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A perifusion method for examining arginine vasopressin (AVP) release from hypothalamo-neurohypophyseal system.*

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Abstract

A perifusion method has been developed using rat hypothalamo-neurohypophyseal system (HNS) or neural lobe to investigate the control mechanism of arginine vasopressin (AVP) release. A specific radioimmunoassay (RIA) for AVP was developed to measure AVP in perifusion medium employing anti-AVP serum which was obtained by immunizing rabbits. At a final dilution of 1/12,000, the antiserum showed less than 0.66 and 0.01% cross reactivity with lysine-vasopressin and oxytocin, respectively. But it did not cross reacted with other peptide hormones. The lowest detectable level of vasopressin was 0.5 pg/tube. The intra-assay coefficient of variation averaged 10.4%. The dilution curve of perifused medium was well paralled to the standard curve of AVP assay. AVP release from HNS or neural lobe gradually declined to the stable level in 90-120 min after the initiation of perifusion. Good repeatability of the AVP release from neural lobe was recognized by repeated stimulation with 10 min perifusion of 60 mM KCl at every 60 min. HNS released AVP in dose related manner to the osmotic challenge of sodium or glucose, and AVP release was stimulated from HNS by prostaglandin E2, but not by dopamine. These results show that the perifusion methods using AVP-RIA is a useful method to examine the AVP release from HNS or neural lobe.

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A PERIFUSION METHOD FOR EXAMINING ARGININE VASOPRESSIN (AVP) RELEASE FROM HYPOTHALAMO-NEUROHYPOPHYSEAL SYSTEM

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Abstract. A perifusion method has been developed using rat hypothalamo-neurohypophyseal system (HNS) or neural lobe to investigate the control mechanism of arginine vasopressin (AVP) release. A specific radioimmunoassay (RIA) for AVP was developed to measure AVP in perifusion medium employing anti-AVP serum which was obtained by immunizing rabbits. At a finial dilution of 1/12,000, the antiserum showed less than 0.66 and 0.01% cross reactivity with lysine-vasopressin and oxytocin, respectively. But it did not cross reacted with other peptide hormones. The lowest detectable level of vasopressin was 0.5 pg/tube. The intra-assay coefficient of variation averaged 10.4%. The dilution curve of perifused medium was well paralled to the standard curve of AVP assay. AVP release from HNS or neural lobe gradually declined to the stable level in 90-120 min after the initiation of perifusion. Good repeatability of the AVP release from neural lobe was recognizied by repeated stimulation with 10 min perifusion of 60 mM KCl at every 60 min. HNS released AVP in dose related manner to the osmotic challenge of sodium or glucose, and AVP release was stimulated from HNS by prostaglandin E_2, but not by dopamine. These results show that the perifusion methos using AVP-RIA is a useful method to examine the AVP release from HNS or neural lobe.

AVP release is known to be controlled not only by osmotic stimuli but also by many non-osmotic factors including blood volume (1), blood pressure (2), and the renin-angiotensin system (3-5). As these factors interact with each other to control AVP release (6-10), it is difficult to clarify the role of each factor in the control of AVP release by *in vivo* experiments. Therefore, *in vitro* methods may be better for clarifying the effect of each factor on AVP release.

In this paper, we report a perifusion method for investigating AVP release from the hypothalamo-neurohypophyseal system or neural lobe. We raised a highly specific AVP antiserum and developed a specific radioimmunoassay to determine the AVP concentration in the medium.

MATERIALS AND METHODS

Perifusion method. Unanesthetized male wister rats weighing 200-250 g were decapitated and the entire brain with pituitary was immediately removed at room temperature. The hypothalamus with neurohypophysis was taken out of the brain. The

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hypothalamus was 5 mm in length, 4mm in width, 4mm in depth and its anterior border was just rostral to the optic chiasm and posterior border of the corpus mamillae. The isolated neurohypophysis was taken out by cutting the infundidular stalk and placed on a small amount of Bio-Gel P_2 (200 μl) in a chamber (700 μl) which prevented tissue

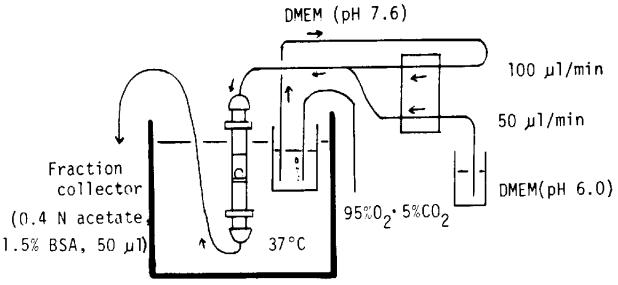

Fig. 1. Perifusion method for examining AVP release from hypothalamo-neurohypophyseal system or neural lobe.

fragments from running through (Fig. 1). This chamber hung in warm water (37° C) was infused with Dulbecco modified Eagles Medium (DMEM: pH 7.4) with a perfusion pump at a flow rate of 150 μl/min. In some experiments, both DMEM (pH 7.8) kept at 37° C and constantly bubbled with a mixture of 95% O_2 and 5% CO_2 and DMEM (pH 6.0) containing test substance and kept at room temperature were perifused at a flow rate of 100 μl/min and 50 μl/min, respectively, and they were joined just before they entered the chamber. By this procedure, the pH of the medium in the chamber could be maintained at pH 7.4. Perifused medium (10 min fraction, 1.5 ml) was fractioned in tubes containing 50 μl of 0.4 N acetate containing 1.5% BSA.

AVP radioimmunoassay (RIA). The concentration of AVP in the medium was determined by RIA. technique.

Antiserum. Synthetic AVP (Sigma grade 1 1U = 2.725 μg) was conjugated to bovine serum albumin (BSA) with glutaraldehyde (11). The complex (1 mg) dissolved in saline (1 ml) was mixed with Freunds complete adjuvant (1 ml) were mixed and injected intradermally into multiple sites on the back of white rabbits for several times over a 2 week interval. Serum was collected periodically for 4 months following the initial injection. The antiserum was diluted to 1/50 with 0.01 M phosphate buffer (pH 7.2) containing (0.15 M NaCl, 0.1% NaN_3, 0.05 M EDTA and 1% normal rabbit serum) and kept at −40° C and further diluted to 1/2000 in the assay.

Labelling. Synthetic AVP was labelled with Na-^{125}I according to a method of modification of the Hunter and Greenwood method without adding metabisulfate (12). The iodination mixture was applied to a Sephadex G-25 superfine column (0.9 × 20 cm) and the iodinated AVP was eluted with 0.01 M acetic acid solution containing 0.5% BSA.

Assay procedure. Fifty μl of diluent containing serially diluted AVP (1-1000 pg), 50 μl

of diluted antiserum (1/2000) and 150 μl assay buffer (0.01 M phosphate buffer containing 0.15 M NaCl, 0.1% NaN_3 and 0.05% BSA) pH 7.2 were mixed and incubated for making a standard curve. A hundred and fifty μl of sample, 50 μl of antiserum and 50 μl of assay buffer were also incubated in plastic tubes. After 24 h incubation at 4° C, 50 μl of ^{125}I-AVP (10,000 cpm) was added to the incubation mixture. After another 48 h incubation at 4° C, 100 μl of 1% bovine gamma globulin and 1 ml of 20% polyethylene glycol were added, mixed and centrifuged at 3000 rpm for at 4° C. After aspirating the supernatant, the radioactivity of precipitated ^{125}I-AVP was counted.

RESULTS

AVP Radioimmunoassay

Standard curve. Fig. 2 shows the standard curve at a finial antiserum dilution of 1/12,000 and 1/24,000. The standard curve at a final antiserum dilution of 1/12,000 shows better sensitivity, and antiserum was usually used at this dilution in RIA. The minimum detectable AVP level was 0.5 pg/tube at this dilution.

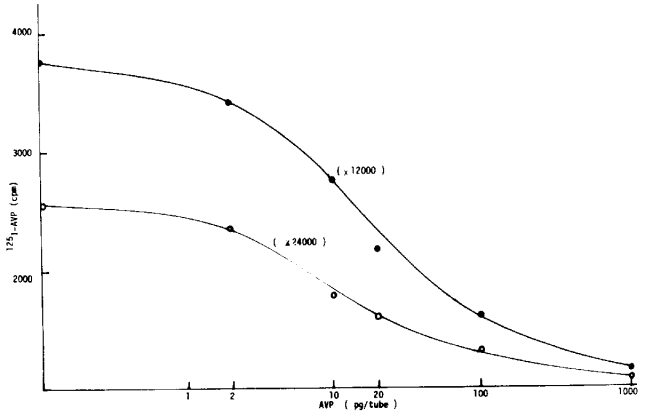

Fig. 2. Effect of antiserum dilution on standard curve for AVP radioimmunoassay.

Specificity. Antiserum obtained was highly specific for AVP with cross reactivity less than 0.66% and 0.01% with lysine vasopressin and oxytocin, respectively. But it exhibited no cross reactivity with ACTH, TRH, LHRH, PRL and GH (Fig. 3). Serially diluted perifusion medium obtained from an experiment was parallel to the standard curve in AVP-RIA (Fig. 4).

Repeatability. The calculated intra-assay coefficient of variation (CV) averaged 10.4% (Table 1.).

Perifusion Method

Basal AVP release. AVP release from HNS declined gradually to settle on a basal release level 90-120 min after initiation of perifusion. Basal AVP release was from 40 pg/tube to 90 pg/tube. AVP was released well from HNS in response to 60 mM KCl at 260 min. This indicates that HNS keeps the ability to release

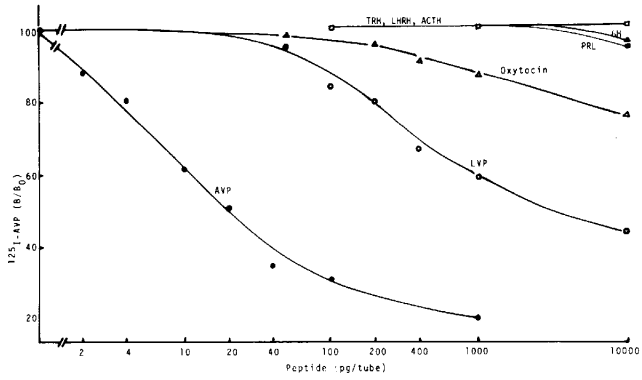

Fig. 3. Cross reactivity studies of AVP antiserum using lysine vasopressin, oxytocin, TRH, LHRH, 1-39 ACTH, rat prolactin and LH.

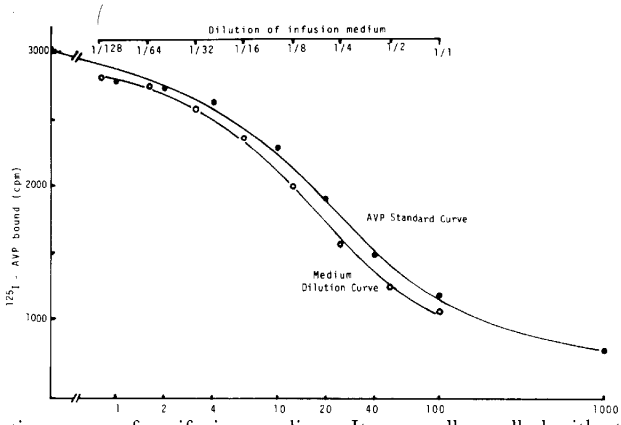

Fig. 4. Dilution curve of perifusion medium. It was well paralled with standard curve.

TABLE 1. REPEATABILITY OF IMMUNOASSAYABLE ARGININE VASOPRESSIN (AVP) IN POOLED SAMPLE

Number	AVP (pg/tube)	
	Pool (a)	Pool (b)
1	246	93
2	240	93
3	258	81
4	228	81
5	246	78
6	216	87
7	204	66
8	252	75
9	234	90
10	228	108
Mean	235.2	85.2
SD	16.7	11.6
CV (%)	7.1	13.6

AVP even 260 min after the initiation of perifusion (Fig. 5).

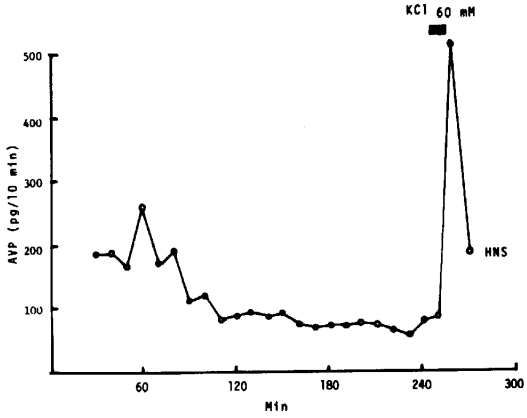

Fig. 5. Basal AVP release from hypothalamo-neurohypophyseal system (HNS) in perifusion system and AVP response to 60 mM KCl.

Repeatability of AVP release. The neural lobe was repeatedly stimulated with 10 min-perifusion of 60 mM KCl in three kinds of medium (Fig. 6). Good

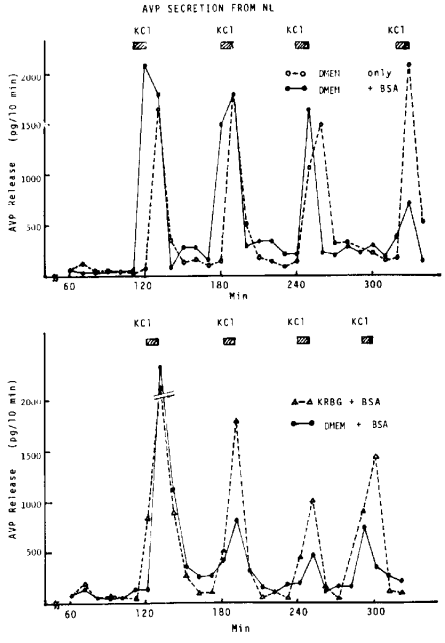

Fig. 6. Repeatability of AVP release from isolated neural lobe when it was stimulated with 10 min perifusion of 60 mM KCl in every 60 min. Perifusion medium used was Dulbecco modified Eagles Medium (DMEM), DMEM +0.25% BSA and Krebs Ringer bicarbonate glucose buffer (KRBG) +0.25% BSA.

repeatability of AVP release was obtained when tissues were perifused with DMEM only. The AVP response to 60 mM KCl decreased gradually when neural lobes were perifused with KRBG or DMEM containing 0.5% BSA. Therefore, DMEM only was used in the perifusion system.

Response to osmotic and non-osmotic stimuli. When 5, 20 and 60 mEq NaCl were added to DMEM of which the osmotic pressure was 268 mosmol/kg H_2O, the osmotic pressure rose to 275, 297 and 363 mosmol/kg H_2O, respectively. When 10, 40 and 120 mEq glucose or urea were added to DMEM, the osmotic pressure rose to 275, 304 and 375, or 273, 302 and 370 mosmol/kg H_2O, respectively. AVP relase from HNS was stimulated in a dose-related manner by administration of these concentrations of sodium or glucose, but not by urea. (Fig. 7) AVP

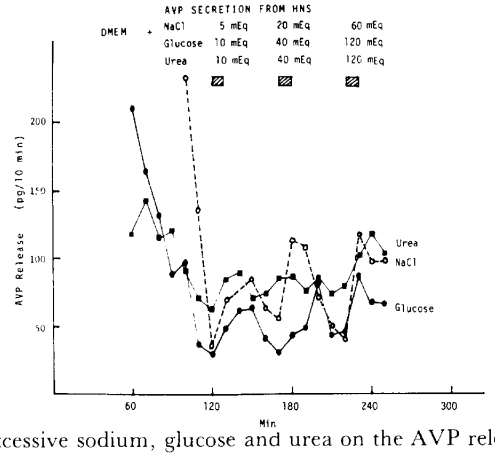

Fig. 7. Effect of excessive sodium, glucose and urea on the AVP release from hypothalamo-neurohypophyseal system (HNS).

was released from HNS by prostaglandin E_2 (PGE_2) at concentrations of 1 μg/ml and 10 μg/ml but not from the neural lobe at these concentrations (Fig. 8).

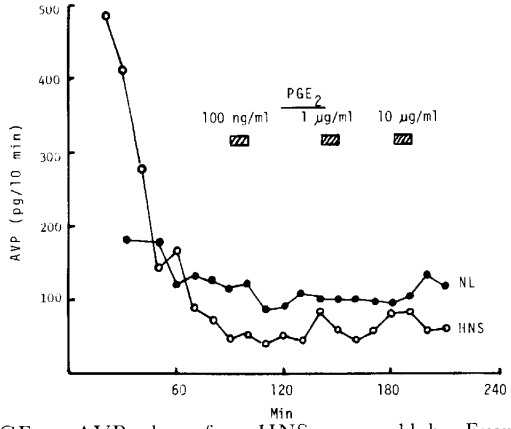

Fig. 8. Effect of PGE_2 on AVP release from HNS or neural lobe. Every perifusion time was 10 min.

Dopamine at concentrations from 10 ng/ml to 10,000 ng did not stimulate AVP release in this system.

DISCUSSION

Antiserum against AVP was produced by immunizing rabbits for 15 weeks using BSA-conjugated AVP and Freunds complete adjuvant. This antiserum had good specificity and sensitivity enough to measure small amount of AVP in perifused medium as shown in Fig. 3 and 4. AVP in the medium was measured without extraction, as the dilution curve of perifused medium was well paralleled the standard curve in AVP-RIA.

In our perifusion method, the neural lobe responded to 60 mM KCl with good reproducibility for 6 h when it was perifused with DMEM, and HNS responded well to osmotic and non-osmotic stimuli. These results suggest that hypothalamo-neurohypophysial system (HNS) maintained in perifused medium can release AVP under various kinds of stimuli for more than 260 min. Although the usefulness of the organ culture system of HNS in studying the mechanism of AVP release has been already reported (13, 14), it is not easy to examine the serial changes of AVP release in the system. As much plasma needs to be withdrawn for the extraction procedure, it is difficult to examine the serial changes of AVP release *in vivo* experiments in small animals. Therefore, the perifusion method is the best way to examine serial changes of AVP release.

The osmotic threshold is an important factor in vasopressin release. A study in rats (6) showed an osmotic threshold of about 292 mosmol/kg H_2O. In our method, AVP responded well to 5 mEq NaCl (275 mosmol/kg H_2O) added to DMEM (268 mosmol/kg H_2O). This indicates that the osmotic threshold of HNS in this system is around 270 mosmol/kg H_2O, which is a little lower than the osmotic threshold reported by other investigators (6). The reason for the discrepancy might be that isolated HNS or posterior pituitary have a lower osmotic threshold for releasing AVP.

The sensitivity of AVP release to osmotic stimuli is another important factor for AVP release (15). Robertson *et al.* (16) reported that an osmotic change in plasma from 285 to 295 mosmol/kg H_2O resulted in a 3 fold increase in plasma AVP levels in humans. In a rat experiment using HNS in organ culture, an osmotic increase from 295 to 305 mosmol/kg H_2O was accompanied by a 2.5 fold increase in AVP release (17). Although short term osmotic stimulation was employed in our perifusion method to investigate the sensitivity of AVP release, the response of AVP to osmotic stimuli seemed to be sensitive enough compared with other methods (16, 17).

There are two hypotheses as for the regulation of AVP release; one is the osmoreceptor concept (18) and the other is the sodium sensitive receptor concept (19-21). The osmoreceptor concept means that the osmotic pressure or tonicity of extracellular fluid changes the volume of cerebral receptor cells and induces AVP

release. In our *in vitro* perifusion method, DMEM can freely come into contact with ventricular wall in the HNS explant. Therefore, the addition of glucose or urea dissolved in DMEM dose not alter the sodium concentration of the intraventricular space but increases the osmotic pressure of the medium. In our study, although not only NaCl but also glucose or urea stimulated AVP release, NaCl was the most effective in stimulating AVP release and urea was the least effective. These findings support Verney's concept of osmoreceptors, but do not exclude the possibility of a specific sodium receptor.

Much evidences indicating that the PGE_2 series can act centrally to stimulate AVP release has been reported (22, 23), but the direct action of PGE on the neural lobe is controversial (24-26). In our perifusion method, PGE_2 may act at the site of hypothalamus to stimulate AVP release.

The present results show that the *in vitro* perifusion method is a useful method for examining the role of osmotic and non-osmotic stimuli in the regulation of AVP release from the HNS or neural lobe.

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