Effect of Vasopressin Antagonist on Antidiuresis by Oxtremorine Microinjected into the Hypothalamic Supraoptic and Paraventricular Nuclei in a Water-Loaded and Ethanol-Anesthetized Rat

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Abstract—Microinjection of the muscarinic agonist oxtremorine into the hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei which contain cell bodies of vasopressinergic neurons induced potent antidiuretic effects in water-loaded and ethanol-anesthetized rats. The effects included both decreases in urine outflow and increases in urine osmotic pressure. However, no significant changes in various visceral functions other than antidiuresis such as mean blood pressure, heart rate, respiration rate and rectal temperature were observed when oxtremorine was microinjected into the SON. Only a slight change in mean blood pressure (approx. 10 mmHg decrease) was observed by the microinjection into the PVN. Intravenous preinjection of a vasopressin (AVP) V1 V2 antagonist that has one of the most potent V2 (antidiuretic)-antagonist activities, d(CH2)5-D-Tyr(Et)VAVP, inhibited nearly completely the antidiuretic effects induced by the microinjection of oxtremorine. The results demonstrated that oxtremorine stimulated muscarinic receptors in the hypothalamic SON and PVN, released AVP and induced an antidiuretic effect through AVP-receptors in the kidney.

The supraoptic (SON) and paraventricular (PVN) nuclei in the hypothalamus contain magnocellular vasopressinergic neurons, the axons of which terminate in the neurohypophysis. Stimulation of the neurons induces release of 8-arginine vasopressin (AVP) in most mammals including rats, from the neurohypophysis into the circulation. AVP in the circulation promotes water reabsorption from cortical and medullary collecting tubules and collecting ducts of the kidney by stimulating AVP V2-receptors, finally causing antidiuresis (1-6).

As neuronal terminals of various neurotransmitters have been immunohistochemically visualized in the SON and PVN, we have been interested in the receptive mechanisms for the neurotransmitters and have demonstrated cholinergic (7), adrenergic (8, 9) and opioid (10) receptive mechanisms in the nuclei by stereotaxically microinjecting drugs into the nuclei and measuring the rate of urine outflow and other visceral functions in water-loaded and ethanol-anesthetized rats. Concerning the cholinergic system, the presence of activities of choline acetyltransferase (11, 12) and acetylcholinesterase (13) and immunohistochemical localization of choline acetyltransferase (14) in the SON and PVN have demonstrated biochemically and morphologically cholinergic innervation which may regulate the nuclei.

Antidiuretic effects induced by microinjection of ACh into the SON (15, 16) and stimulatory effects on vasopressinergic neurons by iontophoretic application of ACh into the SON (17-19) and the PVN (20) have shown that the effects may be mediated through cholinergic receptors in the nuclei.

Oxtremorine, a muscarinic agonist when microinjected into the SON and PVN, causes a very potent decrease in urine outflow in
dose- and time-dependent manners (7). The median effective doses for oxotremorine are as low as 0.4 and 0.2 nmol when it is microinjected into the SON and PVN, respectively. The effects induced by oxotremorine are completely blocked by premicroinjection of atropine, but not by hexamethonium, indicating that they are mediated through muscarinic ACh-receptors in the nuclei (7).

The targets of microinjections, which are the nuclei containing vasopressinergic neurons, and the slow onset (approx. 20 min after microinjection) and long duration (approx. one hour) of the oxotremorine-induced effects strongly suggest that the effects are hormonal, most probably due to the release of AVP from the neurohypophysis (7).

In the present study, we demonstrated that the oxotremorine-induced effects were associated with the facilitation of water reabsorption and were completely blocked by an AVP antagonist administered intravenously, which is evidence that the oxotremorine-induced anti-diuresis was mediated through AVP-receptors in the kidney.

Materials and Methods

Animals and drugs: Male Wistar rats, weighing 280–320 g, were used. Oxotremorine (Aldrich Chemical Co., Inc., Milwaukee, WI) was purchased. Vasopressin antagonist, 1 - (β - mercapto - β ,β - cyclopentamethylene propionic acid) 2-(O-ethyl)-D-tyrosine, 4-valine, arginine vasopressin: d(CH2)5-D-Tyr(Et)VAVP, was kindly provided by Prof. K.G. Hofbauer (Cardiovascular Research Department, Pharmaceutical Division, Ciba-Geigy, Ltd. Basel, Switzerland). The other chemicals used were of the highest analytical grade available.

Measurement of outflow and osmotic pressure of urine: Urine outflow was measured by the method of Dicker with some modifications (7, 21). The rats were starved overnight for approx. 17 hr, but had free access to water. The animals were loaded orally through a catheter with a volume of water equivalent to 5% of the body weight followed by the same volume of 12% ethanol. Cannulae were inserted into the trachea, bladder and external jugular vein. The rat was then immobilized in a stereotaxic instrument for rats (Takahashi Co., Tokyo). Drops of urine flowing from the urinary cannula were counted using a photoelectric drop counter (DCT 102, Unique Medical Inc., Tokyo) and recorded as single pulses. Ethanol (3% in Locke solution) was infused at a constant rate of 0.10 ml/min through the cannula in the jugular vein in order to maintain a constant level of anesthesia and a constant rate of urine outflow. Osmotic pressure of the urine was measured by the freezing point depression method (The Fiske Osmometer, Model G-62, Fiske Associates, Inc., Uxbridge, MA).

Microinjection of oxotremorine: A stainless steel cannula (outer diameter: 200 μm) was unilaterally inserted stereotaxically into the SON (A, 6.28; L, 1.3; H, 8.8) or PVN (A, 5.6; L, 0.24; H, 7.8) (in mm) according to the atlas of König and Klippel (22). Oxotremorine was dissolved in saline (pH, approx. 7). One μl of this solution containing 0.2 to 1.0 nmol oxotremorine was microinjected through a microsyringe connected with the cannula when the urine outflow reached a constant rate of 0.04–0.19 ml/min. Then 2 μl of an artificial cerebrospinal fluid (CSF: 128 mM NaCl, 3.0 mM KCl, 1.2 mM CaCl2, 0.8 mM MgCl2, 0.65 mM NaH2PO4, 4.8 mM NaHCO3, pH 7.4) was injected at a rate of approx. 0.3 μl/min. Effects of oxotremorine on urine outflow were measured at 10 min intervals and expressed as a percent of the initial control urine outflow.

Pretreatment with AVP antagonist: In the experiments to test the effects of pretreatment with the AVP antagonist, d(CH2)5-D-Tyr(Et)VAVP, the first injection of oxotremorine was followed by an intravenous injection of the AVP antagonist (50 μg/kg), and then a second injection of oxotremorine was performed into the same rat. The antagonist dissolved in saline was injected intravenously through the cannula in the external jugular vein when the urine outflow had recovered to the initial level, usually at 60–80 min after the first microinjection of oxotremorine. A second microinjection of oxotremorine was carried out at 40 min after the antagonist was administered. The inhibitory effect of the antagonist was estimated as the change in urine outflow caused by the second injection.
of oxotremorine as compared with that of the first injection.

**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 using, respectively, a pressure transducer (MPU-0.5-290-O-III, Nihon Kohden Kogyo, Co., Tokyo) and an electrocardiograph (Fukuda, FD-14, Tokyo). Respiration rate was measured via a thermister probe (SR-115S, Nihon Kohden Kogyo, Co.) inserted into a tracheal catheter. These three indices were recorded simultaneously 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 cannula insertion:** The position of the tip of the cannula within the SON or PVN was confirmed by the following methods: 1) functionally, by the appearance of an antidiuretic effect by the microinjection of depolarizing doses of KCl (800 and 400 nmol into the SON and PVN, respectively) through the cannula and 2) histochemically, by the localization of the tip of the cannula in a group of magnocellular cells in the SON and PVN positively stained by the method of Gomori (23).

**Statistical analysis:** Significance of differences between mean values was determined by Student’s t-test. Differences were considered significant at P<0.05.

**Results**

**Decreases in urine outflow and increases in urine osmotic pressure by microinjection of oxotremorine into the SON and PVN:** Figure 1a shows the effects of microinjection of oxotremorine into the SON on the outflow and osmotic pressure of urine. The urine outflow decreased to approx. 20% of the control (control: 0.063±0.012 ml/min), and the urine osmotic pressure increased to approx. 230% of the control (control: 300±17 mOsm), at 20 to 30 min after microinjection of oxotremorine (1 nmol) when the urine outflow decreased to a minimal level. Figure 1b demonstrates the recoveries of the effects of the microinjection of oxotremorine. At 60 to 70 min after the microinjection of oxotremorine when the urine outflow recovered to approx. the control value, the osmotic pressure of urine also recovered to approx. the control. Figure 2 shows the similar results obtained by microinjection of oxotremorine into the PVN. The oxotremorine-induced effects on outflow and osmotic pressure of urine as illustrated in Fig. 2a recovered toward the respective control values (Fig. 2b). No significant antidiuretic responses were observed by microinjecting oxotremorine into several sites at a distance of 1 mm to the nuclei: 1 nmol oxotremorine at the sites anterior, posterior, and lateral to the SON and 0.4 nmol oxotremorine at the sites posterior and lateral to the PVN.
Fig. 2. Changes in urine osmotic pressure compared with urine outflow after microinjection of oxotremorine (0.4 nmol) into the PVN. Description for each column is as in Fig. 1. The initial urine outflow and osmotic pressure were 0.085±0.009 ml/min and 227±16 mOsm, respectively. a): when the urine outflow was approx. minimal, i.e., 30 min after microinjection; b): when the outflow recovered to the initial level at 60 min after microinjection. Each column represents the means±S.E. from 4–7 experiments. Significance compared with the value for microinjection of vehicle: *P<0.05.

Effects of microinjection of oxotremorine on various visceral functions: Some visceral functions which might be expected to be responsive to the microinjection of oxotremorine into the hypothalamic nuclei and which might affect the urine outflow were monitored during the experiments (Tables 1 and 2). While the urine outflow decreased to approx. 20% of the control at 30–50 min after microinjection of 0.4 nmol oxotremorine into the SON, mean blood pressure, heart rate, respiration rate and rectal temperature were approx. equal to the control (Table 1). As shown in Table 2, the mean blood pressure decreased to approx. 100 mmHg, which was approx. 10 mmHg lower than the control level at 20 to 40 min after microinjection of oxotremorine into the PVN. However, heart rate, respiration rate and rectal temperature did not change significantly throughout the experiments.

Blockade of antidiuretic effects of microinjection of oxotremorine by AVP antagonist: Figure 3 illustrates the time course of the inhibitory effect of an intravenously preinjected AVP antagonist, d(CH2)5-D-Tyr(Et)VAVP, on antidiuresis induced by microinjection of oxotremorine into the SON. •: first injection of oxotremorine (1 nmol), ○: second injection of oxotremorine (1 nmol) after pretreatment with the AVP antagonist (50 μg/kg, i.v.). The time interval between injection of the AVP antagonist and the second injection of oxotremorine is 40 min. Abscissa indicates time in min after injection of oxotremorine, and ordinate shows urine outflow presented as a percent of the initial control urine outflow (control for the first microinjection of oxotremorine: 0.088±0.029 ml/min). Values for second injection of oxotremorine at 10, 20, 30, 40 and 50 min were 123±8, 117±4, 106±13, 104±12 and 106±13% of the control, respectively (control: 0.094±0.014 ml/min). The points, brackets and the initial urine outflow are the means±S.E. from 3 experiments. Significance compared with the effects of the first microinjection of oxotremorine at the same time after microinjection: *P<0.05.
Table 1. Effects of microinjection of oxotremorine into the SON on various visceral functions

| Visceral functions | n  | 0       | 10      | 20      | 30       | 40      | 50       | 60       | 70       | 90       |
|-------------------|----|---------|---------|---------|----------|---------|----------|----------|----------|----------|
| Urine outflow (% of initial control) | 4  | 100     | 77±6.9  | 26±7.5* | 20±4.5*  | 15±3.0* | 16±4.7*  | 35±18.7* | 67±22.6  | 80±20.5  |
| Blood pressure (mmHg) | 4  | 113±5.2 | 115±8.4 | 120±5.4 | 108±8.5  | 104±7.2 | 105±5.4  | 103±5.2  | 105±5.4  | –        |
| Heart rate (/min) | 4  | 420±20.4| 425±20.2| 390±34.4| 385±36.6 | 388±36.4| 400±32.4 | 398±29.5 | 420±26.5 | –        |
| Respiration rate (/min) | 4  | 116±10.7| 135±5.1 | 136±5.6 | 158±26.4 | 130±8.9 | 126±14.5 | 120±14.5 | 123±19.8 | –        |
| Rectal temperature (°C) | 6  | 34.0±0.2| –       | –       | 34.2±0.2 | –       | –        | 33.9±0.1 | –        | –        |

Significance compared with the initial control values: *P<0.05.

Table 2. Effects of microinjection of oxotremorine into the PVN on various visceral functions

| Visceral functions | n  | 0       | 10      | 20      | 30       | 40       | 50       |
|-------------------|----|---------|---------|---------|----------|----------|----------|
| Urine outflow (% of initial control) | 4  | 100     | 118±9.7 | 71±31.5 | 30±10.0* | 68±35.6  | 82±36.4  |
| Blood pressure (mmHg) | 3  | 112±2.0 | 112±3.8 | 101±2.6*| 101±1.8* | 99±1.8*  | 97±5.5   |
| Heart rate (/min) | 4  | 437±3.3 | 437±3.3 | 433±3.3 | 433±3.3  | 433±3.3  | 443±3.3  |
| Respiration rate (/min) | 3  | 75±9.8  | 81±11.6 | 77±11.0 | 81±13.3  | 83±12.5  | 80±10.5  |
| Rectal temperature (°C) | 4  | 34.9±0.3| 34.9±0.2| 35.1±0.1| 35.2±0.1 | 35.3±0.03| 36.3±0.09|

Significance compared with the initial control values: *P<0.05.
by microinjection of oxotremorine into the PVN. The intravenous injection of the AVP antagonist alone did not significantly change the urine outflow. The values of the urine outflow at 10, 20, 30 and 40 min after intravenous injection of the AVP antagonist were 97±23, 97±29, 101±33 and 104±33% of the control, respectively (control: 0.099±0.021 ml/min). Significance compared with the effects of the first microinjection of oxotremorine at the same time after microinjection: *P<0.05.

Discussion

The decrease in urine outflow by microinjection of oxotremorine into the SON and PVN was accompanied by an increase in urine osmotic pressure, indicating that reabsorption of water was evidently induced in the kidney. However, while the urine outflow decreased to approx. 20% of the control, the increase in the osmotic pressure was approx. 230% of the control, which seemed to be less than the expected increase (approx. 500% of the control). It was suggested that some unknown factors other than water reabsorption might be working in the effects of microinjection of oxotremorine.

No significant effects were observed by microinjecting oxotremorine into several sites at a distance of 1 mm from the SON and PVN. This and the diffusion range after microinjection of methylene blue (1 μl) demonstrated that the distance of simple diffusion of microinjected oxotremorine was less than 1 mm. Oxotremorine may act directly on the nuclei.

No significant changes in various visceral functions such as mean blood pressure, heart rate, respiration rate and rectal temperature, which might be expected to be responsive to the microinjection of oxotremorine and which might affect the urine outflow, were observed when oxotremorine was microinjected into the SON (Table 1). When oxotremorine was microinjected into the PVN, a decrease in mean blood pressure of approx. 10 mmHg was observed, without changes in the other visceral functions (Table 2). However, the approx. 10 mmHg decrease in mean blood pressure at 20 to 40 min after microinjection of oxotremorine was accompanied by different degrees of decrease in urine outflow (Table 2), showing that it is unlikely that the decrease in urine outflow is the result of the decrease in mean blood pressure.

A series of vasopressic V₁- (24) and antidiuretic V₂- (25) AVP antagonists which exert potent inhibitory effects on the each action of AVP have been developed (25–27). The AVP antagonist used in the present study, d(CH₂)₅-D-Tyr(Et)VAVP, is one of the most potent V₂- antagonists (26). Intravenous administrations of 8 and 100 μg/kg of the AVP antagonist (i.v.) are enough to inhibit the antidiuretic effect of 4 and 25 ng/kg AVP (i.v.) for 3 hr, respectively (28, 29). In this study,
the antidiuretic effects of microinjection of oxotremorine into the SON and PVN were almost completely blocked by the AVP antagonist (50 μg/kg) injected intravenously, indicating that the oxotremorine-induced antidiuretic effects were mainly mediated through AVP-receptors in the kidney. As the AVP antagonist (i.v.) alone did not significantly change the urine outflow, various visceral functions which might influence the rate of urine outflow were not likely to be altered by the antagonist. This demonstrated that by the present modified Dicker’s method, which loads a large amount of ethanol and water and induces potent diuresis, the circulating AVP level was so low that the antagonist was not effective on urine outflow. Upon a water-loading when the AVP level is suppressed, the AVP antagonist has been reported to show little effect on urine outflow (29).

In summary, the present study demonstrated that oxotremorine stimulated muscarinic receptors in the SON and PVN, released AVP, and induced antidiuretic effects through AVP-receptors in the kidney. Besides the pharmacological studies using AVP antagonists, direct biochemical evidence of an increase in the plasma AVP level after microinjection of oxotremorine remains to be obtained.

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