Comparative Biological Activities of α -Rat and α -Human Atrial Natriuretic Peptide in the Inhibition of Arginine Vasopressin Release in Conscious Rats

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Abstract

The comparative biological activities of intracerebroventricular (icv) injection of α -rat and α -human atrial natriuretic peptide (rANP and hANP, respectively) in the arginine vasopressin (AVP) release in conscious rats and the binding properties of these peptides to their specific receptors have been investigated. An icv injection of 5 μ g rANP inhibited the AVP release induced by osmotic and hemorrhagic stimuli. In contrast, 20 μ g of hANP was needed to exert an inhibitory effect on the AVP release. The receptor binding studies were carried out by using rat hypothalamic membrane preparations. The binding studies revealed that the potency of rANP was greater than that of hANP in displacing radioligand from its binding sites. Scatchard analysis revealed that the dissociation constant for rANP was significantly lower than that for hANP (0.52 ± 0.04 vs 1.20 ± 0.16 nM, $P < 0.01$). The binding capacity of these peptides was similar. These results suggest that the greater biological potency of rANP compared with hANP in the inhibition of AVP release is caused by the difference in the binding potency of these peptides.

It is well established that atrial natriuretic peptide (ANP) elicits natriuresis, diuresis, vasorelaxation, and inhibits aldosterone secretion (Cantin and Genest, 1985). In addition, a number of reports suggest that ANP in the central nervous system may participate in the control of water and electrolyte balance (Nakao *et al.*, 1987). One of the central effects of ANP is its involvement in the control of arginine

vasopressin (AVP) release from the neurohypophysis. For example, intravenous injection of ANP has inhibited an AVP release that had been stimulated by dehydration and hemorrhage (Samson, 1985; Januszewicz 1986a). An intracerebroventricular (icv) injection of ANP also has attenuated an AVP release induced by dehydration (Iitake *et al.*, 1986), high sodium intake (Poole *et al.*, 1987), and angiotensin II (Takahashi *et al.*, 1986; Yamada *et al.*, 1986; Casto *et al.*, 1987). In *in vitro* studies, ANP has suppressed an AVP release from the isolated posterior pituitary lobes (Obana *et al.*, 1985; Poole *et al.*, 1987) and the

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hypothalamoneurohypophyseal complex in organ culture (Crandall and Gregg, 1986; Januszewicz *et al.*, 1986b). Thus, these previous reports have demonstrated the inhibitory effects of ANP on AVP release with a few exceptions (Januszewicz *et al.*, 1985; Ogawa *et al.*, 1987).

Histoanatomical studies also have indicated the possibility that ANP produced in the central nervous system might regulate the AVP release. Immunoreactive ANP neurons (Kawata *et al.*, 1985; Saper *et al.*, 1985; Skafitsch *et al.*, 1985) and ANP binding sites (Quirion *et al.*, 1984; Bianchi *et al.* 1986; Gibson *et al.*, 1986; Saavedra *et al.*, Kurihara *et al.*, 1987) have been located in areas responsible for the regulation of AVP release.

Little is known, however, about the differences in the structure-activity relationship in α -rat ANP (rANP) and α -human ANP (hANP) in the control of the AVP release. It is well recognized that rANP differs from hANP in the replacement of only a single amino acid residue at position 12 of the ANP molecule. The amino acid residues for rANP and hANP are isoleucine and methionine, respectively. In the present study, we have compared the biological activities of the two peptides in the AVP release. Further, we have determined the properties of the ANP binding receptors in the rat hypothalamus.

Materials and Methods

Indwelling of an icv cannula

Male Sprague-Dawley rats (270–320 g) had an indwelling cannula implanted in the lateral ventricle 4 to 6 days prior to all experiments. The surgery was carried out under pentobarbital anesthesia (40 mg/kg, ip). One stainless steel cannula of 0.6 mm in outer diameter was stereotactically inserted into the lateral ventricle, according to the atlas of Paxinos and Watson (1982). Coordinates for the lateral ventricle were 1.5 mm lateral and 1.0 mm posterior to the

bregma, and 4.6 mm deep. Rats bearing an icv cannula were kept in individual cages at a constant room temperature and humidity with a 12-h light-dark cycle. Standard chow and water were available ad libitum immediately before the experiments. The position of the cannula was confirmed by injection of methylene blue dye at the end of the experiments.

Osmotic stimulation

All animals were in a conscious state. Each rat received two successive icv injections. The first icv injection was 5 μ l of artificial cerebrospinal fluid (ACSF) or the same amount of ACSF containing 5 or 20 μ g ANP (Peptide Institute Inc., Osaka, Japan). One minute later, either 10 μ l of ACSF or the same amount of hypertonic ACSF to which NaCl was added to yield a final concentration of 0.25, 0.5, or 1.0 M was injected. The animals were decapitated one minute after the second icv injection and the trunkal blood was collected into a cold, heparinized beaker. The plasma was separated and stored at -20°C until assayed. The composition of ACSF was as follows (mM): NaCl 121, KCl 3.35, CaCl_2 1.26, MgCl_2 1.16, NaHCO_3 21.0, NaH_2PO_4 0.58, glucose 3.4 and Urea 2.2, pH 7.4, gassed with 95% O_2 and 5% CO_2 .

Hemorrhagic stimulation

Under ether anesthesia, a polyethylene catheter (PE 50, Clay-Adams) was inserted into the femoral artery of rats bearing an icv cannula for monitoring the mean arterial blood pressure (MABP) and for the removal of blood. Experiments were performed with the rats in a conscious state two hours after the withdrawal of ether inhalation. Immediately before the initiation of hemorrhage, 5 μ l of ACSF, or the same amount of ACSF containing 5 or 20 μ g ANP, was injected into the lateral ventricle. Hemorrhaging was carried out by removing 1 or 2 ml/100 g body wt of blood over a 3-min period. The animals were decapitated 10 min after the completion of hemorrhaging and the trunkal blood was collected. The blood was treated in the same manner as described above. MABP was monitored with a strain gage pressure transducer (model PAS-101, Star Medical Co., Tokyo, Japan) throughout the study.

AVP radioimmunoassay

The plasma AVP concentration was determined

by radioimmunoassay as described previously (Ishikawa *et al.* 1986). Briefly, plasma AVP was extracted from acidified plasma using Sep-pak C₁₈ cartridges (Water Associates, Milford, MA). The AVP antiserum was generously supplied by the Mitsubishi Laboratory of Medical Science (Tokyo, Japan). ¹²⁵I-AVP was purchased from New England Nuclear (Boston, MA). Synthetic AVP (grade VI, Sigma, St. Louis, MI) was used for the standard curves. The minimal detection limit of the assay was 0.125 pg/tube. Inter- and intraassay coefficients of variation were less than 10%.

ANP binding assay

Preparation for an ANP binding assay in the rat hypothalamic membrane was carried out according to the method of Quirion *et al.* (1986) with minor modifications. Briefly, Sprague-Dawley rats (240–280 g) were decapitated and seven regions were separated on ice as previously described (Glowinski and Iversen, 1966). Hypothalamic tissues were homogenized in 10 volumes of 150 mM Tris-HCl buffer (pH 7.4 at 4°C) containing 120 mM NaCl and 5 mM KCl using a teflon homogenizer. The homogenates were centrifuged for 10 min at 42,000×g. The pellets were resuspended in 10 volumes of 50 mM Tris-HCl buffer (pH 7.4 at 4°C) containing 300 mM KCl and 10 mM Na₂ EDTA, and incubated on ice for 30 min. After the centrifugation described above, the pellets were resuspended in 20 volumes

of 50 mM Tris-HCl buffer (pH 7.4 at 4°C) and recentrifuged at 42,000×g for 15 min. The final pellets were washed twice with 50 mM Tris-HCl buffer (pH 7.4 at 4°C) and resuspended in 60 volumes of the same buffer.

For binding assays, 200 µl of membrane suspension were incubated for 60 min at 25°C with 400 µl of assay buffer containing various concentrations of unlabeled ANP and 10 pM of ¹²⁵I-ANP (Amersham International plc, Buckinghamshire, England). The assay buffer was 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 5 mM MnCl₂, 40 µg/ml bacitracin (Sigma) and 0.5% bovine serum albumin (fraction V, Sigma). After 60-minute incubation, each assay tube was filled rapidly with 5 ml of the ice-cold assay buffer. Diluted solutions were filtered through whatman GF/C filters (Maidstone, England) presoaked for one hour in 0.1% polyethyleneimine. Each filter was washed three times with 5 ml of the cold assay buffer. Specific binding was calculated as the difference in radioactivity bound in the presence and absence of 2×10^{-6} M unlabeled ANP. Binding of ligands to filters was quantitated by counting the filters in a γ-counter (model ARC-600, Aloka, Tokyo, Japan). All assays were done in triplicate. Protein was determined by the method of Lowry *et al.* (1951).

Statistics

Statistical analysis was performed by analysis

Fig. 1. Plasma arginine vasopressin (AVP) levels after intracerebroventricular injection of hypertonic artificial cerebrospinal fluid (CSF) or hemorrhage. The number of animals in each group is seven. Values are the mean ± SEM. * and ** represent $P < 0.05$ and $P < 0.01$, respectively, compared with 0.14 M sodium-containing CSF injected rats (left panel) and sham operated rats (right panel).

of variance with the multiple comparisons (Dunn and Clark, 1974), and by paired or unpaired *t*-test, where appropriate. A *P* value less than 0.05 was considered significant. Values have been represented as the mean $\pm$ SEM.

Results

Effects of ANP on the AVP release

As shown in the left panel of Fig. 1, the plasma AVP concentration increased in a dose-dependent manner in response to icv injections of hypertonic ACSF. Fig. 2 shows

Fig. 2. Effects of intracerebroventricular (ICV) injection of atrial natriuretic peptide (ANP) on arginine vasopressin (AVP) release induced by icv injection of hypertonic artificial cerebrospinal fluid (CSF) containing 0.25 M sodium. The number of animals in each group is seven. * $P < 0.05$ vs 0.14 M sodium-containing CSF injected rats. rANP and hANP represent α -rat and α -human ANP, respectively.

the effects of icv injection of ANP on plasma AVP. The increase in plasma AVP in response to hypertonic ACSF containing 0.25 M sodium was significantly attenuated by icv injection of 5 μ g rANP. In contrast, an icv injection of 5 μ g hANP did not suppress AVP release stimulated by 0.25 M

Fig. 3. Effects of intracerebroventricular injection of atrial natriuretic peptide (ANP) on arginine vasopressin (AVP) release induced by hemorrhage (1.0 ml/100 g body wt). The number of animals in each group is seven. Values are the mean $\pm$ SEM. * $P < 0.05$ vs sham operated rats. rANP and hANP represent α -rat ANP and α -human ANP, respectively.

Table 1. Mean arterial blood pressure before and after hemorrhage.

Treatment		n	Blood pressure (mmHg)	
Hemorrhage	ICV		Before	After
Sham	ACSF	7	106±1.9	106±2.0
1 ml/100 g BW	ACSF	7	106±1.8	100±1.5*
	5 µg rANP	7	107±2.1	102±2.6*
	5 µg hANP	7	105±2.0	100±1.9*
	20 µg hANP	7	106±2.0	100±2.1*
	ACSF	6	105±1.8	76±7.6**
2 ml/100 g BW	ACSF	6	105±1.8	76±7.6**

*P<0.01, **P<0.001 vs before hemorrhage. Values are the mean±SEM. ACSF, artificial cerebrospinal fluid; ICV, intracerebroventricular injection; n, the number of animals; BW, body weight.

sodium containing hypertonic ACSF. An icv injection of 20 µg hANP, meanwhile, inhibited the AVP release significantly. Neither rANP nor hANP had any effect on the basal AVP release.

As shown in the right panel of Fig. 1, in response to hemorrhage, plasma AVP increased in a dose-dependent manner. Fig. 3 shows the effects of ANP on the AVP release induced by hemorrhage (1.0 ml/100 g body wt). As observed in the osmotic stimuli, an increase in plasma AVP after hemorrhage was attenuated by an icv injection of 5 µg of rANP. Although the icv injection of 5 µg of hANP did not inhibit the AVP release induced by hemorrhage, 20 µg of hANP inhibited the AVP release. The changes in MABP were similar in the groups with 1 ml/100 g body wt of hemorrhage (Table 1).

ANP binding study

Since specific binding of ^{125}I -rANP increased lineary up to 280 µg protein/ml (Fig. 4, upper panel), this membrane concentration was used in the following binding studies. The relationship between the time of incubation and the specific binding of ^{125}I -rANP is shown in the lower panel of Fig. 4. The specific binding reached its maximal within 30 min and then maintained this plateau for at least 30 min. We used a 60-min incubation time in all experiments described hereafter.

Fig. 4. Upper panel; Specific ^{125}I -rat atrial natriuretic peptide (rANP) binding as a protein-dependent reaction in rat hypothalamic membrane preparations.

Lower panel; Time course of specific ^{125}I -rANP binding in rat hypothalamic membrane preparations.

Table 2. Comparative receptor binding properties of rANP and hANP in the rat hypothalamic membrane preparations.

	n	IC ₅₀ (nM)	K _d (nM)	B _{max} (fM/mg protein)
α -rat ANP	6	0.34 $\pm$ 0.17	0.52 $\pm$ 0.04	40.3 $\pm$ 2.5
α -human ANP	5	1.14 $\pm$ 0.35*	1.20 $\pm$ 0.16**	39.1 $\pm$ 6.4

Values are the mean $\pm$ SEM. n, the number of binding studies; IC₅₀, the concentration of unlabeled ANP required to displace 50% of ¹²⁵I-ANP binding; K_d, dissociation constant; B_{max}, maximal binding capacity. *P<0.02. **P<0.01 vs α -rat ANP.

Fig. 5. Displacement curves of specific ¹²⁵I-atrial natriuretic peptide (ANP) binding in response to various concentrations of unlabeled ANP in rat hypothalamic membrane preparations. (●—●), (■—■), (□---□), and (○---○) represent ¹²⁵I-rANP binding displaced by unlabeled rANP, ¹²⁵I-hANP binding displaced by unlabeled hANP, ¹²⁵I-rANP binding displaced by unlabeled hANP, and ¹²⁵I-hANP binding displaced by unlabeled rANP, respectively.

Displacement-curves of ¹²⁵I-ANP binding to the hypothalamic membrane receptor in the presence of unlabeled ANP are shown

in Fig. 5. The concentration of unlabeled rANP required to displace 50% of ¹²⁵I-rANP binding (IC₅₀) was significantly (P<0.02) lower than that of unlabeled hANP (0.34 $\pm$ 0.17 vs 1.14 $\pm$ 0.35 nM, Table 2). Similarly, the IC₅₀ of unlabeled rANP to displace ¹²⁵I-hANP binding was lower than that of unlabeled hANP (0.16 nM and 1.05 nM, respectively.) Scatchard analysis performed by Rosenthal's method (1967) revealed that the hypothalamic ANP receptors consisted of those with a high and a low affinity (Fig. 6). The dissociation constant (K_d) of the high affinity receptor for rANP was significantly (P<0.01) lower than that for hANP (0.52 $\pm$ 0.04 vs 1.20 $\pm$ 0.16 nM, Table 2). The maximal binding capacity (B_{max}) of the high affinity receptors for rANP was not significantly different from that for hANP (Table 2). Since there were considerable variations in the experimental data for the low affinity receptors, it was difficult to analyse its binding parameters. It was also difficult to calculate the average affinity proposed by De Meyts *et al.* (1975), since the abscissa intercept could not be decided.

Discussion

The present results show that centrally administered ANP inhibited the AVP release induced by osmotic and hemorrhagic stimuli. The dose of hANP necessary for this inhibition of the AVP release was greater

Fig. 6. Scatchard analysis of atrial natriuretic peptide (ANP) binding to rat hypothalamic membrane preparations. Data were obtained by ^{125}I -rANP binding displaced by unlabeled rANP (●—●), and by ^{125}I -hANP binding displaced by unlabeled hANP (○---○). rANP and hANP represent α -rat ANP and α -human ANP, respectively.

than that of rANP and the receptor binding potency of rANP was stronger than that of hANP.

The inhibitory effects of ANP on AVP release found in our study are consistent with a number of previous reports. Ogawa *et al.* (1987) reported that neither an icv injection of rANP (0.3 nM/kg) nor an intravenous injection (2.5 nM/kg) has produced any effects on the plasma AVP concentration in the dehydrated rat. They have therefore concluded that the physiological levels of ANP did not affect the AVP release *in vivo*. The dose of ANP administered by Ogawa *et al.* was much smaller than that in our study, so that our dose of ANP might be considered pharmacological. Thus, further investigations are needed to clarify the physiological significance of ANP in the reulation of AVP release.

The present results have revealed that the biological activity of rANP appear to be more potent than that of hANP in the inhibition of the AVP release. This comparative biological potency of the two peptides was confirmed by the receptor binding properties found in the present

study. There have been no reports indicating that the biological potencies of the two peptides differ from each other in the central nervous system. Itoh *et al.* (1987) have reported that centrally administered hANP exerts an equipotent antidipsogenic action compared with rANP in the conscious rat. The reason for the difference between the two remains unclear.

The present receptor binding studies have demonstrated that the affinity constant for rANP was greater than that for hANP, especially in the high affinity binding sites. Quirion *et al.* (1986) have reported that the binding properties of rANP were almost equal to those of hANP in the hypothalamic membrane receptors of the guinea pig. This contradiction may be due to the difference in the species of animal used in the two experiments. On the one hand, the possibility that the oxydation of methionine at position 12 of hANP molecule decreased the binding and the biological activity of hANP cannot be excluded. However, we did not observe a progressive fall in the binding or the biological activity of hANP during the present study.

The presence of specific ANP binding sites in the hypothalamus as observed in our study suggests that the inhibitory effect of ANP on AVP release might be exerted in the hypothalamus. Standaert *et al.* (1987) reported that the firing of the vasopressin neurons in the posterior magnocellular paraventricular subnucleus has been inhibited by the injection of ANP into this area. Wong *et al.* (1986) have also reported that the firing rate of the rat medial preoptic area is decreased by the injection of ANP into this area. Samson *et al.* (1987) have concluded that ANP inhibited the AVP release via the hypothalamus, since ANP inhibited the AVP release from the hypothalamo-neurohypophyseal complex without any effects in the isolated neurohypophysis. However, the possibility that other areas abundant in ANP binding sites, such as the subfornical organ and the area postrema, are responsible for the inhibitory effect of ANP on the AVP release cannot be excluded.

In conclusion, the greater biological potency of rANP compared with hANP in the inhibition of the AVP release is caused by the difference in the receptor binding potency of the two peptides.

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