Antidiuretic Effects of Morphine Microinjected into the Hypothalamic Supraoptic and Paraventricular Nuclei in a Water-Loaded and Ethanol-Anesthetized Rat

Hiromi TSUSHIMA, Mayumi MORI and Tomohiro MATSUDA
Department of Pharmacology, Nagoya City University Medical School, Kawasumi, Mizuho-ku, Nagoya 467, Japan
Accepted August 14, 1987

Abstract—Effects of morphine microinjected into the hypothalamic supraoptic (SON) and paraventricular (PVN) nuclei, which contain neurons producing and releasing antidiuretic hormone (vasopressin), on the outflow and the osmotic pressure of urine and other visceral functions were investigated in a rat which was loaded with water and anesthetized with ethanol. The opioid drug, having predominantly mu-agonist activity, 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 morphine were 19 and 9 nmol when it was microinjected into the SON and PVN, respectively. The antidiuretic effects showed slow onset and long duration, with a minimal outflow at approx. 50 min after microinjection and a return to approx. 50% of the initial control value by 1.5 hr. The morphine-induced effects were inhibited by pretreatment with naloxone or atropine, but not inhibited by 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 morphine.

The supraoptic (SON) and the paraventricular (PVN) nuclei in the hypothalamus contain cell bodies of vasopressinergic neurons which regulate reabsorption of water from the distal tubule and collecting duct of the kidney by controlling release of vasopressin (antidiuretic hormone) from the neurohypophysis into the circulation (1-6).

Immunohistochemical visualization of enkephalinergic nerve terminals in the nuclei (7-9) as well as cholinergic (10, 11) and adrenergic nerve terminals (12, 13) strongly suggested an enkephalinergic regulation of the nuclei. As intracerebroventricular administration of opioid peptides had been shown to affect the release of vasopressin (14-20), it was suggested that enkephalinergic control might play an important part in the release of vasopressin.

Very recently we found that opioid peptides having predominantly delta-agonist activity such as methionine-enkephalin and 2-D-alanine-5-methionine-enkephalaminamide directly microinjected into the SON and PVN induced potent antidiuretic effects, which were inhibited by naloxone (21).

In the present study, we report antidiuretic effects of morphine, which has primarily mu-agonist activity, by microinjecting it into the SON or PVN, which were inhibited by pretreatment with naloxone.

Materials and Methods

Animals and drugs: Male Wistar rats, weighing 280–330 g, were used. Morphine hydrochloride (Shionogi & Co., Ltd., Osaka), phenoxybenzamine hydrochloride (Nakarai Chemicals, Kyoto) and atropine sulfate (Iwaki Co., Tokyo) were purchased. Naloxone hydrochloride and timolol malate were the generous gifts of Sankyo Co. and Nippon Merck-Banyu Co., Tokyo, respectively. The
other chemicals used were of the highest analytical grade available.

Measurement of urine outflow and urine osmotic pressure: Urine outflow was measured by the method of Dicker, with some modifications (22, 23). 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's 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 drugs: A stainless steel cannula (outer diameter: 200 μm) was unilaterally stereotaxically inserted into the SON or PVN according to the atlas of König and Klippel (24). All drugs microinjected were dissolved in saline. Microinjection of 1 μl of each respective drug was performed when the urine outflow reached a constant rate of approx. 0.1 ml/min, which was within one hour after the animal was fixed in the stereotaxic instrument. 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 and 4.8 mM NaHCO3, pH 7.4) was infused at a rate of approx. 0.3 μl/min. Effects of drugs on urine outflow were measured at 10 min-intervals and expressed as a percent of the initial control outflow.

In the experiment to test the effect of pretreatment with naloxone, cholinergic and adrenoceptor antagonists, microinjection of morphine was carried out at 30–70 min after the premicroinjection, when the urine outflow had returned to the initial level. The effect of preinjection of an antagonist was estimated as a change in the antidiuretic effect caused by the injection of morphine without and with the pretreatment.

Identification of the sites of inserted cannula: 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 a depolarizing dose (800 nmol) of KCl through the cannula and 2) histochemically, by localization of the site of the tip of the cannula in a group of magnocellular cells in the SON or PVN positively stained by the method of Gomori (25).

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-0.5–290–0-III, Nihon Kohden Kogyo, Co., Tokyo) and an electrocardiograph (FD-14, Fukuda, 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.

Statistical analysis: Significance of differences between mean values was determined by Student’s t-test. 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 using the least squares method.

Results
Effect of microinjection of morphine on urine outflow: Figure 1 illustrates the effect of microinjection of morphine (8.6 nmol) into the SON on urine outflow, being compared with the effect of microinjection of 4 nmol D-2-alanine-5-methionine-enkephalinamide (DAMEA) into the nucleus.
The urine outflow usually decreased within 20–30 min after microinjection of morphine, with a minimal outflow at approx. 50 min. It returned to approx. 50% of the initial control level at 90 min (Figs. 1 and 3). When vehicle (saline) alone was microinjected into the nuclei, there was no significant change in the urine outflow (Fig. 4).

**Dose-effect curve for morphine:** Figure 2 shows the dose-effect curve for morphine...
compared with the curves for two opioid peptides (21). The approx. median effective doses (ED50) for morphine were estimated to be 19 (3–108) nmol and 9 (3–31) nmol when it was microinjected into the SON and PVN, respectively.

**Effect of microinjection of morphine on urine osmotic pressure:** Table 1 shows the maximal effects of microinjection of 8.6 nmol morphine and intravenous injection of 0.4 and 4 mU 8-arginine vasopressin (AVP) on the outflow and osmotic pressure of urine. When the urine outflow decreased to a minimal value of approx. 27 and 24% of the initial control urine outflow by microinjection of 8.6 nmol morphine into the SON and PVN, respectively, the osmotic pressure increased to a maximal value of 276 and 212% of the initial control osmotic pressure. The urine osmotic pressure was approx. 344% of

| Table 1. Effects of microinjection of morphine into the SON and PVN and intravenous injection of 8-arginine vasopressin on urine outflow and osmotic pressure |
|---|---|---|
| **Urine outflow** | **Urine osmotic pressure** |
| **Initial control values (ml/min)** | **Minimal rate (%)** | **Initial control values (mOsm/kg)** | **At minimal rate of urine outflow (%)** |
| **Vehicle** | **SON** | 3 | 0.047±0.007 | 119±8 | 283±37 | 96±3 |
| | **PVN** | 4 | 0.066±0.012 | 104±12 | 254±33 | 101±11 |
| **Morphine (8.6 nmol)** | **SON** | 4 | 0.105±0.019 | 27±12* | 208±26 | 276±53* |
| | **PVN** | 8 | 0.137±0.018 | 24±6* | 233±21 | 212±29* |
| **Vasopressin (400 µU) i.v.** | **3** | 0.089±0.014 | 31±10* | 297±20 | 344±37* |
| **(4 mU) i.v.** | **4** | 0.067±0.016 | 10±2* | 315±41 | 338±66* |

The minimal rate of urine outflow during the preceding 10 min period at 60–90 min for the control, 60–90 min for morphine, and 20–40 min for vasopressin after injection of drugs is expressed as percentage of each initial control value. The urine osmotic pressure at the minimal rate of urine outflow is expressed as percentage of each initial control value. Values are the mean±S.E.M. Significance compared with the vehicle values: *P<0.05.

Fig. 3. Inhibitory effects of preinjection of naloxone on antidiuretic effects of morphine microinjected into the SON and PVN. a: SON, b: PVN. Solutions of drugs microinjected are as in Fig. 1. Ordinate: rate of urine outflow during the preceding 10 min period expressed as percentage of the initial rate of control outflow (a: 0.088±0.013; b: 0.099±0.019 ml/min). Abscissa: time in min after the injection of 8.6 nmol morphine without (○) and with (closed symbols) preinjection of naloxone. 100 (▲), 150 (●) and 300 (■) nmol of naloxone were preinjected into the same nucleus at 30–70 min before the microinjection of morphine. Symbols are the mean±S.E.M. of 4 to 17 experiments. Significance compared with the effects of the injection of morphine without preinjection of naloxone at the same time point: *P<0.05.
control when the urine outflow was decreased to approx. 31% of the control by intravenous injection of 400 µU AVP. The increase in the osmotic pressure was approx. the same when urine outflow was decreased to approx. 10% of the control by intravenous injection of 4 mU AVP.

Inhibition of morphine-induced effect by naloxone and atropine: As shown in Fig. 3, a premicroinjection of 300 nmol naloxone potently inhibited the antidiuretic effect of morphine (8.6 nmol) microinjected into the SON or PVN. However, the inhibitory effects of pretreatment with 100 nmol naloxone

![Graphs showing effects of preinjection of various antagonists on antidiuretic effects of morphine microinjected into the SON and PVN.](image)

Fig. 4. Effects of preinjection of various antagonists on antidiuretic effects of morphine microinjected into the SON and PVN. a: SON, b: PVN. Ordinate: rate of urine outflow during the preceding 10 min period expressed as percentage of the initial rate of control outflow (a: 0.094±0.009, b: 0.115±0.010 ml/min). Abscissa: time after the injection of 8.6 nmol morphine without (○, n=13, 17) and with (closed symbols) preinjection of various antagonists. △: vehicle (n=10, 8); ●: 300 nmol atropine (n=7, 7); ▲: 80 nmol phenoxybenzamine (n=6, 5); ■: 100 nmol timolol (n=6, 5) were preinjected into the same nucleus at 30-100 min, 30-70 min and 40-70 min before the microinjection of morphine, respectively. The above numbers in the parentheses show the number of experiments when the drugs were premicroinjected into the SON and PVN, respectively. Symbols are the mean±S.E.M. Significance compared with the effects of the microinjection of morphine without preinjection of various antagonists: *P<0.05. Diuretic effect caused by morphine after pretreatment with phenoxybenzamine was compared with the value for vehicle.
were not significant when they were microinjected into both the nuclei. Simultaneous microinjection of 8.6 nmol morphine and 100 nmol naloxone into the SON showed antidiuresis (n=6), which was similar to the effect induced by 8.6 nmol morphine alone. Since the two nuclei are innervated with cholinergic (10, 11) and adrenergic (12, 13) neurons and they are stimulated by cholinergic (22) and adrenergic (26, 27) agonists, the effects of cholinergic and adrenergic agents on the morphine-induced antidiuretic effects were tested. In the both nuclei, a premicroinjection of a muscarinic antagonist, atropine, partially inhibited the morphine-induced effect without any significant effects of premicroinjection of an alpha adrenoceptor antagonist, phenoxybenzamine, and of a beta-adrenoceptor antagonist, timolol (Fig. 4). The length of the preinjection time (30 to 100 min) did not significantly affect the effects of the preinjected drugs.

**Effect of microinjection of morphine on various visceral functions:** Some visceral indices which might be expected to be responsive to the microinjection of morphine into the nuclei and which might affect the urine outflow were also monitored during the experiments. Table 2 is a summary of the results. At 50 min and at 60 min after microinjection of 8.6 nmol morphine when the urine outflow had decreased to 30–40% of control, a slight decrease in mean blood pressure was observed only at 60 min after microinjection of morphine into the SON. However, no significant changes were observed in other visceral functions such as heart rate, respiration rate and rectal temperature, when morphine was microinjected into the SON and PVN.

**Discussion**

Morphine, an opioid drug having predominantly mu-agonist activity induced potent antidiuretic effects in water-loaded rats anesthetized with ethanol when microinjected directly into the SON or PVN. The approx. ED50 values for morphine were 19 and 9 nmol when it was microinjected into the SON and PVN, respectively. As the ED50 values for 2-D-alanine-5-methionine-enkephalinamide (DAMEA) are approx. 1.3 and 0.7 nmol when microinjected into the SON and PVN (21), respectively, morphine was approx. 15 times less effective than the opioid peptide which has primarily delta agonist activity. However, morphine was several times more effective than methionine-enkephalin, the ED50 values of which are approx. 110 and 60 nmol when microinjected into the SON and PVN, respectively (21). Maximal effects induced by microinjection of morphine and the recoveries appeared more slowly than those by DAMEA: The maximal effects by morphine usually appeared at approx. 50 min after the micro-

### Table 2. Effects of microinjection of morphine into the SON and PVN on various visceral functions

<table>
<thead>
<tr>
<th>Nuclei microinjected</th>
<th>n</th>
<th>Initial control values</th>
<th>% of control time after microinjection (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>SON</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine outflow</td>
<td>4</td>
<td>0.105±0.019 ml/min</td>
<td>100</td>
</tr>
<tr>
<td>Blood pressure</td>
<td>4</td>
<td>124±6 mmHg</td>
<td>100</td>
</tr>
<tr>
<td>Heart rate</td>
<td>4</td>
<td>380±13/min</td>
<td>100</td>
</tr>
<tr>
<td>Respiration rate</td>
<td>4</td>
<td>85±10/min</td>
<td>100</td>
</tr>
<tr>
<td>Rectal temperature</td>
<td>4</td>
<td>36.1±0.3°C</td>
<td>100</td>
</tr>
<tr>
<td>PVN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine outflow</td>
<td>3</td>
<td>0.171±0.013 ml/min</td>
<td>100</td>
</tr>
<tr>
<td>Blood pressure</td>
<td>3</td>
<td>122±6 mmHg</td>
<td>100</td>
</tr>
<tr>
<td>Heart rate</td>
<td>3</td>
<td>380±23/min</td>
<td>100</td>
</tr>
<tr>
<td>Respiration rate</td>
<td>3</td>
<td>131±6/min</td>
<td>100</td>
</tr>
<tr>
<td>Rectal temperature</td>
<td>3</td>
<td>36.4±0.2°C</td>
<td>100</td>
</tr>
</tbody>
</table>

8.6 nmol of morphine was microinjected into the SON or PVN. Values (% of control) are the mean±S.E.M. Significance compared with the initial control values: *P<0.05.
injection into the nuclei, while the maximal effects by DAMEA appeared at 20–30 min (21). The effects induced by morphine recovered to approx. 50% of the initial control value at 80 min after microinjection, whereas DAMEA-induced effects recover to approx. 70 and 90% of the control value within 80 min after microinjection into the SON and PVN, respectively (21).

Along with the decreases in urine outflow after microinjections of morphine or intravenous injection of vasopressin, there were increases in urine osmotic pressure, showing 200–350% of the control value. This suggests that the decreases in urine outflow by microinjection of morphine were due to mainly the enhancement of reabsorption of water from the kidney. However, as the increase in the osmotic pressure by morphine microinjected into the PVN was a little less than the increase which was induced by intravenous injection of a similarly antidiuretic dose (400 μU) of vasopressin, factors other than vasopressin may work partly in the decreases in urine outflow induced by morphine.

The antidiuretic effects by morphine (8.6 nmol) were inhibited by pretreatment with naloxone (300 nmol) when they were microinjected into the SON or PVN. The high molar ratios of effective doses of naloxone to morphine suggest that the morphine-induced antidiuretic effects were mediated through an opioid receptor having low sensitivity to naloxone, as the effects induced by DAMEA (21).

The morphine (8.6 nmol)-induced antidiuretic effects were partially inhibited by pretreatment with microinjection of atropine (300 nmol) which does not affect the antidiuresis induced by microinjection of norepinephrine (26, 27), demonstrating that some portions of the morphine-induced effect may also be mediated through muscarinic receptors. Similar partial inhibitions by the muscarinic antagonist are observed in the antidiuretic effects induced by DAMEA (21). The presence of an ACh system in both the nuclei (10, 11), antidiuretic effects of microinjection of muscarinic agonists into the nuclei (22) and presynaptic stimulation (28, 29) and inhibition (29–32) of the release of ACh by opioid agonists in the central nervous system indicate that the present findings may be interpreted as morphine causing the release of ACh from the presynaptic cholinergic terminals and then the released ACh induces the antidiuretic effects through muscarinic receptors in the nuclei (22).

No significant changes which might affect the urine outflow were observed in various visceral functions such as mean blood pressure, heart rate, respiration rate and rectal temperature at 50 min after microinjection of morphine (8.6 nmol) when urine outflow decreased to minimal levels. Therefore, the antidiuretic effects of morphine are not likely to be due to the changes in these visceral functions. Since vasopressin has vasopressive effects when it acts on receptors (V₁) in the vascular smooth muscle, the slight decrease in mean blood pressure observed at 60 min after microinjection of morphine into the SON (Table 1) is not through direct action of released vasopressin on the V₁-receptor, but may be due to side effects on neurons regulating blood pressure.

In the present study antidiuretic effects of morphine microinjected into the SON and PVN were demonstrated in an ethanol-anesthetized rat. As there is a possibility that morphine and ethanol may interact in the nuclei on the release of vasopressin, the effects of morphine in rats anesthetized with anesthetics other than ethanol have to be investigated in order to test the possibility.

The antidiuretic effects of morphine microinjected into the SON and PVN usually appeared at approx. 20 to 30 min after microinjection. Since we had previously reported that KCl, muscarinic, adrenergic and opioid agonists microinjected into the SON and PVN also demonstrated their antidiuretic effects at approx. the same period of time after microinjection (21, 22, 26, 27), it may take 20 to 30 min in order to induce effects in the urinary system after microinjection of agonists in the nuclei in the hypothalamus. The approx. distance of diffusion of methylene blue microinjected into the SON and PVN was less than one mm. Agonists such as norepinephrine and oxotremorine which demonstrate antidiuretic effects when micro-
injected into the SON and PVN do not show any significant antidiuretic effects when they are microinjected into several sites at a distance of one mm from the nuclei (22, 26, 27). The above time lag may not be due to diffusion of morphine to any active sites outside the SON and PVN.

Good evidence for the release of vasopressin into the circulation by microinjection of morphine into the SON and PVN is to demonstrate an increase in the plasma vasopressin; in our laboratory, we are currently investigating a radioimmunoassay procedure for this purpose.

In summary, although effects of systemic administration of morphine on urine outflow which include both central and peripheral actions are controversial (33), as demonstrated in the present study, the effects of morphine directly microinjected into the SON or PVN were antidiuretic. The effects of morphine were inhibited by pretreatment with naloxone or atropine, suggesting that the morphine-induced antidiuretic effects may be mediated through an opioid receptor having low sensitivity to naloxone and partially due to ACh release which stimulates muscarinic receptors in the nuclei (22).

Acknowledgments: The authors thank Dr. Yasuhiro Hasegawa, Department of Physiology in our Medical School, for computing the ED50 values. This work was supported by research grants from the Japanese Ministry of Education, Science and Culture and from the Research Foundation for Oriental Medicine, Nagoya, Japan.

References
11 Abrahams, V.C., Koele, G.B. and Smart, P.: Histochemical demonstration of cholinesterases in the hypothalamus of the dog. J. Physiol. (Lond.) 139, 137–144 (1957)
16 Huidobro-Toro, J.P., Huidobro, F. and Croxatto, R.: Effects of β-endorphin and D-alanine enkephalinamide on urine production and urinary...


