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

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Abstract—The effect of oxotremorine, a muscarinic agonist, on urine outflow compared with the effects of other cholinergic agonists and inhibitory effects of cholinergic antagonists upon the cholinergic actions were studied by microinjecting drugs stereotaxically, unilaterally into the supraoptic (SON) or paraventricular nuclei (PVN) in the hypothalamus of the rat which was loaded with water and anesthetized with ethanol. Oxotremorine decreased the urine outflow in dose- and time-dependent manners when microinjected into these nuclei. The median effective doses (ED50) were approx. 0.3 and 0.2 nmol in SON and PVN, respectively, being much less than ED50 values for nicotine. The time course of the antidiuretic effects was relatively slow, with the minimal urine outflow at approx. 30 min and the duration of one or longer hours. The antidiuretic effects of oxotremorine in these nuclei as well as the effects of acetylcholine and nicotine were completely blocked by pretreatment with atropine. The pretreatment with hexamethonium inhibited partially the effects of nicotine, but was unable to inhibit the effects of oxotremorine and acetylcholine. The data suggest that the antidiuretic effects of cholinergic agonists in SON and PVN are predominantly mediated through a muscarinic type of acetylcholine receptor. A possible mechanism for the antidiuretic effects is discussed.

Conservation of water is one of the most important functions for animals living in an environment outside of water. Antidiuretic hormone (ADH) is the mediator of a regulatory system for the conservation of water. Vasopressin, the mammalian ADH, is known to be synthesized in the magnocellular neurons localized at the two sites in the hypothalamus: the supraoptic (SON) and paraventricular nuclei (PVN). The hormone packaged in granules is transported through the axons to the neurohypophysis. Upon excitation of the neurons, the hormone is released from the neurohypophysis into the circulation and promotes water reabsorption from distal tubules and collecting ducts of the kidney, thus inducing antidiuretic effects (1, 2).

The presence of choline acetyltransferase (3) and acetylcholinesterase (4) in these nuclei and the findings that microinjection of acetylcholine (ACh) into SON induces antidiuresis (5, 6) and iontophoretic application of ACh excites the neurons of SON (7–10) and PVN (11) have suggested that cholinergic innervation may play an important part in the control of these nuclei. However, at present it is not clear which type of ACh receptor is predominantly working in the two nuclei, muscarinic or nicotinic, or both of them, for antidiuresis of the animals.

Oxotremorine is a specific muscarinic agonist which is completely lacking in nicotinic activities (12). Its muscarinic activities are nearly equivalent to ACh with regard to the actions on the peripheral nervous system (13). This has been used as a tool in the studies of the muscarinic
receptor in the central nervous system (14).

In the present study, we investigated the effects of oxotremorine on urine outflow compared with the effects of ACh and nicotine and the effects of cholinergic antagonists upon the cholinergic effects by microinjecting into SON and PVN in order to clarify the type of ACh receptor which controls the urine outflow in the two hypothalamic sites.

Materials and Methods

Animals and drugs: Male Wistar rats, weighing 280 to 320 g, were used in this study. Oxotremorine (Aldrich Chemical Company Inc., Milwaukee, WI), acetylcholine chloride (Katayama Chemical Ind., Osaka), nicotine tartrate (Wako Pure Chemical Ind., Osaka), atropine sulfate (Iwaki, Co., Tokyo), hexamethonium bromide (Nakarai Chemical, Kyoto), and physostigmine salicylate (Sigma Chemical Co., St. Louis, MO) were purchased. The other chemicals used were the analytical grade available.

Measurement of urine outflow: Urine outflow was measured by a modified method of Dicker (15). The animals were starved overnight for approx. 17 hr, having free access to water. They 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 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.

Microinjection of drugs: A stainless cannula (outer diameter: 200 μm) was inserted stereotaxically and unilaterally into SON or PVN according to the atlas of König and Klippel (16). Drugs were dissolved in an artificial cerebrospinal fluid (CSF: NaCl, 128; KCl, 3.0; CaCl₂, 1.2; MgCl₂, 0.8; NaH₂PO₄, 0.65; NaHCO₃, 4.8 in mM, pH 7.4) and injected using a microsyringe in a volume of 1 μl through the cannula. Then 2 μl of the artificial CSF was injected at a rate of approx. 0.3 μl/min. Effects of drugs on urine outflow every 10 min were expressed as a percentage of the initial constant outflow.

In the experiments to test the effect of pretreatment with a cholinergic antagonist, a first injection of a cholinergic agonist was followed by an injection of a cholinergic antagonist, and then a second injection of the same cholinergic agonist was performed, all injections through a single cannula inserted into the nucleus. The injection of the antagonist and the second injection of the agonist were carried out at the time when the urine outflow had recovered to the initial level, usually at 30 to 60 min after micro-injection. The inhibitory effect of an antagonist was estimated as the change in antidiuretic effect caused by the second injection of an agonist as compared with the antidiuretic effect of the first injection of the agonist. In the experiment with an acetylcholinesterase inhibitor, pretreatment was performed with 0.5 nmol physostigmine instead of cholinergic antagonists before the second injection of ACh. One-half nmol physostigmine alone did not change the urine outflow in SON, but decreased it in PVN by approx. 50% of the initial urine outflow (Mori, Tsushima and Matsuda, unpublished). Therefore, the second injection of an agonist into PVN was performed after the urine outflow had recovered to the initial level.

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 a thermister probe (SR-115S, Nihon Kohden Kogyo, Co.) inserted into a tracheal catheter. These three indices were recorded simultaneously on a recticoder (RJG-3004–2, Nihon Kohden Kogyo, Co.). Rectal temperature was monitored by a thermister probe (MGA III-
Identification of the sites of inserted cannula: The position of the tip of the cannula inserted stereotaxically into the nuclei was confirmed by the following methods: 1) functionally, by the appearance of an 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 SON and PVN positively stained by the method of Gomori (17).

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

Results
Effects of microinjection of oxotremorine on urine outflow: Figures 1a and 1b show the effects of various doses of oxotremorine, a muscarinic agonist, on urine outflow as a function of time after microinjection into SON and PVN, respectively. The microinjection of the muscarinic agonist or vehicle, an artificial cerebrospinal fluid, was performed after the urine outflow reached a constant rate of 0.06–0.11 ml/min. Oxotremorine injected into PVN as well as SON caused antidiuretic effects that were dose- and time-dependent. The urine outflow decreased within 20 min, with a minimal outflow at approx. 30 min, and it recovered to the initial level at approx. 1 hr after microinjection of 0.1 to 0.4 nmol of oxotremorine. One nmol oxotremorine microinjected into SON induced a longer duration than 1 hr. Vehicle alone microinjected into these nuclei did not change the urine outflow. The urine outflow at 30 min after injection of the vehicle into SON and PVN were 110±4% (n=7) and 97±4% (n=7) of the initial urine outflows, respectively.

Dose-effect curves for various cholinergic agonists: Figure 2a demonstrates the dose-effect curve for the antidiuretic effect of oxotremorine compared with the dose-effect curves for ACh and nicotine when microinjected into SON. The median effective doses (ED50) were estimated to be approx. 0.3 nmol for oxotremorine and 120 nmol for nicotine. Although 5–80 nmol of ACh microinjected into SON significantly caused antidiuretic effects, the dose-effect curve was not sufficiently dependent on doses over 20 nmol and therefore, no estimate could be made of the ED50 value. The pretreatment by microinjection of 0.5 nmol physostigmine, an acetylcholinesterase inhibitor, did not shift the curve for ACh significantly. The dose-effect curves for the cholinergic agonists microinjected into PVN are shown in Fig. 2b. In PVN, the ED50 values were approx. 0.2, 20 and 35 nmol for oxotremorine, ACh and
Fig. 2. Dose-effect curves for the effects of microinjection of cholinergic agonists into supraoptic nucleus (a) and paraventricular nucleus (b). The abscissa indicates the doses of cholinergic agonists microinjected as a 1 μl solution in the artificial cerebrospinal fluid (pH 7.4), and the ordinate presented as percentage of the initial urine outflow shows the minimal urine outflow at 30 min after microinjection of oxotremorine and at 20 min after microinjection of acetylcholine (ACh) and nicotine. The points and brackets are the mean±S.E. from 3-15 experiments.

Fig. 3. Effects of pretreatment with atropine and hexamethonium on antidiuresis induced by microinjection of 0.4 nmol oxotremorine into supraoptic nucleus (a) and 0.2 nmol oxotremorine into paraventricular nucleus (b). ○: first injection of oxotremorine, ● second injection of oxotremorine after pretreatment of microinjection of 2 nmol atropine (in the upper frame) and 20 nmol (a) or 10 nmol (b) hexamethonium (in the lower frame). Drugs were injected as a 1 μl solution in the artificial cerebrospinal fluid (pH 7.4). The abscissa indicates time in min after the first (○) and second injections (●), and the ordinate urine outflow presented as percent of the initial urine outflow (a: 0.056±0.013; b: 0.058±0.014 ml/min). The points, brackets and the initial urine outflows are the mean±S.E. from 4 experiments. Significance compared with the effects of the first injection of oxotremorine at the same time after microinjection: *P<0.05.
nicotine, respectively. The dose-effect curve for ACh in PVN was not influenced by the pretreatment with 0.5 nmol physostigmine.

Effects of pretreatment with cholinergic antagonists: The anti-diuretic effect of the second injection of a cholinergic agonist into SON or PVN was approx. equal to that of the first injection of the agonist, showing that the effects of cholinergic agonists were reproducible. Therefore, the effects of oxotremorine were compared before and after pretreatment of microinjection of muscarinic and nicotinic antagonists in order to test the type of cholinergic receptor at which the cholinergic agonists act in the two nuclei. Figure 3a illustrates that oxotremorine (0.4 nmol)-induced antidiuresis by microinjecting into SON was completely blocked by the pretreatment with 2.0 nmol atropine. However, the effects were not inhibited by the pretreatment with 20 nmol hexamethonium. The antidiuresis induced by 0.2 nmol oxotremorine microinjected into PVN was also completely blocked by the pretreatment with 2.0 nmol atropine, but it was not affected at all by 10 nmol hexamethonium (Fig. 3b). The results of the effects of pretreatment with cholinergic antagonists on ACh- and nicotine-induced antidiuresis are summarized in Tables 1 and 2. The antidiuretic effects of 80 nmol ACh microinjected into SON or PVN were blocked nearly completely by the pretreatment with 300 nmol atropine. The antidiuretic effects, however, were not inhibited by the pretreatment with the same dose of hexamethonium. The antidiuretic effects of 100 nmol nicotine into SON were completely blocked by the pretreatment with 300 nmol atropine, and the effects of 40 nmol nicotine were inhibited partially by the same dose of hexamethonium. The effects of 40 nmol nicotine microinjected into PVN were inhibited completely by 300 nmol of atropine and partially by the same dose of hexamethonium. Three hundred nmol of atropine was not able to inhibit the antidiuretic effects of norepinephrine which induced as potent an antidiuresis as ACh (Tsushima, Mori and Matsuda, unpublished). Neither 2 nmol atropine nor 10 to 20 nmol hexamethonium alone decreased it by 30% of the initial urine outflow.

Effects of microinjection of oxotremorine on visceral indices: Some visceral indices which might be expected to be responsive to the hypothalamic cholinergic microinjections and which might affect the urine outflow were monitored during the experiments. When urine outflow was decreased to 17±5% of the initial outflow (±S.E.M., n=3) at 30 min after microinjection of 0.4 nmol oxotremorine into SON, mean blood pressure, heart rate, respiration rate and rectal temperature were 113±9 mmHg (117±4 mmHg), 350±15 /min (400±6 /min), 133±13 /min (111±14 /min), and 34.2±0.4°C (34.1±0.3°C), respectively (the values in the parentheses: the initial control values). When urine outflow was decreased to 29±10% of the initial outflow (±S.E.M., n=4) at 30 min after microinjection of 0.2 nmol oxotremorine into PVN, the rectal temperature was 34.2±0.3°C (the initial value: 34.4±0.5°C). Thus, by the microinjection of oxotremorine, the mean blood pressure, respiration rate, and rectal temperature did not change significantly, but the heart rate decreased slightly.

Discussion

The present paper is, to our knowledge, the first published demonstration of antidiuretic effects of oxotremorine microinjected into SON and PVN and of the effects of cholinergic drugs microinjected into PVN. Oxotremorine microinjected into these nuclei induced anti-diuretic effects which were dose- and time-dependent. The median effective doses (ED50) were approx. 0.2 and 0.3 nmol when microinjected into SON and PVN, respectively. The time course of the effects was relatively slow, with the minimal urine outflow at approx. 30 min and the duration of approx. 1–2 hr, suggesting that the effects are hormonal rather than through neuronal pathways.

The order of the potency inducing antidiuresis of various cholinergic agonists microinjected into SON or PVN was oxotremorine>ACh>nicotine, indicating that the muscarinic agonist is much more potent than the nicotinic agonist. The effects of oxotremorine were more than two hundred
Table 1. Effects of pretreatment with atropine and hexamethonium on antidiuresis induced by microinjection of acetylcholine (ACh) and nicotine into supraoptic nucleus

<table>
<thead>
<tr>
<th>Drugs microinjected</th>
<th>n</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 nmol ACh</td>
<td>4</td>
<td>100</td>
<td>75±10</td>
<td>15±4</td>
<td>42±12</td>
<td>78±10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>300 nmol atropine and 80 nmol ACh</td>
<td></td>
<td>100</td>
<td>88±8</td>
<td>86±10*</td>
<td>91±12*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80 nmol ACh</td>
<td>3</td>
<td>100</td>
<td>88±5</td>
<td>27±13</td>
<td>53±22</td>
<td>80±7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>300 nmol hexamethonium and 80 nmol ACh</td>
<td></td>
<td>100</td>
<td>66±10</td>
<td>18±2</td>
<td>20±7</td>
<td>48±19</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 nmol nicotine</td>
<td>4</td>
<td>100</td>
<td>100±11</td>
<td>26±8</td>
<td>13±3</td>
<td>13±4</td>
<td>20±5</td>
<td></td>
<td>42±7</td>
<td>71±4</td>
</tr>
<tr>
<td>300 nmol atropine and 100 nmol nicotine</td>
<td></td>
<td>100</td>
<td>110±6</td>
<td>110±7*</td>
<td>120±10*</td>
<td>130±14*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 nmol nicotine</td>
<td>6</td>
<td>100</td>
<td>82±5</td>
<td>22±7</td>
<td>31±14</td>
<td>42±17</td>
<td>55±16</td>
<td>63±15</td>
<td>68±13</td>
<td>74±13</td>
</tr>
<tr>
<td>300 nmol hexamethonium and 40 nmol nicotine</td>
<td></td>
<td>100</td>
<td>100±6</td>
<td>62±20</td>
<td>44±18</td>
<td>90±22</td>
<td>100±14</td>
<td>120±6*</td>
<td>120±6*</td>
<td></td>
</tr>
</tbody>
</table>

The initial control urine outflow values before microinjection of acetylcholine and nicotine were 0.11±0.01 (n=6) and 0.10±0.02 (n=7) ml/min, respectively. Atropine and hexamethonium were microinjected into supraoptic nucleus before microinjection of the agonists. All values are the mean±S.E. n: number of experiments. *P<0.05: Significance compared with the effects of the first injection of the agonists at the same time after microinjection.
Table 2. Effects of pretreatment with atropine and hexamethonium on antidiuresis induced by microinjection of acetylcholine (ACh) and nicotine into paraventricular nucleus

<table>
<thead>
<tr>
<th>Drugs microinjected</th>
<th>n</th>
<th>Time, min after microinjection of agonists</th>
<th>Urine outflow (% of initial control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>80 nmol ACh</td>
<td>3</td>
<td>100</td>
<td>73± 9</td>
</tr>
<tr>
<td>300 nmol atropine and 80 nmol ACh</td>
<td></td>
<td>100</td>
<td>100±4</td>
</tr>
<tr>
<td>80 nmol ACh</td>
<td>3</td>
<td>100</td>
<td>110±21</td>
</tr>
<tr>
<td>300 nmol hexamethonium and 80 nmol ACh</td>
<td></td>
<td>100</td>
<td>94±6</td>
</tr>
<tr>
<td>40 nmol nicotine</td>
<td>5</td>
<td>100</td>
<td>100±4</td>
</tr>
<tr>
<td>300 nmol atropine and 40 nmol nicotine</td>
<td></td>
<td>100</td>
<td>110±3</td>
</tr>
<tr>
<td>40 nmol nicotine</td>
<td>3</td>
<td>100</td>
<td>95±17</td>
</tr>
<tr>
<td>300 nmol hexamethonium and 40 nmol nicotine</td>
<td></td>
<td>100</td>
<td>100±11</td>
</tr>
</tbody>
</table>

The initial control urine outflow values before microinjection of ACh and nicotine were 0.098±0.009 (n=6) and 0.081±0.001 (n=3) ml/min, respectively. Atropine and hexamethonium were microinjected into paraventricular nucleus before microinjection of the agonists. All values are the mean±S.E. n: number of experiments. *P<0.05: Significance compared with the effects of the first injection of the agonists at the same time after microinjection.
times as potent as that of nicotine in both nuclei. Oxotremorine was more potent than ACh in these nuclei as was found in other parts of the central nervous system (18), although it has approx. equal activities to that of ACh in the peripheral nervous system (13). No shifts of the dose-effect curves for ACh by the pretreatment with physostigmine suggest that acetylcholinesterase may not significantly influence the effects of microinjected ACh in these nuclei. Studies from other laboratories using various animals and methods of experiments such as peripheral administration (19, 20), intracerebroventricular injection (21–23), microinjection into SON (5, 6), iontophoretic application on the neurosecretory cells in SON (7–10) and PVN (11), organ-culture of the hypothalomo-neurohypophyseal system (24), and neuronal culture from the SON area (25) have shown that muscarinic and/or nicotinic receptors may mediate the excitation of the neurons in SON and PVN, directly or indirectly. Our observation that nicotine by direct microinjection into SON and PVN had much less effects than oxotremorine support the idea that nicotine may excite indirectly the neurons in these nuclei.

Antidiuretic effects of oxotremorine as well as the effects of ACh and nicotine were completely blocked by atropine, a muscarinic antagonist. A nicotinic antagonist, hexamethonium, inhibited partially the effects of nicotine, but it was unable to inhibit the effects of oxotremorine and ACh. From the study using cholinergic antagonists, the antidiuretic effects of cholinergic agonists were also found to be mediated through a muscarinic type of ACh receptor in the two nuclei. The effects of nicotine were inhibited both by muscarinic and nicotinic antagonists. One possible explanation for this observation is that nicotine may release ACh through the nicotinic receptor from presynaptic cholinergic terminals on the neurons in the nuclei as demonstrated recently in a synaptosomal preparation of the myenteric plexus (26) or through the nicotinic receptor on any of the short cholinergic interneurons, and the released ACh may excite the neurons through the muscarinic receptor. This suggestion, however, must await further substantiation.

It is interesting that PVN which are located at a different site in the hypothalamus and also have ADH-secreting neurons responded to various cholinergic agents in a pattern similar to that seen in the SON.

The ED50 values for depolarizing the neurons by microinjecting of high KCl doses in SON and PVN were 846±13 and 320±78 nmol, respectively (Tsushima, Mori and Matsuda, unpublished). Since the ED50 values for oxotremorine were 0.3 and 0.2 nmol when microinjected into SON and PVN, respectively, oxotremorine was a thousand-fold more potent than KCl. The median effective concentration for oxotremorine can be roughly estimated to be less than 30 μM, provided that central neurons are depolarized by KCl at approx. 30 mM concentration. In the present experiments, animals were loaded with water and ethanol in order to keep the urine outflow constant and at a measurable flow rate. As a lower extracellular osmotic pressure with water-loading (27) and ethanol-anesthesia are known to inhibit the neurons in these nuclei (15, 28), the true effective concentrations for oxotremorine in unanesthetized animals without water- and ethanol-loading could be lower than the apparent values estimated above.

The observation that no significant antidiuretic responses were found by microinjecting oxotremorine into several sites at a distance of 1 mm from SON suggested that the distance of simple diffusion of microinjected oxotremorine was less than 1 mm. Other visceral indices which might be expected to be responsive to hypothalamic cholinergic microinjections and might affect the urine outflow were monitored during the experiments. When oxotremorine microinjected into SON caused maximal antidiuretic effects, mean blood pressure, respiration rate and rectal temperature demonstrated no significant changes. It is suggested that the antidiuretic effects of oxotremorine may not be due to the changes in these indices.

Since our targets of microinjections were SON and PVN which contain neurons releasing ADH, slow onset and long duration of the antidiuretic effects strongly suggest
that the effects are hormonal, most probably due to the release of ADH. We are measuring the concentration of the plasma ADH by radioimmunoassay method and the osmotic clearance, which demonstrate the release of ADH.

In conclusion, the results presented suggest that antidiuresis induced by microinjection of cholinergic agonists into PVN as well as SON may be mediated mainly through a muscarinic type of ACh receptor, although the precise mechanism of the antidiuresis is not yet determined at present.

References

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