Antidiuretic Effects of Methionine-Enkephalin and 2-D-Alanine-5-Methionine-Enkephalinamide Microinjected into the Hypothalamic Supraoptic and Paraventricular Nuclei in a Water-Loaded and Ethanol-Anesthetized Rat

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Abstract—Effects of methionine-enkephalin (ME) and 2-D-alanine-5-methionine-enkephalinamide (DAMEA) microinjected into the hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei, which contain neurons synthesizing and releasing antidiuretic hormone, upon the outflow and the osmotic pressure of urine and the other visceral functions were studied in a rat which was loaded with water and anesthetized with ethanol. These opioid peptides when microinjected into the SON or PVN induced potent antidiuretic effects in dose-dependent and time-dependent manners with no significant effects on the other visceral functions. The approx. ED50 values for DAMEA were 1.3 (in the SON) and 0.7 (in the PVN) nmol, and the values for ME were 110 (in the SON) and 60 (in the PVN) nmol. The antidiuretic effects showed slow onset and long duration, with a minimal urine outflow at approx. 0.5 hr after microinjection and an approx. 2 hr-duration. The effects induced by the opioid peptides were inhibited by pretreatment with naloxone or atropine, without effects of pretreatment with alpha- or beta-adrenoceptor antagonists, suggesting that the antidiuretic effects were mediated through an opioid receptor having low sensitivity to naloxone and also possibly mediated through a muscarinic receptor which was stimulated probably by the ACh released by the opioid peptides.

The supraoptic (SON) and the paraventricular (PVN) nuclei in the hypothalamus contain the cell bodies of the magnocellular neurons which synthesize antidiuretic hormone (ADH, vasopressin). When a group of neurons in the nuclei are stimulated by neurotransmitters, ADH is released into the circulation from the nerve terminals which are located in the neurohypophysis. The circulating ADH enhances the reabsorption of water from the distal tubule and collecting duct of the kidney, inducing an antidiuretic effect and thus playing a key role in osmoregulation of the body fluids (1-3).

Cholinergic and adrenergic innervations to the SON and PVN are suggested morphologically and biochemically (4-7). We demonstrated that microinjection of cholinergic and adrenergic agonists induced antidiuretic effects which were mediated through muscarinic receptors (8) and alpha- and beta-adrenoceptors (9, 10) in the nuclei, respectively.

Effects of intracerebroventricular administration of opioid peptides on the release of vasopressin (11-17) have suggested that enkephalinergic control may play an important part in the release of vasopressin. However, at present, it is not clear whether or not the opioid peptides act directly on the SON and PVN. Recent immunohistochemical studies have visualized enkephalinergic nerve terminals in the nuclei (18-20), strongly supporting enkephalinergic innervation to the vasopressinergic magnocellular neurons.

In the present study, we investigated the
effects of direct microinjection of methionine-enkephalin and its analog into the SON and PVN on various visceral functions. The opioid peptides induced strong antidiuretic effects which were inhibited by pretreatment with opioid and muscarinic antagonists.

Materials and Methods

Animals and drugs: Male Wistar rats, weighing 280–350 g, were used. The following were obtained from commercial sources: Methionine-enkephalin (ME), 2-D-alanine-5-methionine-enkephalinamide (DAMEA) (Sigma Chemical Co., St. Louis, MO); atropine sulfate (Iwaki Co., Tokyo); and phenoxybenzamine hydrochloride (Nakarai Chemicals, Kyoto). Naloxone hydrochloride and timolol malate were the generous gifts of Sankyo Co., Tokyo and Nippon Merck-Banyu Co., Tokyo, respectively. The other chemicals used were the analytical grade available.

Measurement of urine outflow and urine osmotic pressure: Urine outflow was measured by the method of Dicker (21), with some modifications (8–10). The rats were starved overnight for approx. 17 hours, having free access to water. The animals were 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, bladder and external jugular vein, respectively. The rat 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 using a photoelectric drop counter (DCT 102, Unique Medical Inc., Tokyo) and recorded as signal pulses. Three percent ethanol in Locke solution was infused at a constant rate of 0.10 ml/min through the cannula inserted into the jugular vein in order to maintain a constant level of anesthesia and a constant rate of urine outflow. The osmotic pressure of the urine was measured by the freezing point depression method (The Fiske Osmometer, Model G-62, Fiske Associates, Inc., Uxbridge, MA).

Administration of drugs: A stainless cannula (outer diameter: 200 μm) was inserted stereotaxically and unilaterally into the SON or PVN, according to the atlas of König and Klippel (22). Drugs were dissolved in saline and injected using a microsyringe in a volume of 1 μl through the cannula. Then 2 μl of the artificial cerebrospinal fluid (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) was injected at a rate of approx. 0.3 μl/min. The microinjection was performed when the urine outflow reached a constant rate of 0.07–0.13 ml/min within one hour after the animal was fixed in the stereotaxic instrument. Effects of drugs on urine outflow during every 10 min were expressed as a percent of the initial constant outflow. Effects of drugs on urine osmotic pressure were presented as a percent of the initial osmotic pressure.

In the experiment to test the effect of pretreatment with an antagonist, the first microinjection of an agonist was followed by microinjection of an antagonist, and then the second microinjection of the same agonist was performed. All microinjections were through a single cannula inserted into the nucleus. The microinjection of the antagonist and the second microinjection of the agonist were carried out at the time when the urine outflow had recovered to the initial level, usually at approx. 1.5 hr after the first microinjection of the agonist and at 30 to 80 min after the microinjection of the antagonist, respectively. The inhibitory effect of an antagonist was estimated as the change in antidiuretic effect caused by the second microinjection of an agonist compared with the antidiuretic effect of the first microinjection of the agonist. The antidiuretic effect of the second microinjection of ME or DAMEA was approximately equal to the effect induced by the first microinjection through the same cannula.

When the effect of systemic administration of naloxone was investigated, naloxone (10 mg/kg) was injected through the cannula inserted into the jugular vein. The interval between the intravenous injection of naloxone and the second administration of DAMEA was 20–40 min.

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-O-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 a 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 SON or PVN was confirmed by the following methods: 1) functionally, by the appearance of the antidiuretic effect by microinjecting a depolarizing dose (400 or 800 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 SON or PVN which were positively stained by the method of Gomori (23).

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

Effects of microinjection of ME and DAMEA on urine outflow: One hundred and sixty nmol ME microinjected into the SON and PVN, respectively, decreased the urine outflow within 20 min after microinjection, the urine outflow recovering toward the initial rate at approx. 2 hr (open circle in Fig. 1). Four nmol DAMEA microinjected into the nuclei induced a similar time-dependent decrease in urine outflow (open circle in Fig. 2).

Dose-effect curves for ME and DAMEA: Figure 3 demonstrates the dose-effect curves for the antidiuretic effects of ME and DAMEA when microinjected into the SON and PVN.

The ED50 values for ME microinjected into the SON and PVN were estimated to be 111 (69–178) nmol and 61 (38–99) nmol, respectively. The ED50 value for DAMEA microinjected into the SON was 1.3 (0.6–2.7) nmol and that for DAMEA microinjected into the PVN, 0.7 (0.3–1.7) nmol.

Effects of pretreatment with naloxone: The antidiuretic effect of the second microinjection of ME or DAMEA was approx. equal to that of the first microinjection, demonstrating that the effects of the opioid
peptides were reproducible. Therefore, the effects of the opioid peptides were compared before and after pretreatment with microinjection of the specific opioid antagonist naloxone.

Figure 1a shows that ME (100 nmol)-induced antidiuresis by microinjection into the SON tended to be inhibited by the pretreatment with 600 nmol naloxone. ME (60 nmol)-induced antidiuresis by microinjection into the PVN was significantly inhibited by the pretreatment with 300 nmol naloxone (Fig. 1b).

Figure 2 illustrates that the antidiuretics induced by 4 nmol DAMEA microinjected into the SON and PVN were also partially inhibited by the pretreatment with microinjection of 300 nmol naloxone.

The antidiuresis induced by microinjection of 4 nmol DAMEA into the SON and PVN were nearly completely blocked by a pretreatment with 10 mg of naloxone per kg of body weight which was injected intravenously at 20–40 min before the microinjection of DAMEA: The urine outflow which was 30±10% of the control at 30 min after the microinjection of DAMEA into the SON without the pretreatment of naloxone was 82±2% of the control with the pretreatment.
of naloxone (n=4), and the outflow that was 21±12% of the control at 30 min after the microinjection of DAMEA into the PVN without the pretreatment was 98±14% of the control with the pretreatment (n=3).

Naloxone alone microinjected or injected intravenously did not show any changes on urine outflow significantly.

Effects of pretreatment with adrenoceptor and muscarinic antagonists: Figure 4c illustrates that the antidiuresis induced by DAMEA (4 nmol) microinjected into the SON was blocked nearly completely by pretreatment with atropine (300 nmol), a muscarinic antagonist. Pretreatment with this dose of atropine did not affect antidiuretic effects induced by microinjection of norepinephrine (9). Neither pretreatment with phenoxybenzamine (80 nmol), an alpha-adrenoceptor antagonist nor timolol (100 nmol), a beta-adrenoceptor antagonist which completely blocked norepinephrine- or isoproterenol-induced antidiureses (9, 10) had any influence on the DAMEA-induced antidiuresis (Fig. 4a and b).

Similarly, the antidiuretic effects of the same dose of DAMEA microinjected into the PVN was inhibited by pretreatment with atropine (300 nmol), but not by pretreatment with these adrenoceptor antagonists (Fig. 5).

Effects of microinjection of DAMEA on urine osmotic pressure: Figure 6 compares the effects of microinjection of DAMEA on the urine osmotic pressure with the effects on the urine outflow, showing also those effects of vasopressin injected intravenously. Vasopressin (400 µU, i.v.) induced a decrease in urine outflow to approx. 30% of the control and an increase in urine osmotic pressure to approx. 340% of the control. A higher dose of vasopressin (4 mU, i.v.) decreased the urine outflow to approx. 10% of the control and increased the urine osmotic pressure to approx. 340% of the control (Fig. 6a).

By microinjection of DAMEA (4 nmol) into the SON, when the urine outflow was decreased to approx. 40% of the control, the urine osmotic pressure was increased to approx. 260% of the control (Fig. 6b). When DAMEA (4 nmol) was microinjected into the PVN, a decrease in urine outflow to approx. 30% of the control was accompanied by an increase in urine osmotic pressure to approx. 160% of the control (Fig. 6c).

Effects of microinjection of DAMEA on various visceral functions: Some visceral indices which might be expected to be responsive to the microinjection of DAMEA into the hypothalamic nuclei and which might affect the urine outflow were monitored.
During the experiments. At 30 and 40 min after microinjection of 4 nmol DAMEA, when it induced a marked decrease in the urine outflow to approx. 30% of the control, there were no significant changes in mean blood pressure, heart rate, respiration rate and rectal temperature (Table 1).

### Discussion

ME and DAMEA microinjected into the hypothalamic SON and PVN induced strong antidiuretic effects. The non-metabolizable analog caused much more potent antidiuresis than ME in dose-dependent and time-dependent manners; the ED50 value for DAMEA was approx. 1.3 nmol versus that for ME which was approx. 110 nmol when microinjected into the SON, and the ED50 values were approx. 0.7 nmol for DAMEA and 60 nmol for ME when microinjected into the PVN. The antidiureses showed slow onset and long duration, with a minimal urine outflow at approx. 20 to 40 min and a duration of approx. 2 hr after microinjection, suggesting that the effects are hormonal, most probably due to the release of ADH. They showed similar time-courses of antidiuretic effects induced by microinjection of muscarinic and adrenoceptor agonists (8-
Table 1. Effects of microinjection of 2-D-alanine-5-methionine-enkephalinamide into the supraoptic and paraventricular nuclei on various visceral functions

<table>
<thead>
<tr>
<th>Nuclei microinjected</th>
<th>n</th>
<th>Control values</th>
<th>Time after microinjection (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Urine outflow</td>
<td>3</td>
<td>0.099±0.017 ml/min</td>
<td>100</td>
</tr>
<tr>
<td>Blood pressure</td>
<td>3</td>
<td>107±10 mmHg</td>
<td>100</td>
</tr>
<tr>
<td>Heart rate</td>
<td>3</td>
<td>367±32/min</td>
<td>100</td>
</tr>
<tr>
<td>Respiration rate</td>
<td>3</td>
<td>138±2/min</td>
<td>100</td>
</tr>
<tr>
<td>Rectal temperature</td>
<td>6</td>
<td>35.7±0.3°C</td>
<td>—</td>
</tr>
<tr>
<td>Urine outflow</td>
<td>3</td>
<td>0.113±0.030 ml/min</td>
<td>100</td>
</tr>
<tr>
<td>Blood pressure</td>
<td>3</td>
<td>130±8 mmHg</td>
<td>100</td>
</tr>
<tr>
<td>Heart rate</td>
<td>3</td>
<td>333±15/min</td>
<td>100</td>
</tr>
<tr>
<td>Respiration rate</td>
<td>3</td>
<td>97±6/min</td>
<td>100</td>
</tr>
<tr>
<td>Rectal temperature</td>
<td>3</td>
<td>35.4±0.2°C</td>
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</table>

Values are the mean±S.E.M. Significance compared with the initial control values: *P<0.05.

10). Intracerebroventricular injections of ME and DAMEA also induced antidiureses and changed the plasma ADH concentration (11–17). In these cases, the drugs may diffuse to the brain tissues around the cerebroventricle and indirectly affect the vasopressinergic neurons such as the SON and PVN through intervening neurons. However, drugs microinjected into the nuclei demonstrate direct effects upon these nuclei. Therefore, it is possible that the effects of drugs administered by the two methods are different.

The antidiuretic effects of DAMEA (4 nmol) microinjected into the SON and PVN and the effect of ME (60 nmol) microinjected into the PVN were inhibited by pretreatment with naloxone (300 nmol), the effect of ME (100 nmol) microinjected into the SON also tending to be inhibited by pretreatment with naloxone (600 nmol). This suggests that antidiuretic effects induced by the opioid peptides may be mediated at least partly through an opioid receptor having a low sensitivity to naloxone blockade. Antidiuretic effects induced by DAMEA (4 nmol) microinjected into the SON and PVN were also inhibited by naloxone (10 mg/kg) injected intravenously. This dose of naloxone is approx. 30-times higher than 300 nmol naloxone microinjected. The inhibitory effect on DAMEA-induced antidiuresis of naloxone which was microinjected intravenously appears to be more potent than that of naloxone which was microinjected directly into the nuclei.

Considerable portions of the DAMEA-induced antidiuretic effects were inhibited by pretreatment with microinjection of atropine (300 nmol) which does not affect the antidiuresis induced by microinjection of norepinephrine (9), showing that some portions of the DAMEA-induced effects may also be mediated through a specific muscarinic receptor. The presence of an ACh system in the SON and PVN (4, 5), antidiuretic effects of microinjection of muscarinic agonists into the nuclei (8), inhibition (24–28) and facilitation (27, 29, 30) of presynaptic release of neurotransmitters by opioid peptides in some parts of the central nervous system and the present result suggest that the opioid peptides released ACh from the presynaptic cholinergic terminals and the released ACh induced antidiuretic effects through muscarinic receptors in the nuclei (8).

As shown in Fig. 6, the decreases in the urine outflow after intravenous injection of vasopressin or microinjection of DAMEA appeared with concomitant increases in the urine osmotic pressure, demonstrating that the antidiuretic effects induced by the opioid peptide were related to the increase in reabsorption of water. When the urine outflow decreased down to approx. 40% of the
control by the microinjection of the opioid peptide into the SON, the urine osmotic pressure increased up to approx. 260%, indicating that the decrease in urine outflow was mainly regulated by the increase in water reabsorption. However, in the PVN, when the maximal decrease in the urine outflow was approx. 30% of the control after micro-injection of the opioid peptide, the urine osmotic pressure was only approx. 160% of the control. This suggests that some unknown factors other than water reabsorption might be mediating in the effect of the opioid peptide microinjected into the PVN. The antidiuretic effect induced by DAMEA injected intracerebroventricularly was suggested not to be due to the enhanced release of ADH (13, 15). However, as discussed above, the effect of the drug injected intracerebroventricularly may be different from the effect of that microinjected into the nuclei directly. During the DAMEA-induced antidiuresis in the present study, no significant changes were observed in mean blood pressure, respiration rate and rectal temperature which might affect the urine outflow. Therefore, the antidiuretic effect of the opioid peptide is not likely due to the changes in these visceral functions.

In summary, this is the first study reporting the antidiuretic effects of ME and DAMEA microinjected into the hypothalamic SON and PVN which contain vasopressinergic magnocellular neuronal cell bodies and enkephalinergic and cholinergic nerve terminals. The mechanism of the effects of the opioid peptide may be mediated through an opioid receptor and at least partially due to ACh release which stimulates muscarinic receptors, in the nuclei.

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