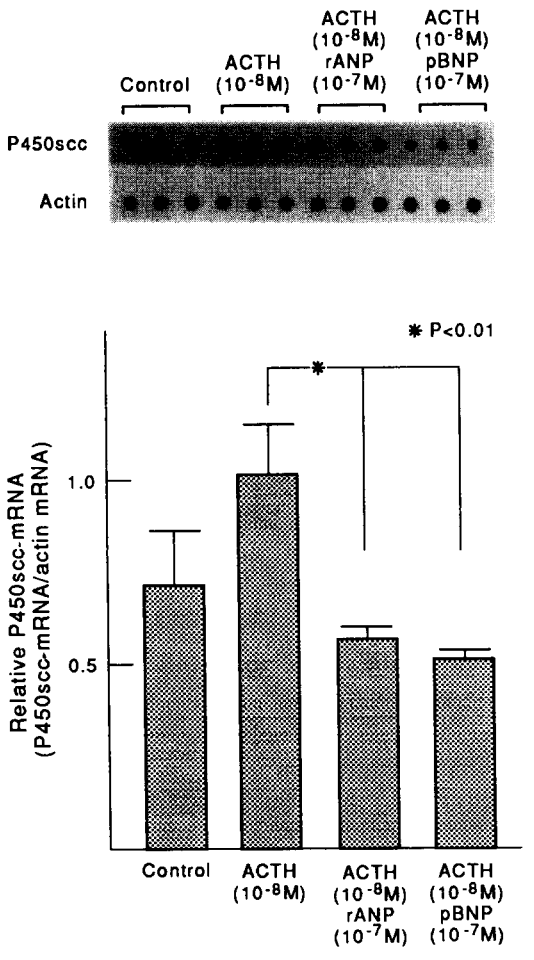


Fig. 7. Dot blot analysis of *P*450scc-mRNA in cultured bovine adrenal cells treated by ANP or BNP.

plasma levels of hANP, under physiological conditions. *In vitro* receptor autoradiography demonstrated specific binding sites for [^{125}I]pBNP by which unlabeled pBNP or α-hANP can take place at these sites [24]. A specific pBNP receptor was identified in the human adrenal tissues. Affinity labeling of [^{125}I]pBNP showed 2 specific binding sites for pBNP of 140 and 67 kDa (Fig. 10) [16]. We purified ANP receptor by ANP-agarose, GTP-agarose and wheat germ agglutinin-Sepharose chromatography and found 2 subtypes of ANP receptor guanylate cyclase-containing and free receptor (Fig. 11) [10]. A [^{125}I]pBNP binding study of the human and bovine adrenal membrane fraction demonstrated the presence of high-affinity and low-capacity binding sites for pBNP [16]. Moreover, these binding sites for [^{125}I]pBNP were displaced by unlabeled α-rANP as well as pBNP [24]. The α-hANP required for 50% inhibition of specific [^{125}I]pBNP binding was almost identical to that for pBNP [24]. These results suggest that pBNP exerts a suppressive effect on bovine adrenocortical steroidogenesis via a receptor which may be shared with ANP.

The effects of α-hANP on aldosteronogenesis in APA cells in primary monolayer cultures were studied. α-hANP had no inhibitory effect on aldosterone secretion from APA cells and had no effect on intracellular cGMP

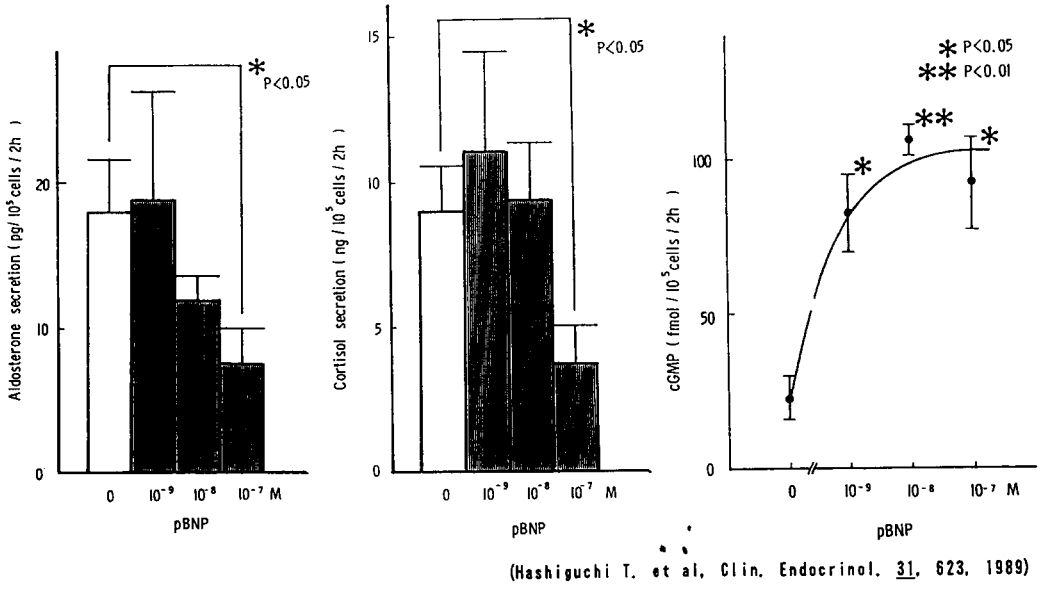


Fig. 8. Effect of pBNP on aldosterone and cortisol secretion and cGMP formation in cultured human adrenal cells.

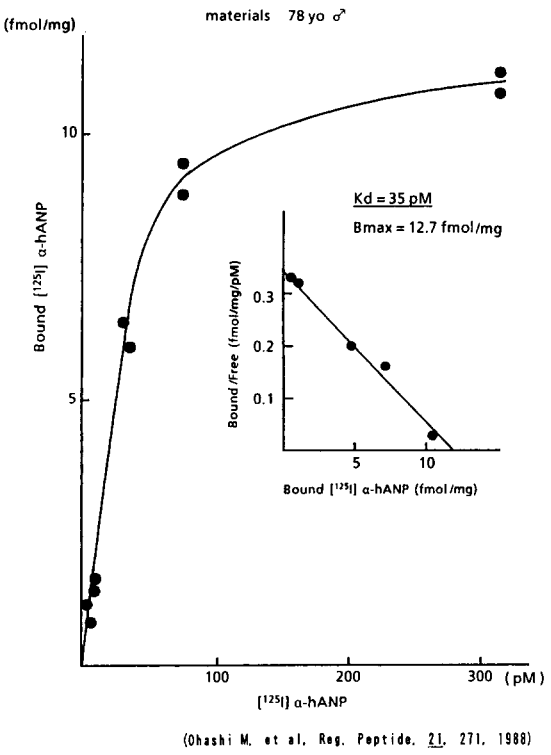
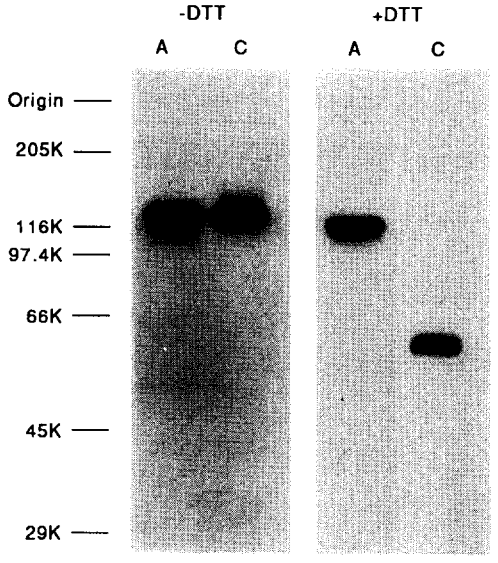


Fig. 9. [^{125}I]α-hANP receptor in human adrenal cell membranes.

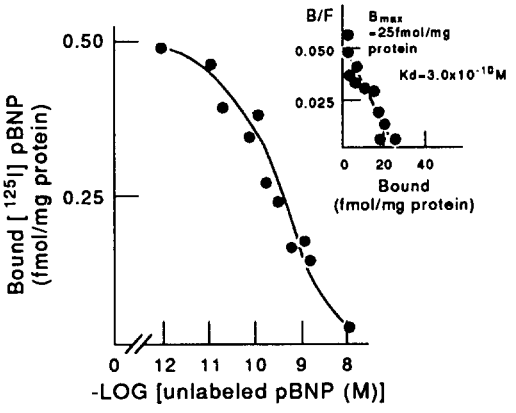


(Takayanagi et al. J.Biol.Chem. (1987)262, 12104)

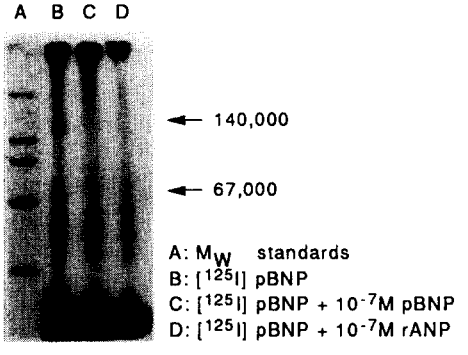
Fig. 11. SDS–PAGE of purified bovine adrenocortical ANP receptors.

in APA cells (Fig. 12) [25]. Visualization of [^{125}I]α-hANP-specific binding sites in APA and adjacent normal adrenal tissues by an *in vitro* receptor autoradiography showed that these sites were localized only in normal adrenal tissue, but not in APA tissue (Fig. 13). The relative binding capacity for [^{125}I]α-hANP in the APA membrane fractions was only half of that in the normal adrenal membrane fractions.

The radioactive bands indicating specific binding sites for [^{125}I]α-hANP were mainly visualized at the 140 kDa and faintly at 67–70 kDa regions, in the affinity labeling of the normal adrenal tissues. No radioactive band could be discerned in the case of the adenoma tissues (Fig. 14). These results demonstrate that the lack of an inhibitory effect of α-hANP on aldosteronogenesis in APA cells may be due to the absence of α-hANP-specific receptor sites in APA cells.



Competitive binding of [125I] pBNP to human adrenal membrane fractions by unlabeled pBNP (inset) Scatchard plot of the data.



Affinity labeling of [125I] pBNP to human adrenal membrane fractions.

Fig. 10. Binding of [^{125}I]pBNP (left) and affinity labeling (right) of [^{125}I]pBNP to human adrenal membrane.

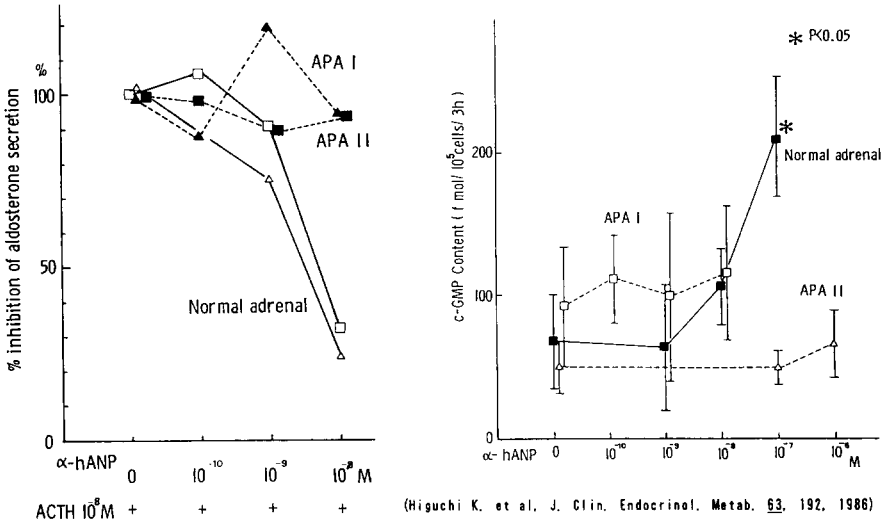


Fig. 12. Effect of α-hANP on the aldosterone secretion (left) and intracellular cGMP (right) in cultured human normal adrenal cells and APA cells.

Plasma and adrenomedullary ANP and BNP

The mean basal plasma α-hANP level was 25 ± 5 (± SEM) pg/ml in the young men and 120 ± 22 pg/ml in the elderly men [17]. Using a specific RIA for pBNP, we identified BNP in the extracts of bovine and porcine adrenal medulla. The mean contents of ANP and BNP in the acid extracts of bovine adrenal medulla were 8.42 ± 1.63, and 0.59 ± 0.10 pmol/g wet tissue, respectively. BNP and ANP in the extract of bovine adrenal medulla in reversed-phase HPLC showed 3 major peaks of ANP, α-ANP and γ-ANP. BNP was also eluted as 3 molecular forms: pBNP-26 or pBNP-32 and γ-BNP.

DISCUSSION

We elucidated the physiological role of ANP and BNP of vascular smooth muscle relaxation, diuretic and natriuretic effects and suppression of AVP release in the regulation of fluid and electrolyte homeostasis to lowering blood pressure [12]. Another important factor which regulates fluid and electrolyte homeostasis is mineralocorticoid steroid hormone. The *in vivo* effect of α-hANP on aldosterone secretion was controversial; synthetic auriculin causes a significant decrease in plasma renin activity and plasma aldosterone concentrations in anaesthetized dogs [26]. α-hANP infusion caused a considerable reduction in plasma aldosterone concentrations in the human subject [27]. On the other hand, neither plasma renin activity nor plasma aldosterone concentrations show any remarkable change by synthetic α-hANP infusion in human subjects [27, 28]. On the latter case aldosterone levels tended to fall after ANP infusion. These results must be interpreted cautiously because of the small number of observations and the discrepancy due to the different modes of α-hANP administration. In the present study, the mean plasma aldosterone concentration was significantly suppressed during α-hANP infusion despite the change in plasma renin activity.

cGMP may play an important role in the metabolic effects of α-hANP in humans, from the result of the significant decrease in the mean blood pressure followed by a rise of plasma cGMP, and the marked diuresis and natriuresis

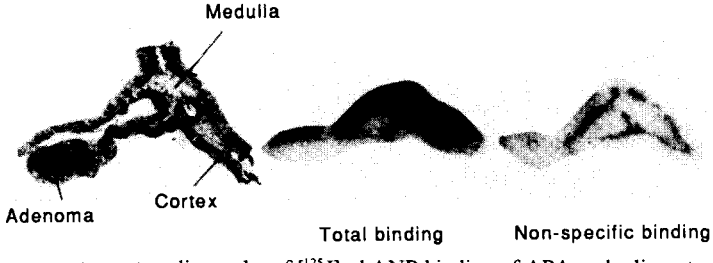


Fig. 13. *In vitro* receptor autoradiography of [^{125}I]α-hANP binding of APA and adjacent adrenal tissues.

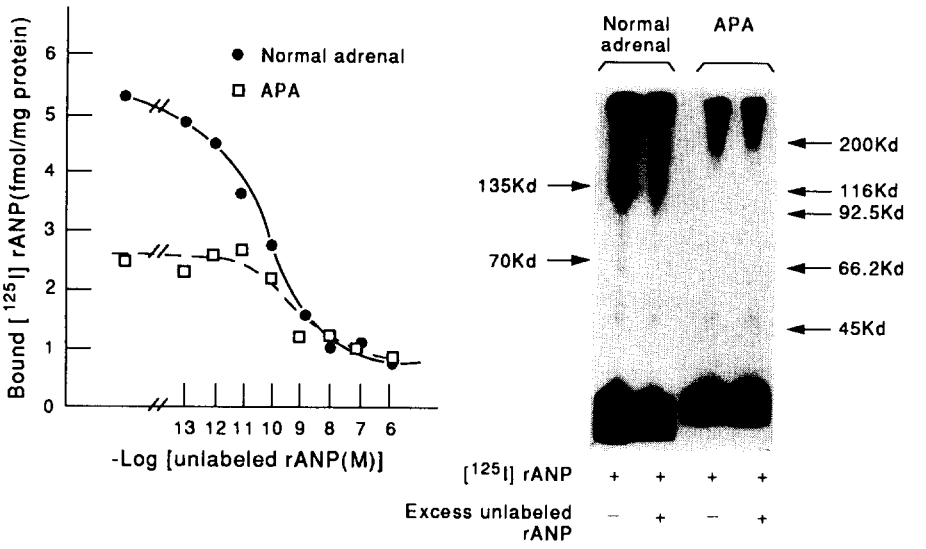


Fig. 14. Binding of [^{125}I]ANP (left) and affinity labeling of [^{125}I]α-hANP (right) to normal human adrenal and APA membranes.

in parallel with the increase of urinary cGMP excretion during α-hANP infusion [19].

The direct effect of α-hANP on adrenal glands was determined by using dispersed adrenocortical cells in culture or suspension. The present findings, of the inhibitory effect of α-hANP on aldosterone secretion from human and bovine adrenocortical cells in culture [14, 22] are in agreement with a number of studies in rat, bovine and human adrenal cells [30–33]. All these studies showed either decreased aldosterone secretion or decreased aldosterone and corticosterone or cortisol secretions. We found that α-hANP inhibited not only aldosterone but also cortisol, DHEA and 19-OH-androstenedione secretion by monolayer culture of human and bovine adrenal cells [14, 22, 23]. In bovine glomerulosa cells, ANP inhibited the production of pregnenolone [34]. These results indicate that ANP acts on an early step of steroidogenesis, to a step before cholesterol side chain cleavage [35]. We demonstrated the significant *P*450scc-mRNA inhibition by α-hANP. These results suggest that α-hANP suppresses the rapid reaction of cholesterol uptake before cholesterol scc and the late reaction of *P*450scc-mRNA inhibition. Autoradiography of [^{125}I]α-hANP specific binding sites in bovine adrenal glands demonstrated that these sites were found not only in zona glomerulosa but also in zona fasciculata and zona reticularis [14]. We found that the whole zone of adrenal cortex was a target organ for α-hANP. We also found that α-hANP directly stimulated accumulation of intracellular cGMP in a dose-responsive manner in human and bovine adrenal cells [16, 22, 24]. The accumulation of cGMP shows a good biological marker of hANP. The evidence that α-hANP significantly induced the cGMP formation suggests that human and bovine adrenal glands might possess the functional α-hANP receptor, possibly coupled with guanylate cyclase. We characterized the binding sites for α-hANP in human adrenal membrane fractions for the first time.

We detected a high-affinity, limited-capacity binding site for [^{125}I]α-hANP, as shown in bovine adrenocortical or glomerulosa tissue [36, 37]. Meloche *et al.* [37] demonstrated the presence of 2 classes of binding sites with a K_d of 40 and 6000 pM in the solubilized ANP receptor from bovine adrenal zona glomerulosa. The K_d obtained from saturation experiments on human adrenal membrane fractions may represent the higher-affinity binding sites. We demonstrated that plasma hANP concentration in healthy subjects is 25 ± 5 to 120 ± 20 pg/ml [17]. Thus, half of the binding sites for [^{125}I]α-hANP in the human adrenal membrane fractions may be occupied by circulating hANP. Therefore, we speculate that the adrenocortical steroidogenesis in humans may be regulated by the circulating level of hANP under physiological conditions.

In the present study, we clearly demonstrated that pBNP also had a potent suppressive effect on cortisol and aldosterone secretion of cultured human and bovine adrenal cells [16, 24]. It is noted that the mode of suppression by pBNP

was similar to that in α-hANP. We confirmed the presence of the binding sites for [^{125}I]pBNP, of which Scatchard plot analysis characterized the high-affinity and low-capacity nature in human and bovine adrenocortical cells membrane [16, 24]. Moreover, such specific binding for [^{125}I]pBNP was completely substituted by unlabeled α-hANP, in addition to unlabeled pBNP in human and bovine adrenal cortex. The molecular weights of the major binding sites for [^{125}I]pBNP were 140 and 67 kDa, as determined by the affinity cross-linking technique, in the human and bovine adrenal membrane fractions. ANP and BNP apparently share common receptors and stimulate the intracellular production of cGMP as a biological marker. Molecular cloning has defined 3 types of natriuretic peptide receptors: the ANP-C receptor (60–70 K), which is not coupled to cGMP production and whose function is to absorb excess ANP and either to eliminate it from circulation or store and slowly release it [11] and the ANP-A and ANP-B receptors (120–140 K), which are membrane forms of guanylate cyclase in which ligand binding to the extracellular domain activates the cytoplasmic domain of protein kinase and guanylate cyclase. The ANP-B receptor is preferentially activated by pBNP rather than α-hANP, whereas the ANP-A receptor responds similarly to both natriuretic peptides [9]. Guanylate cyclase exists in at least 2 different molecular forms: a soluble enzyme consisting of 2 subunits and a transmembrane protein having a single subunit [38]. It is the membrane form of guanylate cyclase that is activated following binding of ANP to target cells. The ANP receptor/guanylate cyclase represents a new class of mammalian cell-surface receptors which contain an extracellular ligand binding domain and an intracellular guanylate cyclase catalytic domain [9].

Although ANP receptors are distributed extensively in many types of organs and ANP elicits cGMP production in these organs, it is not clear if ANP-induced actions can be explained by a cGMP-mediated mechanism. Atriopeptin significantly elevates cGMP and activates cGMP-dependent protein kinase and causes vascular relaxation [39]. In adrenal zona glomerulosa cells, ANP elicits vigorous production of cGMP, and inhibition of aldosterone occurs. However, sodium nitroprusside, which also induces cGMP production, did not inhibit aldosterone secretion. Moreover, 8-bromo-cGMP and 8-chlorophenylthio-cGMP did not inhibit the aldosterone release [5, 40]. The present *in vivo* and *in vitro* studies of the patients with primary aldosteronism [25] and Cushing's syndrome [41] clearly showed that ANP could not suppress aldosterone secretion and increase intracellular cGMP in APA cells because of the significant decrease in the number of ANP receptors in APA cell membranes [25].

Several investigators have already reported that ANP is synthesized in and secreted by mammalian adrenal chromaffin cells [42], postulating that it might play some role in the regulation of adrenocortical functions. Aldosterone production may be affected not only by circulating ANP but also by adrenomedullary ANP. In the present study, appreciable amounts of ANP and BNP were detected in the extracts of bovine and porcine adrenal medulla. It has already been reported that bovine chromaffin cells are capable of synthesizing and subsequently secreting ANP with nicotine or high potassium treatment. The presence of appreciable amounts of BNP as well as ANP suggests that both natriuretic peptides play some physiological role in adrenal and other endocrine functions.

Very recently C-type natriuretic peptide (CNP), the 3rd member to join the natriuretic peptide family was identified in the porcine brain [6]. This may be a candidate of adrenomedullary natriuretic peptide. With the identification of CNP, the natriuretic peptide family is shown to comprise of at least 3 different peptides, implying that physiological functions so far attributed mainly to ANP and its receptor are controlled by a combination of a series of natriuretic peptides, ANP, BNP and CNP and their respective receptors.

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