Antidiuretic Effects of ATP Induced by Microinjection into the Hypothalamic Supraoptic Nucleus in Water-Loaded and Ethanol-Anesthetized Rats

Mayumi Mori, Hiromi Tsushima and Tomohiro Matsuda

Department of Pharmacology, Nagoya City University Medical School, Kawasumi, Mizuho-ku, Nagoya 467, Japan

Received July 13, 1994 Accepted September 17, 1994

ABSTRACT—The effects of microinjection of purinoceptor agonists into the hypothalamic supraoptic nucleus (SON) on urination were examined in water-loaded and ethanol-anesthetized rats. Adenosine triphosphate (ATP), but neither adenosine diphosphate (ADP), adenosine monophosphate (AMP) nor adenosine, concentration-dependently decreased the urine outflow with concomitant increase in the urine osmotic pressure. The ED50 value for ATP was approx. 60 nmol. The antidiuretic effect of ATP was blocked either by prior injection of theophylline (an antagonist of the P1-type purinoceptor) or by intravenous administration of an arginine vasopressin (AVP)-receptor antagonist, d(CH2)5-D-Tyr(Et)-valine-arginine-vasopressin (VAVP). These results suggest that ATP injected into the SON has antidiuretic effects due to release of AVP through an activation of theophylline-sensitive purinoceptors.

Keywords: ATP, Antidiuresis, Supraoptic nucleus, Vasopressin

The hypothalamic supraoptic nucleus (SON), as well as the paraventricular nucleus (PVN), contains cell bodies of the magnocellular vasopressergic neurons that synthesize and release arginine vasopressin (AVP). Stimulation of the AVP neurons by individual neurotransmitters and neuromodulators can induce the release of AVP, eventually causing antidiuretic effects through an activation of renal AVP (V2) receptors (1, 2). Electrophysiological and immunohistochemical examinations (3–5) indicate that the AVP neurons receive inputs from cholinergic and adrenergic neurons.

Using the microinjection technique, the cholinergic (muscarinic) and adrenergic (α- and β-subtypes) receptors in both the SON and PVN are shown to be involved in the urine outflow (6–8). It is known that adenosine triphosphate (ATP) is co-localized with acetylcholine (ACh) and noradrenaline (NA) in cholinergic and adrenergic nerve terminals, respectively, and this purine is also co-released with these neurotransmitters (9, 10). It is, thus, expected that ATP may act as a neurotransmitter or neuromodulator in the peripheral and central nervous system (11–13). We previously found that ATP injected into the PVN induced antidiuretic effects through an activation of P2-purinoceptors (14). Therefore, such may also be the case in the SON, and ATP may act on the AVP neurons through an activation of a specific receptor. ATP will then modulate cholinergic and adrenergic agonists-induced antidiureses.

In the present studies, we measured the effects of ATP injection into the SON on the urine outflows, urine osmotic pressure and the visceral functions. The effects of antagonist pretreatment of the AVP receptor, purinoceptor and muscarinic receptor on these ATP actions were also examined, the objective being to determine the possible involvement of these individual receptors on the effect of ATP. The effects of some purine compounds (ADP, AMP and adenosine) on urine outflows were also compared with those of ATP to characterize the purinoceptor subtype involved in the effects of ATP.

MATERIALS AND METHODS

Drugs

Adenosine 5′-triphosphate sodium salt (ATP), adenosine 5′-diphosphate sodium salt (ADP), adenosine 5′-monophosphate sodium salt (AMP), adenosine hemi-sulfate, atropine sulfate (Sigma Chemical Co., St. Louis, MO, USA) and theophylline (Wako Pure Chemical Indus-

\(^{\text{deceased}}\)
tries, Ltd., Osaka) were used. The AVP 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 Pro-
Fessor K.G. Hofbauer (Department of Pharmacology, 
Heidelberg University, Germany and Cardiovascular 
Research Department, Pharmaceutical Division, Ciba-
Geigy, Ltd., Basel, Switzerland). The other reagents used 
were of the highest analytical grade available.

Measurement of outflow and osmotic pressure of urine

The urine outflow was measured by Dicker’s method, 
with some modifications (6, 15). Male Wistar rats, weigh-
ing 280–320 g, were starved overnight for approx. 17 hr 
but allowed free access to water. The animals were loaded 
orally through a catheter with a volume of water equiva-
 lent to 5% of their body weight. Then they were anesthe-
tized by oral administration of a volume of 12% ethanol, 
equivalent to 5% of their body weight. Cannulae were in-
serted into the trachea, bladder, and external jugular 
vein. The rats were fixed in a stereotaxic instrument (Taka-
hashi Co., Tokyo). The ethanol-anesthetized rats did not 
move during the experiments. Drops of urine, flowing 
from the urinary cannula, were counted by a photoelec-
tric 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. The Fiske Osmometer (Model G-
62; Fiske Associates, Inc., Uxbridge, MA, USA) was used 
to measure urine osmotic pressure by the freezing point 
depression method. Urine, which was collected by a 
catheter in the bladder and then diluted to 10-fold by dis-
tilled water, was used (minimum volume: 200 μ1) for mea-
suring osmotic pressure. The initial control osmotic pres-
sure was 278 ± 34 mOsm. Vehicle alone, when injected 
into the SON, changed neither the urine outflow nor the 
urine osmotic pressure.

Injection of drugs

A stainless steel cannula (outer diameter: 200 μm) was 
unilaterally inserted stereotaxically into the SON (A, 
6.28; L, 1.3; H, 8.8 mm) according to the atlas of König 
and Klippel (16). The position of the tip of the cannula 
within the SON was histochernically confirmed by locali-
ization in a group of magnocellular cells, positively stained 
by the method of Gomori (17). Injection of a 1-μ1 volume 
of the effective doses of antidiuretic agonists, such as oxo-
tremorine (0.4 nmol) or NA (80 nmol), at a distance of 
1 mm outside the SON did not induce any significant 
effects on the urine outflow. Therefore, the spread of the 
drug to sites in the brain outside the SON by injection of 

A 1-μ1 volume of the drug was estimated to be less than ap-
prox. 1 mm. The pH of the solutions containing adenine 
nucleotides and adenosine were adjusted to approx. 7.0 
by adding NaOH. After the urine outflow had reached a 
constant rate of 0.078–0.133 ml/min, 1 μ1 of the drug sol-
ution was injected through a microsyringe connected to 
the cannula. Then 2 μ1 of an artificial cerebrospinal fluid 
(C.S.F.: 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 μ1/min for 
10 min (total of 3 μ1). The outflow of urine was measured 
every 10 min before and after the injection of drugs, and 
it was expressed as a percentage of the control outflow. In 
the experiments to test the effect of pretreatment with 
theophylline or atropine, ATP, theophylline or atropine, 
and ATP were injected into the SON in turn. All the injec-
tions were performed after the urine outflow had re-
covered to the initial level.

Pretreatment with an AVP antagonist

The effect of pretreatment with d(CH2)5-D-Tyr(Et)- 
VAVP (an AVP receptor antagonist) was tested in order 
to check the release of AVP. The effect of microinjection 
of ATP into the SON was first observed, and then the 
AVP antagonist (50 μg/kg) was applied intravenously 
through a cannula in the external jugular vein 40 min 
before the second injection of ATP. The urine outflows 
were not detectably changed after injection of the AVP 
antagonist. The inhibitory effects of the antagonist were 
estimated as described above for the inhibitory effects of 
theophylline or atropine.

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, Co., Tokyo) and an electrocardiograph 
(FD-14; Fukuda, Tokyo). Respiration rate was measured 
by a thermistor 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 thermistor probe (MGA III-219, Nihon 
Kohden Kogyo, Co.) inserted into the rectum.

Statistical analyses

Student's t-test was used for the statistical analysis of 
paired tests. Differences were considered significant at 
P < 0.05. The ED50 values and 95% confidence limit of the 
ED50 values for ATP were calculated from dose-response 
curves (Fig. 2), drawn by the least squares method.
RESULTS

Effects of injection of ATP on the urine outflow

Figure 1 shows the effect of single injections of ATP (30–150 nmol) into the SON on the urine outflow. ATP decreased the urine outflow in a dose-dependent manner. The injection of ATP induced a decrease in urine outflow; the decrease reached the maximal at 20 min after injection of ATP at any concentration of ATP. The effects of ATP injection on the urine outflow were reversible, and the initial level of urine outflow was produced at approx. 1 hr after the injection of lower concentrations of ATP (<100 nmol). The injection of 150 nmol ATP caused a slower recovery of urine outflow, and the recovery remained incomplete even at 100 min after the injection.

Figure 2 shows the dose-response relationship of the peak value of urine outflow inhibited by a single injection of ATP into the SON. The relationship was linear, and the median effective dose (ED50 value) estimated from the relationship was 56 nmol (values ranged from 38 to 84 nmol, n = 3–8) nmol.

Effects of injection of ATP on the urine osmotic pressure

Figure 3 shows the effects of injection of ATP (100 nmol) into the SON on both the urine osmotic pressure and the urine outflow measured at 20 and 60 min after the injection (n = 6). At 20 min after the injection of ATP, the osmotic pressure was increased to 210%, and the urine outflow was decreased to 34 ± 7% of the control. At 60 min, when the urine outflow had recovered to close to the initial level (63 ± 11%), osmotic pressure was nearly recovered to the initial control level (135 ± 24%).

---

**Fig. 1.** Effects of various doses of ATP on the urine outflow, as a function of time, after the injection into the SON. Abscissa: time in min after injection. Ordinate: the urine outflow presented as a percent of the initial control urine outflow during the 10 min before the injection of ATP (○=30 nmol: 0.135 ± 0.019 ml/min, △=50 nmol: 0.089 ± 0.018 ml/min, ×=100 nmol: 0.111 ± 0.015 ml/min, □=150 nmol: 0.123 ± 0.044 ml/min). Symbols that represent the urine outflow during the immediately preceding 10-min period are the means ± S.E. from 3–11 experiments.

**Fig. 2.** Dose-response curve of the antidiuretic effect of ATP into the SON. Abscissa: the dose of ATP. Ordinate: the minimal urine outflow at 20 min after the injection, expressed as a percent of the initial urine outflow during 10 min before the injection of ATP. Symbols represent the mean ± S.E. from 3–11 experiments.

**Fig. 3.** Effects of ATP, injected into SON on the urine outflow and osmotic pressure. Closed column: urine outflow at 20 min or 60 min after the injection, expressed as a percent of the initial control during 10 min before the injection of ATP (0.102 ± 0.022 ml/min); hatched column: urine osmotic pressure at 20 min or 60 min after the injection, expressed as a percent of the initial control values (278 ± 34 mOsm). Abscissa: vehicle or ATP (100 nmol) injected into the SON and time after the injections. Columns represent the means ± S.E. of 6 experiments. Significance compared with the value for the injection of vehicle: *P < 0.05.
Inhibition of ATP-induced antidiuretic effect by intravenous injection of AVP antagonist

Intravenous administration of d(CH2)5-D-Tyr(Et)VAVP (50 μg/kg), an AVP-receptor antagonist, by itself did not significantly change the urine outflow (129±5, 137±11, 108±3, 103±4, 100±4 and 104±2% at 10, 20, 30, 40, 50 and 60 min after intravenous injections of the AVP-receptor antagonist, respectively, n=3), but completely blocked the antidiuretic effect of ATP (100 nmol) injected into the SON (Fig. 4).

Effects of pretreatment with theophylline or atropine on the antidiuretic effect of ATP

Theophylline (50 nmol, a P1-purinoceptor antagonist), injected into the SON significantly inhibited the antidiuretic effect of ATP (100 nmol) (Fig. 5). Atropine (300 nmol), a muscarinic receptor antagonist when injected into the SON, also inhibited the effect of ATP (100 nmol) (Fig. 6). Neither 50 nmol theophylline nor 300 nmol atropine alone changed the urine outflow. The urine outflows, expressed as a percentage of the control, at 10, 20 and 30 min after the injection of theophylline into the SON were 103±5, 96±7 and 93±4, respectively (n=3). The urine outflows at 10, 20 and 30 min after the injection of atropine into the SON were 83±19, 94±29 and 114±28% of the control, respectively (n=5).

Effects of injection of various adenine nucleotides and adenosine

Figure 7 shows the effects of microinjection of ADP, AMP and adenosine (100 nmol) into the SON on the urine outflow measured at 20–30 min after the injections. None of the three caused a significant change in the urine outflow.
Cardiovascular and respiratory effects of the injection of ATP

The effects of injection of ATP into the SON were observed on blood pressure, heart rate, respiration rate and rectal temperature. The injection of ATP (100 nmol) into the SON had no detectable effect on these parameters. The blood pressure, heart rate and respiration rate were 112±17 (116±9) mmHg, 420±24 (425±13) /min and 87±13 (81±9) /min at 20 min after injection of ATP, respectively, when the urine outflow was 37±10% of the initial urine outflow (100%) (each control value during the 10 min before the injection is indicated in parentheses).

Fig. 7. Effects of injection of adenine nucleotides and adenosine on the urine outflow, compared with the effects of ATP. Ordinate: minimal urine outflow at 20 min after the injection, expressed as a percent of the initial control during 10 min before the injection of drug (CSF: 0.113±0.015 ml/min, ATP: 0.111±0.015 ml/min, ADP: 0.122±0.024 ml/min, AMP: 0.133±0.032 ml/min, adenosine: 0.116±0.021 ml/min). Abscissa: CSF or drugs (100 nmol) injected into the SON. The number of experiments is indicated in the parentheses. Columns represent the means±S.E. of experiments. Significance compared with the values for injection of CSF: *P<0.05.

DISCUSSION

The present experiments showed that injection of ATP into the SON caused a marked decrease in urine outflow with a concomitant increase in the urine osmotic pressure, in a concentration-dependent manner. These effects of ATP on urine outflow appeared slowly, and the time-courses were comparable to those induced by cholinergic (6) or adrenergic agonists injected into either the SON or PVN (7, 8) and also those for ATP injected into the PVN (14). The ATP-induced increase in urine osmotic pressure indicated that the decrease in urine outflow by ATP implied an antidiuretic effect probably due to a release of AVP. No significant change was found in the cardiovascular and respiratory parameters following the injection of ATP into the SON. Following the injection of ATP into the SON, the urine osmotic pressure was increased, and the antidiuretic effect of ATP was completely blocked by an intravenous injection of d(CH2)5-D-Tyr(Et)AVP, an AVP-receptor antagonist (50 μg/kg). The small distance of the diffusion of drugs microinjected into the SON (less than 1 mm) suggests that the action site of ATP is inside the SON. These results suggest that microinjected ATP activates the magnocellular cells and then elicits a release of AVP from the neurohypophysis into the circulation, causing an antidiuretic effect through an activation of the renal AVP (V2) receptors.

The results suggest that the antidiuretic effect of ATP injected into the SON may be mediated through an activation of P1-purinoceptors since the antidiuretic effect of ATP (100 nmol) was antagonized by pretreatment with theophylline (50 nmol), a P1-purinoceptor antagonist (12). On the other hand, the absence of any effect by ADP, AMP or adenosine injected into the SON on the urine outflow suggests that the effects of ATP are mediated mainly through an activation of P2-purinoceptors (11, 18, 19). These results also contrasts with those observed in the PVN, in which microinjection of ATP causes a more potent antidiuretic effect than that of adenosine and is not antagonized by theophylline (14). The purinoceptor involved in the antidiuretic effect of ATP in the SON may not be a typical P1- or P2-subtype. Further studies using a P2-purinoceptor selective antagonist are needed to characterize the subtype of purinoceptor in the SON.

The antidiuretic effect of ATP was inhibited by the pre-injection of atropine into the SON, suggesting that the effect may be mediated through an activation of muscarinic receptors. We previously reported that oxotremorine injected into the SON causes an antidiuretic effect, possibly through elevated AVP release (6). The presence of choline acetyltransferase and acetylcholinesterase (4, 5) in the SON and the findings that iontophoretic application of ACh excites the vasopressinergic neurons of the SON (20, 21) suggested that the cholinergic neurons may play important roles in the AVP release. It has been recently reported that ATP potentiates a release of neurotransmitters such as ACh (22, 23), NA (24) and glutamate (25). Therefore, it is speculated that ATP microinjected into the SON may activate purinoceptors on the presynaptic terminals of cholinergic neurons, and enhance the release of ACh, and thus elevate the release of AVP through an activation of muscarinic receptors.

In conclusion, ATP injected into the SON induced a
potent antidiuretic effect. The results suggest that ATP stimulates a theophylline-sensitive purinoceptor, promotes the release of AVP and then induces the antidiuretic effect through a mediation of renal AVP (V₂) receptors. It is also suggested that cholinergic neurons in the SON may participate in this ATP-induced antidiuretic effect.

Acknowledgments
We would like to thank Professor K.G. Hofbauer (Ciba-Geigy Ltd., Basel, Switzerland) for providing the AVP-receptor antagonist. We thank Professor Dr. H. Suzuki, Department of Physiology, and Professor Dr. T. Itoh, Department of Pharmacology, Nagoya City University Medical School for their advice during the preparation of this manuscript.

REFERENCES
1 Zimmerman EA: Oxytocin, vasopressin and neurophysins. In Brain Peptides, Edited by Kreiger DT, Brownstein MJ and Martin JB, pp 597–611, Wiley, New York (1983)
2 Manning M and Sawer WH: Development of selective agonists and antagonists of vasopressin and oxytocin. In Vasopressin, Edited by Schrier RW, pp 131–144, Raven, New York (1985)
3 McNell TH and Sladek JR Jr: Simultaneous monoamine histofluorescence and neuropeptide immunocytochemistry. II. Correlative distribution of catecholamine varicosities and magnocellular neurosecretory neurons in rat supraoptic and paraventricular nuclei. J Comp Neurol 193, 1023–1033 (1980)
4 Kimura H, MacGeer PL, Reng JH and MacGeer EG: The central cholinergic system studies by choline acetyl-transferase immunohistochemistry in the cat. J Comp Neurol 200, 151–201 (1981)
5 Swanson LW and Sawchenko PE: Hypothalamic integration: organization of the paraventricular and supraoptic nuclei. Annu Rev Neurosci 6, 269–324 (1983)
6 Mori M, Tsushima H and Matsuda T: Antidiuretic effects of oxytocin microinjected into the hypothalamic paraventricular nucleus of water-loaded, ethanol-anesthetized rats. Neuropharmacology 31, 585–592 (1992)
7 Dicker SE: A method for the assay of very small amounts of antidiuretic activity with a note on the antidiuretic titre of rat’s blood. J Physiol (Lond) 122, 149–157 (1953)
8 König JFR and Klippel RA: The Rat Brain, a Stereotaxic Atlas of the Forebrain and Lower Parts of the Brain Stem. The Williams and Wilkins, Baltimore (1963)
9 Bargman W: Über die neurosekretorisch Verknüpfung von Hypothalamus und Neurohypophyse. Z Zellforsch 34, 610–634 (1949) (in German)
10 Paton DM and Taaerum T: A comparison of P1 and P2-purinoceptors. Ann NY Acad Sci 603, 165–171 (1990)
11 Olsson RA and Pearson JD: Cardiovascular purinoceptors. Physiol Rev 70, 761–845 (1990)
12 Dreifuss JJ and Kelly JS: The activity of identified supraoptic neurons and their response to acetylcholine applied by iontophoresis. J Physiol (Lond) 220, 105–118 (1972)
13 Barker JL, Crayton JW and Nicoll RA: Supraoptic neurosecretory cells: Adrenergic and cholinergic sensitivity. Science 171, 208–210 (1971)
14 Vizi ES, Sperlagh B and Lajtha A: Evidence for a presynaptic P2x-purinoceptor involved in facilitation of acetylcholine release. Ann NY Acad Sci 603, 500–502 (1990)
15 Fu W-M and Poo M-M: ATP potentiates spontaneous transmitter release at developing neuromuscular synapses. Neuron 6, 837–843 (1991)
16 Inoue K, Nakazawa K, Fujimori K and Takenaka A: Extracellular adenosine 5’-triphosphate-evoked norepinephrine secretion not relating to voltage-gated Ca channels in pheochromocytoma PC12 cells. Neurosci Lett 106, 294–299 (1989)
17 Inoue K, Nakazawa K, Fujimori K, Watano T and Takenaka A: Extracellular adenosine 5’-triphosphate-evoked glutamate release in cultured hippocampal neurons. Neurosci Lett 134, 215–218 (1992)