Antidiuretic Effects of Alpha- and Beta-Adrenoceptor Agonists Microinjected into the Hypothalamic Paraventricular Nucleus in a Water-Loaded and Ethanol-Anesthetized Rat

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Abstract—Effects of catecholamines microinjected into the paraventricular nucleus in the hypothalamus on urine outflow in a rat which was loaded with water and anesthetized with ethanol were studied. L-Norepinephrine, L-epinephrine and L-isoproterenol induced potent antidiureses with similar time courses to each other. The ED50 values for L-norepinephrine, L-epinephrine and L-isoproterenol were approximately 5, 10 and 5 nmol, respectively. The D-isomer of isoproterenol demonstrated no significant antidiuretic activity. The effect of L-norepinephrine was inhibited strongly by premicroinjection of alpha- and beta-adrenoceptor antagonists. The effect of DL-isoproterenol was inhibited strongly by beta-adrenoceptor antagonists, but not affected by alpha-adrenoceptor antagonist. Premicroinjection of a muscarinic antagonist, atropine, partially inhibited antidiuretic effects induced by L-norepinephrine and DL-isoproterenol. Visceral functions other than urine outflow such as mean blood pressure, respiration rate, heart rate and rectal temperature were not significantly altered when the urine outflow decreased down to 20–30% of the control by microinjection of L-norepinephrine and DL-isoproterenol. The results demonstrated that stimulation of alpha- and beta-adrenoceptor in the paraventricular nucleus induced potent antidiuretic effects, partial inhibition of which by atropine suggested a possible presynaptic facilitation of the release of ACh by the stimulation of the adrenoceptors.

The paraventricular nucleus (PVN) in the hypothalamus as well as the supraoptic nucleus (SON) is one of the main nuclei containing magnocellular vasopressinergic neurons which synthesize and upon excitation release vasopressin, antidiuretic hormone (ADH), from neurohypophysis into the general circulation. The circulating ADH promotes water reabsorption from the renal distal tubles and collecting ducts, inducing an antidiuretic effect (1–5). The PVN has been shown to contain one of the most dense catecholaminergic nerve terminal fields in the central nervous system (6). Histofluorescence of catecholamine-containing varicosities and immunohistochemical visualization of catecholamine-synthesizing enzymes in the nerve terminals on the magnocellular neurons in the PVN have suggested adrenergic innervation upon the nucleus (7–12). Three administering methods have been tried to test the central effects of catecholamines, that is, intracerebroventricular injection (13, 14), microinjection into the nuclei (15, 16) and iontophoretic application on the neurons (17). Among them, the second method of microinjection used to measure the effects on various visceral functions in vivo may inform us about effects on physiological functions in living whole animals. By microinjecting catecholamines and their antagonists into the SON, we recently demonstrated that the stimulation of α- and β-adrenoceptors in the SON induced potent antidiuretic effects (18). In the present study, we investigated the
effects of microinjection of catecholamines into the PVN on urine outflow and on other visceral functions. The type of adrenoceptors and a possible cholinergic mechanism mediating the effects were also studied.

**Materials and Methods**

**Animals and drugs:** Male Wistar rats weighing 280–330 g were used. L-Nor-epinephrine (NE) bitartrate, L-epinephrine (EPI) bitartrate, DL-isoproterenol (DL-ISO), L-isoproterenol (ISO), D-isoproterenol (D-ISO) hydrochloride (Sigma Chemical Co., St. Louis, MO), phenoxybenzamine hydrochloride (Nakarai Chemicals, Kyoto), and atropine sulfate (Iwaki Co., Tokyo) were purchased. Propranolol hydrochloride and timolol malate were the generous gifts of Sumitomo Chemical and Industrial Co., Tokyo and Nippon Merck-Banyu Co., Tokyo, respectively. The other chemicals used were the analytical grade available.

**Measurement of urine outflow:** Urine outflow was measured by the method of Dicker, with modifications (19), as described elsewhere (18, 20). Rats were starved overnight for approximately 17 hours, having free access to water. The rat was loaded orally through a catheter with a volume of water equivalent to 5% of the body weight and then the same volume of 12% ethanol. The cannulae were inserted into the trachea, urinary bladder and external jugular vein, respectively. The animal was then fixed in a stereotaxic instrument for rats (Takahashi Co., Tokyo). The number of drops of urine outflow from the cannula inserted into the bladder was counted and recorded as signal pulses using a photoelectric drop counter (DCT 102, Unique Medical Inc., Tokyo). Three percent ethanol in Locke solution was infused at a constant rate of 0.10 ml/min through the cannula inserted into the vein, in order to maintain a constant level of anesthesia and urine outflow. The urine outflow usually reached a constant rate of 0.05–0.1 ml/min within one hour after the animal was fixed in the instrument.

**Microinjection of drugs:** A stainless cannula (outer diameter: 200 μm) was inserted stereotaxically and unilaterally into the PVN according to the atlas of König and Klippel (21). Using a microsyringe, 1 μl of the drugs dissolved in an artificial cerebrospinal fluid (CSF: 128 mM NaCl, 3.0 mM KCl, 1.2 mM CaCl₂, 0.8 mM MgCl₂, 0.65 mM NaH₂PO₄ and 4.8 mM NaHCO₃, pH 7.4: solution for isoproterenol) or in saline was injected through the cannula, and then 2 μl of the artificial CSF was injected at a rate of approximately 0.3 μl/min. Effects of drugs on urine outflow during every 10 min were expressed as percentage of the initial control outflow.

In the experiments to test the effect of premicroinjection of an antagonist, the first injection of an agonist was followed by an injection of an antagonist, and then the second injection of the same agonist was performed through one cannula being kept inserted into the PVN at approximately 30–50 min after the injection of an antagonist when the urine outflow had recovered to the initial level. Inhibitory effect of an antagonist was estimated as the change in antidiuretic effect of the second injection of an agonist compared with the antidiuretic effect of the first injection of the agonist. Antidiuretic effect of the second injection of NE or DL-ISO without preinjection of an antagonist was approximately equal to the effect of the first injection. The effect of the second injection of EPI without preinjection was apparently less than the effect of the first injection (tachyphylaxis).

**Measurement of blood pressure, heart rate, respiration rate and rectal temperature:** Mean blood pressure and heart rate were measured through a cannula inserted into the carotid artery with a pressure transducer (MPU-0.5-290-0-III, Nihon Kohden Kogyo, Co., Tokyo) and by an electrocardiograph (FD-14, Fukuda, Tokyo), respectively. Respiration rate was measured by using a thermister probe (SR-115S, Nihon Kohden Kogyo, Co.) inserted into the trachea cannula. These indices were recorded simultaneously, blood pressure and respiration rate being recorded on a recticorder (RJG-3004-2, Nihon Kohden Kogyo, Co.). Rectal temperature was monitored by a thermister probe (MGA III-219, Nihon Kohden Kogyo, Co.) inserted into the rectum.

**Identification of the sites of inserted**
cannula: The position of the tip of the cannula inserted stereotaxically into the PVN was confirmed by the following methods: 1) functionally, by the appearance of the antidiuretic effect by microinjecting a depolarizing dose (400 nmol) of KCl through the cannula, and 2) histochemically, by the localization of the tip of the cannula in a group of magnocellular cells in the PVN which were positively stained by the method of Gomori (22).

**Statistical analysis:** Significance of differences between mean values was determined by Student’s t-test. The differences were considered significant at P<0.05. The ED50 values and 95% confidence limit of the ED50 values were computed from dose-effect curves drawn by the least squares method.

**Results**

**Antidiuretic effects of catecholamines microinjected into the PVN:** Effects of microinjection of several doses of L-norepinephrine (NE), L-epinephrine (EPI) and L-isoproterenol (ISO) into the PVN on the rate of the urine outflow are illustrated in Fig. 1. The antidiuretic effects of the three catecholamines showed similar time courses to each other. The catecholamines decreased the rate of the urine outflow within 20 min after microinjection. The rates of the urine outflow were minimal at 20–40 min and recovered toward the initial rate at 1–2 hours after the microinjections.

In Fig. 2 are shown the dose-antidiuretic effect curves for various drugs microinjected into the PVN. High doses of KCl and NaCl microinjected into the PVN induced antidiuretic effects with similar time courses to those induced by microinjection of catecholamines in Fig. 1. The dose-effect curves for KCl and NaCl are shown in Fig. 2a. The ED50 value for KCl was 204 (91–456) nmol and that for NaCl was 1.1 (0.8–1.5) μmol. The curves for EPI and ISO were nearly parallel to each other, but the curve for NE was less dependent on the dose microinjected than that for EPI or ISO. The ED50 values for NE, EPI and ISO were computed to be 4.4 (0.8–24), 10 (4–24) and 4.6 (2.8–7.7) nmol, respectively. As illustrated in Fig. 2d, the L-isomer of isoproterenol was more potent in inducing antidiuresis than the DL-
form, the ED50 values for the L-isomer being 4.6 (2.8–7.7) nmol versus 17 (11–27) nmol for the DL-form, the D-isomer demonstrating no significant antidiuretic activity. Vehicle, an artificial CSF or saline, alone, did not affect the rate of the urine outflow. No significant antidiuretic effects were observed by microinjecting NE (20 nmol) into several sites at a distance of one mm from the PVN.

Effects of pretreatment with various antagonists on catecholamine-induced antidiureses: As summarized in Table 1, the antidiuretic effect of 10 nmol NE microinjected into the PVN was inhibited by premicroinjection of 20 and 80 nmol phenoxybenzamine, an alpha-adrenoceptor antagonist, into the nucleus; the preinjection of phenoxybenzamine alone showing no significant effect on the urine outflow. Premicroinjection of a beta-adrenoceptor antagonist, propranolol (150 nmol) or timolol (100 nmol), also inhibited the NE-induced antidiuretic effect. The pretreatment with propranolol alone slightly, but not significantly, increased the urine outflow, and that with timolol alone increased the urine outflow to 146±19% (n=7) of the control at 40 min after microinjection (not shown).

Premicroinjection of a muscarinic antagonist, atropine (300 nmol), partially inhibited the antidiuretic effect induced by NE (20 nmol).

Table 2 demonstrates the effects of these antagonists on antidiuresis induced by microinjection of 20 nmol DL-ISO into the PVN. The two beta-adrenoceptor antagonists and the muscarinic antagonist, but not the alpha-adrenoceptor antagonist inhibited the antidiuretic effects of DL-ISO. Atropine (300 nmol) which is enough to block nearly completely the potent antidiuretic effect of ACh (80 nmol) (20) only partially inhibited the DL-ISO-induced antidiuretic effect. Timolol (100 nmol), however, approximately totally blocked the effect of DL-ISO.

Effects of microinjection of catecholamines on the other visceral functions: As shown in Table 3, several visceral functions which might be expected to be responsive to microinjection of catecholamines into the PVN were monitored during the experiments. At 20 and 30 min after microinjection of 40 nmol NE or DL-ISO when the urine outflow decreased to their minimal levels of less than 1/3 of the control, there were no alterations in mean blood pressure, heart rate, respiration rate and rectal temperature except in the following cases: At 30 min after microinjection of DL-ISO, mean blood pressure decreased to approximately 76% of the initial control. At 30 min after microinjection of NE, heart rate decreased to approximately 84% of the initial control and at 20–30 min after microinjection of DL-ISO, heart rate increased up to approximately 120% of the initial control.

Discussion

This is the first publication demonstrating...
Table 1. Effects of pretreatment with various antagonists on antidiuresis induced by microinjection of L-norepinephrine into the PVN

| No. of exp. | Pretreatment     | n | Dose of NE microinj. (nmol) | Control initial urine outflow (ml/min) | Urine outflow (% of control) |
|-------------|------------------|---|-----------------------------|----------------------------------------|-----------------------------|
|             |                  |   |                            | 0                          | 10                         | 20 | 30 | 40 | 50 | 60 |
| 1           | None             | 5 | 10                          | 0.112±0.017                 | 100                        | 83±11 |       |    |    |    |
|             | 20 nmol phenoxybenzamine | 5 | 10                          | 0.122±0.030                 | 100                        | 69±20 |       |    |    |    |
| 2           | None             | 4 | 10                          | 0.109±0.021                 | 100                        | 90±15 |       |    |    |    |
|             | 80 nmol phenoxybenzamine | 4 | 10                          | 0.094±0.020                 | 100                        | 96±2  |       |    |    |    |
| 3           | None             | 4 | 10                          | 0.084±0.011                 | 100                        | 91±5  |       |    |    |    |
|             | 150 nmol propranolol | 4 | 10                          | 0.135±0.021                 | 100                        | 100±8 |       |    |    |    |
| 4           | None             | 6 | 10                          | 0.107±0.017                 | 100                        | 90±9  |       |    |    |    |
|             | 100 nmol timolol | 6 | 10                          | 0.158±0.021                 | 100                        | 101±5 |       |    |    |    |
| 5           | None             | 8 | 20                          | 0.088±0.013                 | 100                        | 95±10 |       |    |    |    |
|             | 300 nmol atropine| 8 | 20                          | 0.062±0.005                 | 100                        | 101±5 |       |    |    |    |

Drugs were dissolved in an artificial cerebrospinal fluid containing the ingredients described in "Materials and Methods". None: first microinjection of norepinephrine. Antagonists were premicroinjected at 30 to 50 min before the second injection of norepinephrine. Values for urine outflow are the means ±S.E.M. Significance compared with the effects of the first injection of norepinephrine at the same time point after microinjection: *P<0.05.

Table 2. Effects of pretreatment with various antagonists on antidiuresis induced by DL-isoproterenol microinjected into the PVN

| No. of exp. | Pretreatment     | n | Dose of DL-ISO microinj. (nmol) | Control initial urine outflow (ml/min) | Urine outflow (% of control) |
|-------------|------------------|---|-----------------------------|----------------------------------------|-----------------------------|
|             |                  |   |                            | 0                          | 10                         | 20 | 30 | 40 | 50 | 60 | 70 |
| 1           | None             | 5 | 20                          | 0.087±0.009                 | 100                        | 100±1 | 37±9 | 14±6 | 15±4 | 27±5 | 46±8 | 66±8 |
|             | 80 nmol phenoxybenzamine | 5 | 20                          | 0.097±0.023                 | 100                        | 83±7  | 12±4 | 16±5 | 26±7 | 45±10 | 78±17 |
| 2           | None             | 5 | 20                          | 0.079±0.026                 | 100                        | 89±14 | 15±10| 10±2 | 20±4 | 44±10 | 75±10 | 100±5 |
|             | 150 nmol propranolol | 5 | 20                          | 0.146±0.033                 | 100                        | 89±8  | 59±16*| 53±17*| 55±17*| 71±19*| 88±14 |
| 3           | None             | 4 | 20                          | 0.110±0.016                 | 100                        | 80±3  | 10±6 | 11±7 | 17±7 | 33±11 | 61±10 | 76±20 |
|             | 100 nmol timolol | 4 | 20                          | 0.178±0.020                 | 100                        | 97±4  | 97±7  | 97±6  | 88±4  | 94±6  | 93±6  | 110±6*|
| 4           | None             | 5 | 20                          | 0.092±0.021                 | 100                        | 88±7  | 30±12 | 7±2  | 12±3 | 31±11 | 54±14 | 80±19 |
|             | 300 nmol atropine| 5 | 20                          | 0.099±0.029                 | 100                        | 74±10 | 25±12 | 30±11| 49±13*| 75±14*| 93±6  | 110±6*|

Drugs were dissolved and antagonists were pretreated as in Table 1. None: first microinjection of isoproterenol. Values for urine outflow are the means ±S.E.M. Significance compared with the effects of the first injection of isoproterenol at the same time point after microinjection: *P<0.05.
Table 3. Effects of L-norepinephrine and DL-isoproterenol microinjected into the PVN on various visceral functions

| Microinjection | Various visceral functions | n | Control initial values | % of control min after microinjection |
|----------------|---------------------------|---|------------------------|--------------------------------------|
|                |                           |   | 0 | 20 | 30 |
| 40 nmol L-NE   | Urine outflow             | 4 | 0.070±0.015 ml/min     | 100  | 32±8* | 25±14* |
|                | Blood pressure            | 4 | 111±15 mmHg            | 100  | 101±1  | 90±7  |
|                | Heart rate                | 4 | 400±15 /min            | 100  | 90±2   | 84±3* |
|                | Respiration rate          | 4 | 108±7 /min             | 100  | 118±6  | 90±16 |
|                | Rectal temperature        | 4 | 35.7±0.3°C             |       | 35.6±0.3°C | 35.5±0.3°C |
| 40 nmol DL-ISO | Urine outflow             | 4 | 0.083±0.016 ml/min     | 100  | 21±9*  | 3±2*  |
|                | Blood pressure            | 4 | 131±11 mmHg            | 100  | 89±4   | 76±4* |
|                | Heart rate                | 4 | 380±16 /min            | 100  | 117±2* | 120±4* |
|                | Respiration rate          | 4 | 122±5 /min             | 100  | 114±2  | 101±10|
|                | Rectal temperature        | 4 | 35.5±0.1°C             |       | 35.5±0.1°C | 35.7±0.1°C |

Visceral functions were measured simultaneously in one animal. Urine outflows were usually minimal at 20 or 30 min after microinjections. Drugs were dissolved as in Table 1, and one μl of the solution of L-norepinephrine or DL-isoproterenol was microinjected into the PVN. Values for urine outflow, mean blood pressure, heart rate and respiration rate at 20 and 30 min were expressed as % of control initial values, and values for rectal temperature were expressed as centigrade (°C). All values are the means±S.E.M. Significance compared with control initial values: *P<0.05.
that the catecholamines, NE, EPI and ISO, microinjected into the PVN in the hypothalamus induced potent antidiuretic effects. The antidiuretic effects appeared relatively slowly, the onset and the duration of which were at 10–20 min and 1–2 hours after microinjection, respectively, suggesting that the effects were mediated by a release of hormone, probably ADH, rather than through neuronal pathways. However, a possible neuronal regulation of the urine outflow could not be ruled out in the present study.

The mean ED50 values for NE and ISO were approximately 5 nmol, indicating that the alpha- and the beta-adrenoceptor agonists had similar antidiuretic activities in the PVN. The effect of NE was inhibited by pretreatment with alpha- or beta-adrenoceptor antagonists, indicating that the NE-induced antidiuresis was mediated through both alpha- and beta-adrenoceptors. In contrast, the effect of DL-ISO was nearly completely inhibited by a beta-antagonist, but not affected by an alpha-antagonist. The DL-ISO-induced effect, therefore, was mediated only through beta-adrenoceptors in the nucleus. Presynaptic facilitation of the release of NE through beta-adrenoceptors (23) may not be working significantly in this nucleus. The results demonstrated that alpha- and beta-adrenoceptors in the PVN as well as the SON (18) induced antidiureses together.

Dose–effect curves for L-, DL- and D-forms of ISO in Fig. 1d indicated that the beta-adrenoceptor mediating the antidiuretic effect had a rigid stereospecificity, which is one of the criteria for a specific receptor. Timolol alone, a beta-adrenoceptor antagonist having no local anesthetic activity, when microinjected into the PVN as well as into the SON (18), induced a significant diuresis of approximately 150% of the control, suggesting a physiological role of beta-adrenoceptors in the nucleus in vivo. Inhibitory effect of pretreatment with atropine on the effects of NE and DL-ISO might demonstrate a possible presynaptic facilitation of the release of ACh as reported in other tissues (24–27). The effect of atropine was observed only in the PVN, suggesting a greater contribution of cholinergic mechanisms to the PVN. The neurons in the PVN are known to project to various regions in the central nervous system regulating autonomic functions, particularly vasomotor activities (28–30). As shown in Table 3, mean blood pressure decreased to approximately 75% of the control at 30 min after microinjection of DL-ISO (40 nmol), when the urine outflow was reduced to approximately 3% of the control. However, the mean blood pressure was approximately equal to the control at 20 min after microinjection of the same agonist and at 20–30 min after microinjection of NE (40 nmol), when the urine outflow decreased to 20–30% of the control. Therefore, the moderate antidiuretic effects induced by NE and DL-ISO were not the results of any decrease in mean blood pressure. On the other hand, heart rate decreased to approximately 85% of the control at 30 min after microinjection of NE, which had not been significantly altered at 20 min, when the urine outflow was approximately 20–30% of the control. At 20–30 min after microinjection of DL-ISO, when the urine outflow was approximately 20% of the control, heart rate oppositely increased up to approximately 120% of the control. This indicated that any changes of approximately 20% of the control in heart rate might not be a cause of the antidiuretic effects induced by NE and DL-ISO.

The antidiuretic effect of high doses of KCI microinjected into the PVN was more potent than the effect of NaCl, the ED50 value for KCl being approximately 200 nmol versus the ED50 value for NaCl, approximately 1 μmol. The effect of KCl may be due to depolarization of the neurons rather than an increase in extracellular ionic strength and in the concentration of chloride ions. Comparing the ED50 values for NE and EPI (approximately 5 and 10 nmol, respectively) with the ED50 value for KCl, the catecholamines which are candidates of neurotransmitters in the PVN, were about 20–40 fold more potent than KCl. The median effective concentrations for the catecholamines would be roughly estimated to be approximately 1.5–3 mM, if the neurons in the PVN could be stimulated by approximately 60 mM KCl (31). A few millimolar
concentration of the catecholamines is extremely higher than the level in the circulation (32), but the concentration of the catecholamines in the synaptic cleft could increase up to several mM, considering that the concentration of NE in the varicosities is calculated to be 6–18 mM (33). As the animals were loaded with water and ethanol in the present study, the neurons in the PVN may be less sensitive than the neurons in unloaded intact animals (34).

No significant antidiuretic effects were observed after microinjection of 20 nmol NE into several sites at a distance of one mm from the PVN. The distance of the effect of diffusion of the microinjected NE may be less than one mm. The possible presence of neurons having alpha- and beta-adrenoceptors which regulate neurons in the PVN within the distance of diffusion, however, could not be excluded in the present study.

Comparing the present data with the previous data studied in the SON (18), generally the effects of microinjection of the catecholamines were surprisingly similar to each other. In both nuclei, the three catecholamines induced potent antidiuretic effects mediated through alpha- and beta-adrenoceptors. As the ED50 values for NE and ISO microinjected into the SON are 25 (11–50) and 5.3 (4.5–6.2) nmol, respectively (18), and the two values when microinjected into the PVN were 4.4 (0.8–24) and 4.6 (2.8–7.7) nmol, respectively, in the present study, the effect of NE is less potent than that of ISO in the SON, but in the PVN, the two catecholamines showed approximately equal potency. In other words, the sensitivity to NE was a little higher in the PVN than in the SON. A similar relationship in the sensitivity to ACh has been found between the PVN and SON (20). Pretreatment with atropine partially inhibited the effects of NE and DL-ISO when microinjected into the PVN, which are not observed when the drugs are microinjected into the SON (18). Therefore, in the PVN, the antidiuretic effects induced by the catecholamines may be only partially mediated by the presynaptic release of ACh, which is not observed in the SON.

With minor differences in the sensitivities to the catecholamines between the SON and PVN, it is of great interest that the two nuclei may be similarly regulated by the catecholamines.

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