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Porcine brain natriuretic peptide, another modulator of bovine adrenocortical steroidogenesis

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Porcine brain natriuretic peptide (pBNP) significantly inhibited aldosterone production stimulated by an angiotensin II analog and ACTH-stimulated cortisol secretion, together with simultaneously increasing the formation of cGMP in dispersed bovine adrenocortical cells. Receptors for pBNP were identified in bovine adrenal gland using an in vitro receptor autoradiographic technique and studies of ^{125}I-pBNP binding. In vitro receptor autoradiography demonstrated specific binding sites for ^{125}I-pBNP in bovine adrenal cortex. Complete displacement of ^{125}I-pBNP by unlabeled pBNP or human atrial natriuretic peptide (hANP) can take place at these sites. Analysis of ^{125}I-pBNP binding to bovine adrenocortical membrane fractions showed that the adrenal cortex had high-affinity, low-capacity pBNP-binding sites, with a dissociation constant (K_d) of $2.32 \pm 0.33 \times 10^{-10}$ M (mean $\pm$SE) and a maximal binding capacity (B_{max}) of 36.7 ± 1.6 fmol/mg protein. Moreover, the specific binding sites for ^{125}I-pBNP were completely displaced not only by unlabeled pBNP but also by unlabeled hANP. The hANP dose required for 50% inhibition of specific ^{125}I-pBNP binding was almost identical to that for pBNP (IC_{50} values for hANP and pBNP: 8.5×10^{-10} and 6.5×10^{-10} M, respectively). These results suggest that pBNP exerts a suppressive effect on bovine adrenocortical steroidogenesis via a receptor which may be shared with ANP.

Natriuretic peptide; Adrenal gland; (Porcine brain)

1. INTRODUCTION

A newly discovered peptide comprising 26 amino acid residues, termed porcine brain natriuretic peptide (pBNP), has been identified and sequenced in porcine brain [1]. The complete amino acid sequence of BNP is remarkably similar to, but definitely distinct from that of human atrial natriuretic peptide (hANP) [1,2]. pBNP exerts natriuretic, diuretic and hypotensive effects on anesthetized rats and displays chick rectum relaxant activity to the same extent as rat ANP [1]. The natural occurrence of BNP within the mammalian brain suggests the possibility that its physiological functions may be regulated through a dual mechanism involving both ANP and BNP [1]. Recently, Song et al. [3] reported the presence of a pBNP-sensitive guanylate cyclase and ^{125}I-pBNP-binding sites in cultured bovine aortic smooth muscle cells and endothelial cells. However, no studies have been published as yet on the mode of action and receptor binding of pBNP in adrenal gland. It is well known that ANP inhibits the secretion of aldosterone and other steroids, and simultaneously elevates cGMP levels in rat, bovine and human adrenal cortex [4-8]. Here, we have attempted to ascertain the effects of pBNP on the secretion of aldosterone and cortisol and on formation of cGMP, using dispersed cells of bovine adrenal cortex. Moreover, we have tried to determine the presence of a specific pBNP-binding site in bovine adrenocortical tissue by performing a ^{125}I-pBNP binding analysis on bovine adrenocortical membrane fractions and an in vitro receptor autoradiographic technique for bovine adrenal tissue.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Synthetic pBNP was produced using solid-phase techniques and its purity was checked by reverse-phase HPLC [1]. Synthetic α-hANP was obtained from the Peptide Institute (Osaka). Bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF), leupeptin and chymostatin were purchased from Sigma (St. Louis, MO). Dithiothreitol was purchased from Katayama (Osaka). Collagenase and Hepes were purchased from Wako (Osaka). [Asn^1,Val^5]-angiotensin II was obtained from Ciba-Geigy (Basel). ACTH (1–18) was obtained from Shionogi (Osaka).

2.2. *Materials*

Bovine adrenal glands used in these experiments were collected from a local abbatoir immediately after slaughter of the animals.

2.3. *Cell suspension*

The method of preparing dispersed adrenocortical cells was that in [8]. The preparation dispersed cells was resuspended in modified Krebs' solution (135 mM NaCl, 3.5 mM KCl, 2.0 mM $CaCl_2$, 1.0 mM $MgSO_4$, 20 mM Hepes, pH 7.4; 2 mg/ml glucose, 1 mg/ml BSA. The cells were transferred to a polypropylene tube (Corning Glass Works, New York). at a density of 10^5 cells per tube. Preincubations of the dispersed bovine adrenocortical cells were carried out for 90 min at 37°C. 5 min after the addition of various amounts of pBNP, the dispersed cells were stimulated with angiotensin II analog (final concentration, 10^{-7} M) or ACTH (final concentration, 10^{-8} M) for 90 min at 37°C. Incubations were terminated by centrifugation for 10 min at $1000 \times g$. Supernatants were collected for radioimmunoassay of steroids. To assay cGMP formation, preincubations were for 10 min at 37°C with 0.5 mM 1-methyl-3-isobutylxanthine, then cells were incubated with various amounts of pBNP for 20 min at 37°C. After centrifugation for 10 min at $1000 \times g$, the supernatants were discarded. A 1 ml aliquot of 6% trichloroacetic acid was added to the cell pellets, samples then being sonicated briefly using a Branson Sonifier (Danbury, CT). Following centrifugation at $3000 \times g$ for 15 min, the supernatants were collected and lyophilized for radioimmunoassay.

2.4. *Radioimmunoassay*

Aldosterone and cortisol in the medium were directly measured using an RIA kit from Diagnostic Products (Los Angeles, CA), cGMP in dispersed cells was assayed with an RIA kit from Yamasa Shoyu (Chiba) after syccinylation. The intra-assay coefficient of variation was 7% for aldosterone, 8% for cortisol and 3.2% for cGMP.

2.5. *^{125}I-pBNP in vitro receptor autoradiography of bovine adrenal gland*

In vitro receptor autoradiography was performed as in [8,9]. Briefly, adrenal tissues were rapidly isolated and frozen in OCT compounds (Division Miles Labs, Naperville, IL) at −80°C. Sections of 25 μm thickness were then cut in a cryostat at −20°C and collected on cold microscope slides. Some of the slides were used for staining with hematoxylin-eosin. Others were dessicated 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 PMSF, 40 μg/ml bacitracin, 4 μg/ml leupeptin, 2 μg/ml chymostatin and 0.5% bovine serum albumin at room temperature for 1 h with 50 pM ^{125}I-pBNP for total binding, and 50 pM ^{125}I-pBNP plus 100 nM unlabeled pBNP for nonspecific binding. Another slide section was incubated with 50 pM ^{125}I-pBNP plus 100 nM unlabeled α-hANP. At the end of the incubation period, slides were rinsed three times in cold 50 mM Tris-HCl buffer and rapidly dried. These slides were tightly juxtaposed with Kodak XAR film and stored at −80°C for 6 days. After exposure films were processed. Specific binding was evluated from the difference in ^{125}I-pBNP bound in the absence and presence of 100 nM unlabeled pBNP.

2.6. *Binding of ^{125}I-pBNP to bovine adrenocortical membrane fractions*

Bovine adrenocortical tissue was minced finely and homogenized with a Polytron (Kinematica Kriens, Switzerland) in 10 vols ice-cold 50 mM Tris-HCl buffer (pH 7.6), containing 1 mM EDTA, 1 mM dithiothreitol and 0.1 mM PMSF. The homogenate was centrifuged at $800 \times g$ for 10 min, the resultant pellet being suspended in 3 vols of the same buffer and centrifuged under identical conditions. The supernatant fractions were combined and centrifuged at $30000 \times g$ for 30 min. The resultant pellet was stored at −80°C. All operations were conducted at 4°C. pBNP was iodinated using the lactoperoxidase method and purified by reverse-phase HPLC. The specific activity of ^{125}I-pBNP amounted to 4 mCi/nmol.

Binding of ^{125}I-pBNP to bovine adrenocortical membrane fractions was determined as in [10]. The standard binding assay was conducted at a final pH of 7.6 in 250 μl of 50 mM Tris-HCl, containing 1 mM EDTA, 1 mM cystamine, 150 mM NaCl, 5 mM $MnCl_2$, 0.1 mM PMSF, 50–200 μg adrenocortical membrane fractions and 5–300 pM ^{125}I-pBNP, with or without 100 nM BNP. Incubation was carried out at 22°C for 45 min in a polystyrene culture test tube (12 × 75 mm) (Elkey Products, Shrewsbury, MA). Bound ^{125}I-pBNP was separated from free ligand by filtration on a 934 AH glass-fiber filter (Whatman, Clifton, NJ) which had been pretreated with 0.3% (w/v) polyethylenimine. The filters were washed 3 times with 3 ml phosphate-buffered saline and counted using a gamma-counter (Aloka auto well gamma-system ARC-60, Tokyo, Japan). Specific binding was calculated by subtraction from the total binding of binding obtained in the presence of 100 nM unlabeled pBNP. The protein concentration of the suspension was determined according to Lowry et al. [11] using BSA as standard.

3. RESULTS

3.1. *Effect of pBNP on formation of cGMP and secretion of steroids*

As shown in fig.1, pBNP stimulated cGMP formation in bovine adrenocortical cells in a dose-dependent manner. The basal level of cGMP formation was 24 fmol/10^5 cells per 20 min. With

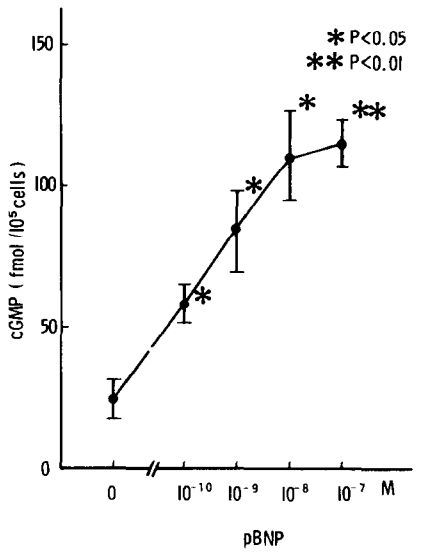

Fig.1. Effect of pBNP on formation of cGMP in bovine adrenocortical cells. Each point represents the mean ± SE ($n=3$).

10^{-8} M pBNP, intracellular cGMP formation increased 5-fold over the unstimulated level (128 fmol/10^5 cells per 20 min). The EC_{50} of pBNP in cGMP formation was approx. 3×10^{-10} M in dispersed bovine adrenocortical cells.

Fig.2 shows the effect of pBNP on the secretion of aldosterone and cortisol stimulated by 10^{-7} M angiotensin II analog or 10^{-8} M ACTH. With 10^{-7} M angiotensin II analog, aldosterone secretion of the dispersed bovine adrenocortical cells was 0.77 ng/10^5 cells per 90 min. Apparently, pBNP inhibited aldosterone secretion dose-dependently. Maximum inhibition was obtained at 10^{-8} M pBNP, with which aldosterone secretion decreased to 0.3 ng/10^5 cells per 90 min. The IC_{50} of pBNP in the suppression of aldosterone secretion was approx. 3×10^{-10} M (fig.2A). With 10^{-8} M ACTH, secretion of aldosterone and cortisol amounted to 0.75 and 5.9 ng/10^5 cells per 90 min, respectively. pBNP significantly inhibited secretion of both aldosterone and cortisol in a dose-dependent manner. Maximum inhibition of secretion of aldosterone and cortisol was observed with 10^{-8} M pBNP, which resulted in the level of aldosterone secretion being reduced to 0.22 ng/10^5 cells per 90 min and in cortisol secretion falling to 40% of that in the absence of pBNP. The IC_{50} of pBNP for the suppression of aldosterone secretion was approx. 3×10^{-11} M (fig.2B,C).

3.2. *In vitro receptor autoradiography of ^{125}I-pBNP in bovine adrenal gland*

Fig.3 shows the localization by autoradiography of ^{125}I-pBNP-binding sites in cross-sections of adrenal gland. ^{125}I-pBNP-binding sites were

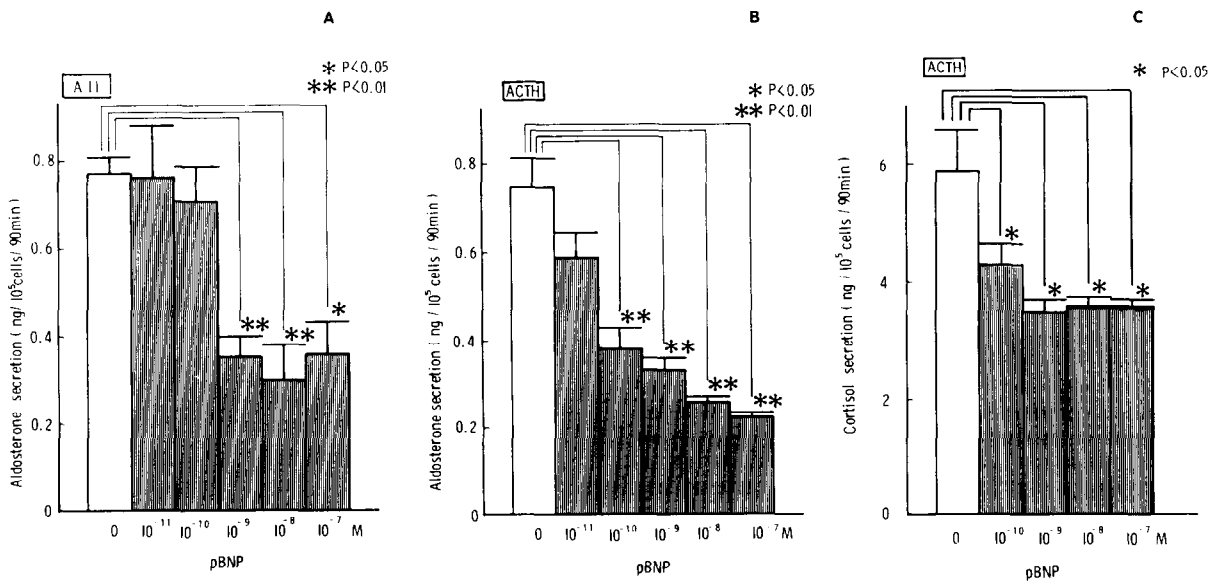

Fig.2. Effect of pBNP on aldosterone (A,B) and cortisol (C) production stimulation by angiotensin II or ACTH in bovine adrenocortical cells. Each point represents mean ± SE ($n=3$).

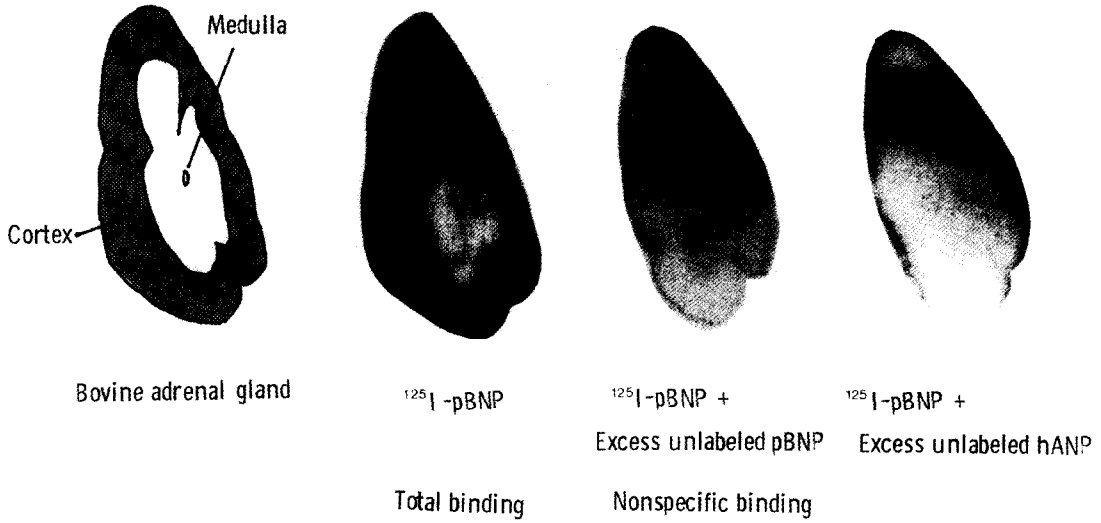

Fig.3. Autoradiography of ^{125}I-BNP binding to bovine adrenal gland. On the left a schematic illustration of a section of bovine adrenal gland is shown.

recognized throughout the entire adrenal cortex. In particular marked uptake of ^{125}I-pBNP was noted in the zona glomerulosa of adrenal cortex. Excess amounts of unlabeled pBNP significantly inhibited ^{125}I-pBNP binding of the adrenocortical region. In addition, the ^{125}I-pBNP-binding site was also displaceable by unlabeled hANP. On the other hand, we found no difference in ^{125}I-pBNP binding in the presence or absence of 100 nM unlabeled pBNP in adrenal medulla.

3.3. *Binding of ^{125}I-pBNP to adrenocortical membrane fractions*

Fig.4 shows saturation data for ^{125}I-pBNP binding to adrenocortical membrane fractions. 50 μg cell membranes was incubated with ^{125}I-pBNP ranging from 5 to 300 pM in the presence or absence of 100 nM unlabeled pBNP at 22°C for a period of 45 min. Specific binding reached a plateau around 150 pM ^{125}I-pBNP. Scatchard plots of ^{125}I-pBNP specific binding gave a straight line, from which the K_d and B_{max} were estimated to be $2.32 \pm 0.33 \times 10^{-10}$ M and 36.7 ± 1.6 fmol/mg protein, respectively. Fig.5 shows the displacement curve for ^{125}I-pBNP in adrenocortical membrane fractions by the various concentrations of unlabeled hANP and pBNP. Unlabeled pBNP inhibited ^{125}I-pBNP binding to adrenocortical membrane fractions in a dose-dependent manner. The actual dose of unlabeled pBNP required for 50% inhibition was estimated to be 6.5×10^{-10} M. Unlabeled hANP also showed equipotent inhibitory effect on ^{125}I-pBNP specific binding to unlabeled pBNP.

4. DISCUSSION

It has been reported that synthetic α-hANP significantly inhibits secretion of aldosterone, cortisol and DHEA in cultured bovine adrenocortical cells [8]. Moreover, specific binding sites for ^{125}I-hANP were visualized in the glomerulosa and fasciculata-reticularis zones of bovine adrenal gland by in vitro ^{125}I-hANP receptor autoradiography [8].

The present study clearly shows that pBNP exerts a suppressive effect on angiotensin II analog-stimulated secretion of aldosterone, and on secretion of aldosterone and cortisol stimulated by ACTH in bovine adrenocortical cells. Although it is questionable whether cGMP functions as a second messenger in the action of ANP in adrenocortical cells, Matsuoka et al. [6] demonstrated that the suppresive effect of ANP

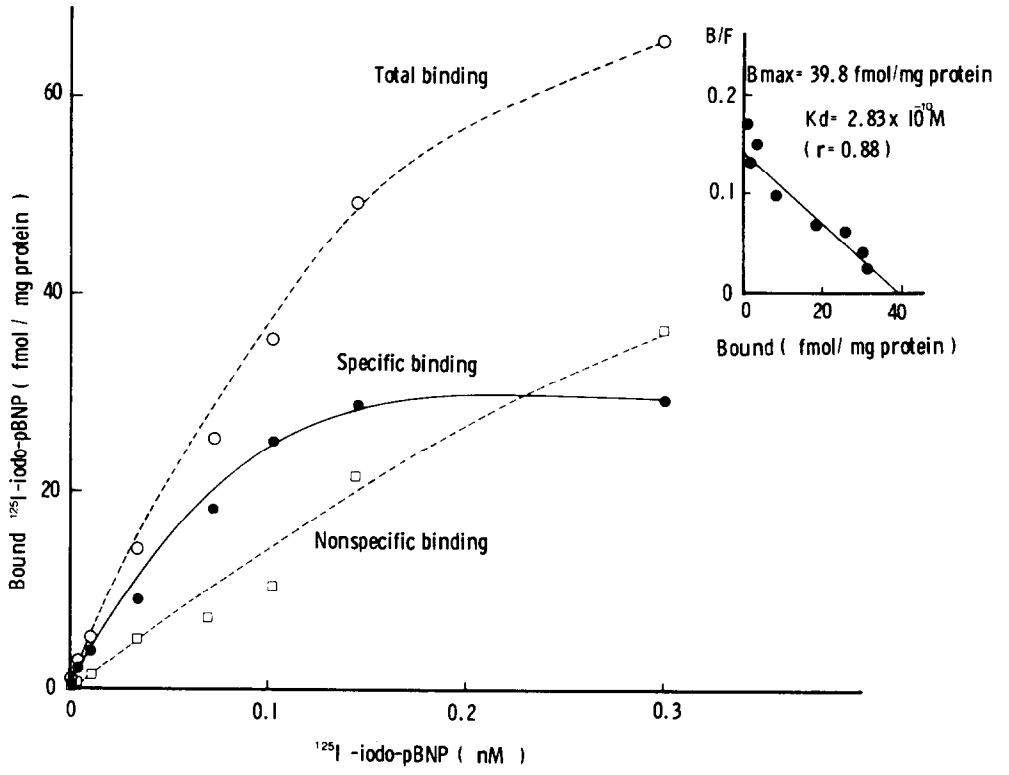

Fig.4. Saturable binding of ^{125}I-pBNP to adrenocortical membrane fractions. Specific binding in the presence of 100 nM unlabeled pBNP. Each point is the mean of two experiments. (Inset) Scatchard plot of the binding data. Ratio of bound to free ^{125}I-pBNP is plotted vs concentration of bound ^{125}I-pBNP.

was inversely related to the increase in cGMP formation in the dispersed bovine adrenocortical cells, suggesting that the formation of cGMP may provide a good metabolic indication of the action of the ANP in aldosteronogenesis. The present study also shows that the EC_{50} value of pBNP for the increase in cGMP formation was almost identical to the IC_{50} values for the suppression of secretion of aldosterone and cortisol, suggesting that the increase on cGMP formation reflects the metabolic effect of pBNP in bovine adrenocortical cells.

Since pBNP is as potent as α-hANP in inhibiting secretion of aldosterone and cortisol in bovine adrenocortical cells, pBNP may act on a bovine adrenocortical cells in the same manner as that in α-hANP. The number of amino acid residues of pBNP is different from that of α-hANP, however, the highest homology was observed between pBNP and α-hANP (4–28) [1]. This structural resemblance led us to investigate the possibility of whether ^{125}I-pBNP could bind functional hANP receptor.

We have demonstrated that the bovine adrenal cortex possesses pBNP-binding sites by using an in vitro receptor autoradiographic technique and performing binding studies on ^{125}I-pBNP. Scatchard plots of the binding data gave a straight line, indicating a single population of pBNP receptors with a K_d of 2.32×10^{-10} M and B_{max} of 36.7 fmol/mg protein. The pattern of distribution of ^{125}I-pBNP-binding sites visualized by autoradiography was very similar to that in ^{125}I-hANP of bovine adrenal gland and the ^{125}I-pBNP specific binding site was displaceable by unlabeled hANP as well as pBNP [8], suggesting that the bovine adrenal cortex may possess a common receptor for both hANP and pBNP. In hANP, the 17-amino-acid ring structure enclosed by a cysteine-cysteine disulfide bridge may be important for the receptor

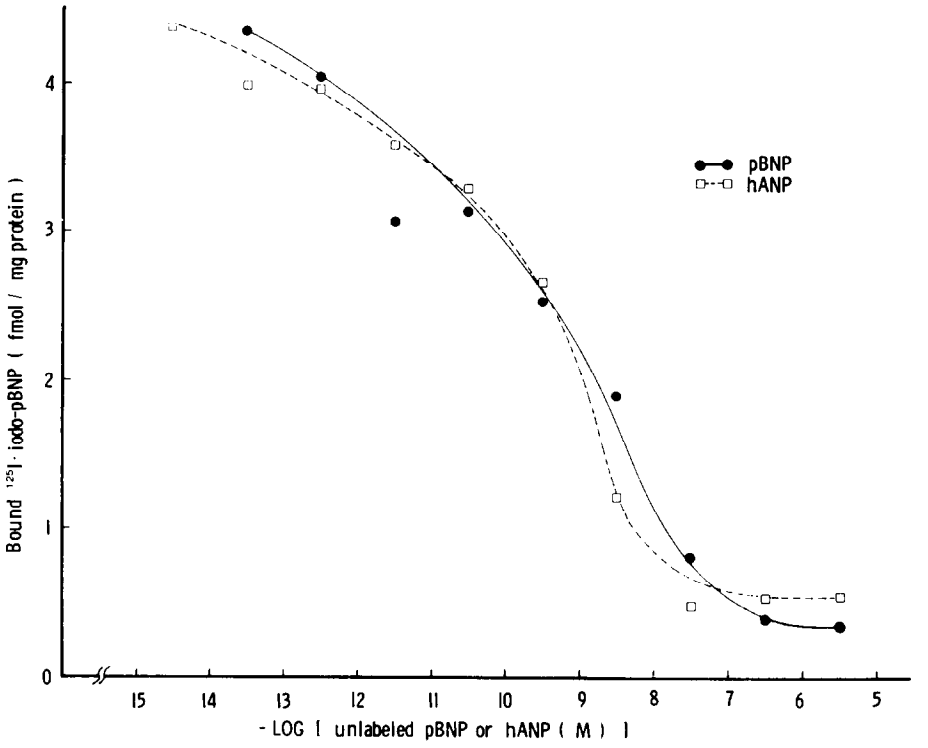

Fig.5. Competitive binding of ^{125}I-pBNP to adrenocortical membrane fractions by unlabeled hANP and pBNP.

binding and biological activity of hANP [12-15]. The present data suggest that the most important region in the binding of hANP to the receptor may be 13 amino acid residues except at least 4 amino acid sequences (α-hANP 7,9,14,15). It has been reported that the specific ANP receptor sites are localized in some area of mammalian brain, and may be important sites for the central regulation of water and electrolyte metabolism [16]. The specific binding sites for hANP in brain tissues may function as a pBNP receptor and subsequently modulate cardiovascular and fluid balance regulation.

In conclusion, pBNP has an inhibitory effect on bovine adrenocortical steroidogenesis. The high-affinity-low-capacity pBNP receptor, which may be identical to the ANP receptor, could mediate such a specific, hormonal action of pBNP in bovine adrenal cortex.

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