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Effects of Arginine-Vasopressin Fragment 4–9 on Rodent Cholinergic Systems

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TANABE, S., Y. SHISHIDO, Y. NAKAYAMA, M. FURUSHIRO, S. HASHIMOTO, T. TERASAKI, G. TSUJIMOTO AND T. YOKOKURA. *Effects of arginine-vasopressin fragment 4–9 on rodent cholinergic systems.* PHARMACOL BIOCHEM BEHAV **63**(4) 549–553, 1999.—Arginine-vasopressin fragment 4–9 (AVP_{4-9}) has been demonstrated in animal studies to facilitate learning and memory. To clarify the mechanisms of this facilitation, we focused on the effects of AVP_{4-9} on rodent cholinergic systems. AVP_{4-9} (0.1 μM) enhanced the basal and the high-potassium–evoked acetylcholine (ACh) release from rat hippocampal slices (122.4 and 120.0% of control, respectively) in the presence of 1.3 mM calcium (physiological level) at 60 min after the incubation at 37°C. The AVP_{4-9}-stimulated basal ACh release was inhibited by a V_1-selective antagonist ([(β-mercapto-β,β-cyclopentamethylene propionic acid)^1, *O*-methyl-Tyr^2, Arg^8] vasopressin), but not by a V_2-selective antagonist ([adamantaneacetyl^1, *O*-ethyl-D-Tyr^2, Val^4, aminobutyryl^6, Arg^{8,9}]-vasopressin). In addition, AVP_{4-9} did not affect the basal ACh release under the calcium-free condition at 37°C or in the presence of 1.3 mM calcium at 4°C. However, AVP_{4-9} facilitated the passive-avoidance response of scopolamine (a cholinergic blocker)-induced memory-deficient mice. These findings demonstrate that AVP_{4-9} stimulates ACh release via mediation by V_1-like vasopressin receptors, and shows dependence on calcium ion and temperature. The results also suggest that the mechanism of the facilitative effects of AVP_{4-9} on learning and memory consist of the observed stimulation of cholinergic systems and other parallel pathways that would not be inhibited by cholinergic blocking. © 1999 Elsevier Science Inc.

Arginine-vasopressin fragment 4–9 Passive avoidance Acetylcholine release Hippocampal slice

ARGININE-VASOPRESSIN fragment 4–9 (AVP_{4-9}), a major proteolytic metabolite of arginine-vasopressin (AVP) (4,5), is known as a potent memory facilitative peptide (9,14,23). Based on this potent effect, AVP_{4-9} analogues (15,28) and peptidase inhibitors designed to protect AVP_{4-9} from further disposition (21,29) have recently been developed for potential clinical use for dementia. However, the mechanisms of the facilitative effects of AVP_{4-9} on learning and memory have not yet been clarified. The identification of the mechanisms will contribute to not only the basic studies but also the clinical uses of AVP_{4-9} and related compounds for dementia.

The important roles of cholinergic systems in learning and memory processes have been widely accepted (2). It was recently suggested that nerve growth factor (NGF) and thyrotropine-releasing hormone (TRH) affect learning and memory via stimulation of the acetylcholine (ACh) release in the central nervous system (CNS) (16,24,26). However, little attention has been given to the cholinergic effect of AVP_{4-9}.

The present study was thus conducted to clarify the mechanisms of the facilitative effects of AVP_{4-9} on learning and memory from the aspect of the effects of this peptide on the cholinergic systems with an *in vitro* investigation and an *in vivo* behavioral experiment.

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METHOD

Peptides

AVP_{4-9} ([pGlu[4], Cyt[6], Arg[8]]-vasopressin fragment 4–9) and [adamantaneacetyl[1], *O*-ethyl-D-Tyr[2], Val[4], aminobutyryl[6], Arg[8,9]]-vasopressin (a V_2-selective receptor antagonist) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). [(β-mercapto-β, β-cyclopentamethylene propionic acid)[1], *O*-methyl-Tyr[2], Arg[8]]-vasopressin (a V_1-selective receptor antagonist) was obtained from Peninsula Laboratories (Belmont, CA).

Acetylcholine Release From Rat Hippocampal Slices

The ACh release from the CNS was examined with hippocampal slices from 7- to 9-week-old male Wistar rats (CLEA Japan, Tokyo, Japan). Rats were decapitated, and the brains were rapidly and carefully placed in chilled (4°C) standard incubation buffer (in mM: 120 NaCl, 3.5 KCl, 1.3 $CaCl_2$, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 25 $NaHCO_3$, 10 glucose, 5×10^{-2} physostigmine sulfate (Wako Pure Chemical Industries, Osaka, Japan), 1×10^{-4} atropine sulfate (Wako), and 2×10^{-3} choline (Ch) chloride (Sigma), insufflated with 95% O_2 and 5% CO_2) or Ca^{2+}-free incubation buffer (in the Ca^{2+}-free study; the $CaCl_2$ of the standard incubation buffer was replaced with equimolar $MgCl_2$, and 1 mM EGTA was added). Physostigmine sulfate and atropine sulfate were added to each incubation buffer to avoid the change of the disposition of ACh by choline esterase and the muscarinic autoinhibition of ACh release, respectively (25). The dorsal cerebrums were carefully cut into 300-μm slices with a McIlwain tissue chopper (Mickle Laboratory Engineering, Surrey, UK). Preincubation and incubation were carried out with a slight modification of the method of Silva *et al.* (22). Four dorsal hippocampal slices were carefully placed in the net of a Netwell® (74-μm Mesh, Costar, Cambridge, MA) to allow free contact with an excess volume of standard incubation buffer or Ca^{2+}-free incubation buffer, and the slices were then preincubated in 95% O_2 and 5% CO_2 at 4 or 37°C for 10 min. The slices were then transferred with the nets to incubation wells (12-well cell culture dishes) and incubated with or without peptides in 4.0 ml standard incubation buffer, high-K^+ incubation buffer (in the high-K^+ study; the 120 mM NaCl and 3.5 mM KCl of the standard incubation buffer were replaced with 93.5 mM NaCl and 30 mM KCl, respectively), or Ca^{2+}-free incubation buffer in 95% O_2 and 5% CO_2 at 4 or 37°C for 60 min. In the experiments concerning the antagonists, Ca^{2+} and temperature dependency, 20 μM V_1- or V_2-selective receptor antagonists and 0.1 μM AVP_{4-9} were used.

ACh Analysis

The incubation supernatant (600 μl) was recovered at 60 min after the start of the incubation. Then, internal standard solution (200 μl) [1.0 μM ethylhomocholine (EHC) dissolved in 100 mM $NaH_2PO_4 \cdot 2H_2O$) containing 100 μM physostigmine sulfate and 40 mM EDTA-2Na (pH 3.5)] was added, followed by centrifugation ($10{,}000 \times g$) to remove debris at 4°C for 10 min, and 200 μl aliquots of centrifugation supernatants were applied on a high-performance liquid chromatography electrochemical detector (HPLC-ECD) as follows. HPLC-ECD was carried out with a slightly modified version of the method of Damsma *et al.* (7). For separation, an AC-GEL column (6 × 150 mm, EICOM, Kyoto, Japan) was used. An enzymatic postcolumn reactor (AC-ENZ, EICOM) containing immobilized ACh esterase (EC3.1.1.7) and Ch oxidase (EC 1.1.3.17) converted ACh, Ch, and EHC to hydrogen peroxide. The generated hydrogen peroxide was detected with an ECD (ECD-300, EICOM) by a platinum electrode (450 mV) (WE-PT, EICOM). The temperature of the columns was maintained at 35°C. The columns were eluted with 0.1 M phosphate buffer containing 65 mg/l tetramethylammonium chloride and 200 mg/l sodium l-decane-sulfonate (pH 8.5) at a flow rate of 1.0 ml/min.

Passive-Avoidance Test

The effects of AVP_{4-9} on learning and memory were determined by a slightly modified version of the method of Ader *et al.* (1) with 5-week-old male ICR mice (CLEA Japan), using a step-through apparatus equipped with a shock generator (SGS-001; Muromachi Kikai, Tokyo). For the learning trial, each mouse was placed in an illuminated box attached to a large dark compartment and allowed to enter the dark compartment. As soon as the mouse entered the dark compartment, an unavoidable scrambled foot shock (0.5 mA, 50 Hz) was delivered through the grid floor until the mouse escaped from the dark compartment to the illuminated compartment. Twenty-four hours after the learning trial, each mouse was placed in the illuminated compartment, and the passive avoidance latency (max.; 300 s) was recorded (retention test).

Treatment of Mice for the Passive-Avoidance Test

AVP_{4-9} and scopolamine (Sigma) were dissolved in saline before use and administered at 1 ml/kg. Each concentration of AVP_{4-9} was subcutaneously (sc) administered and scopolamine hydrobromide (1.5 mg/kg) was intraperitoneally (ip) administered, at 60 and 30 min prior to the learning trial, respectively. In the vehicle-treated mice, the same volume of saline was substituted for AVP_{4-9} and scopolamine. As a control, mice received scopolamine and saline as a substitute for AVP_{4-9}.

Data Analysis

The released ACh level was analyzed by the Kruskal–Wallis test and subsequently with Dunnett's test. Differences in the passive-avoidance response were analyzed by Wilcoxon's test or the Kruskal–Wallis and subsequently with the Schally–Williams test.

RESULTS

Effects of AVP_{4-9} on the Basal ACh Release From Rat Hippocampal Slices

The basal ACh release from the control slices in the presence of 1.3 mM Ca^{2+} at 37°C was increased in a dose-dependent manner. The basal ACh release (control; 35.5 ± 2.4 pmol/mg protein) was significantly elevated by AVP_{4-9} at the concentrations of 0.1 and 1 μM (43.5 ± 2.0 and 47.4 ± 2.5 pmol/mg protein, 122.4 ± 5.5 and 133.4 ± 6.9% of control, respectively) (Fig. 1).

Effects of V_1- and V_2-Selective Receptor Antagonists on the AVP_{4-9}-Stimulated ACh Release From Rat Hippocampal Slices

As shown in Fig. 2, both the V_1- and V_2-selective receptor antagonists showed no significant effects on the basal ACh release compared with the control slices in the presence of 1.3 mM Ca^{2+} at 37°C. The AVP_{4-9}-stimulated ACh release was

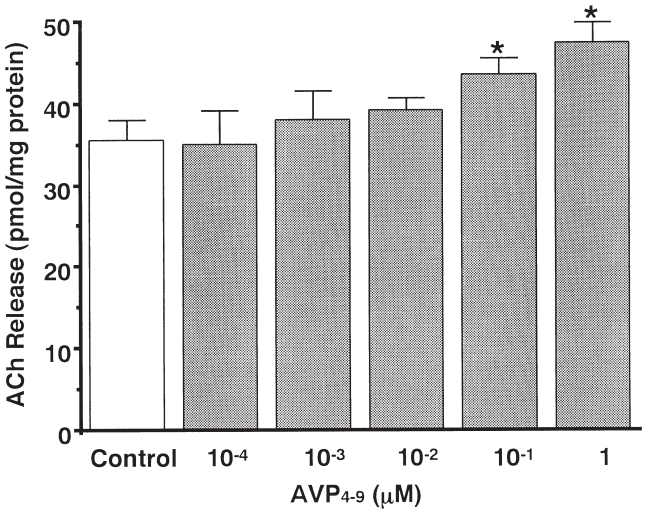

FIG. 1. Effects of AVP_{4-9} on ACh release from rat hippocampal slices. Rat dorsal hippocampal slices were incubated with the indicated concentration of AVP_{4-9} in the presence of 1.3 mM Ca^{2+} at 37°C for 60 min. The ACh released in the incubation supernatant was quantified with HPLC-ECD as described in the Method section. Each column presents the mean ± SEM of five or six separate experiments. *$p < 0.05$ vs. control.

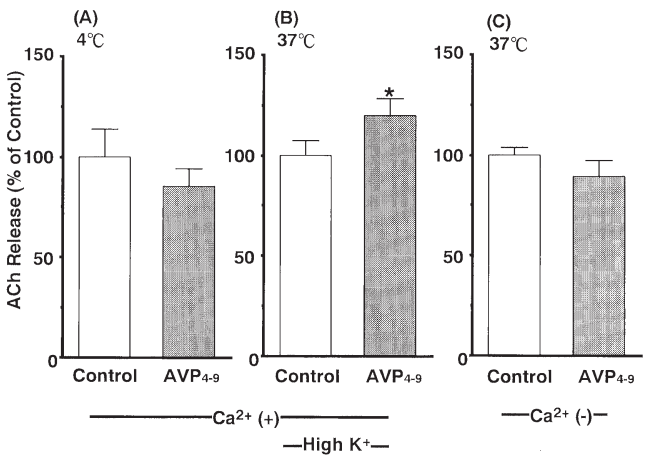

FIG. 3. Effects of Ca^{2+} and temperature on AVP_{4-9}-stimulated ACh release from rat hippocampal slices. Rat dorsal hippocampal slices were incubated with or without 0.1 μM AVP_{4-9} in the presence of 1.3 mM Ca^{2+} at 4°C for 60 min (A). Other slices were incubated with or without 0.1 μM AVP_{4-9} under the high-potassium (30 mM) condition in the presence of 1.3 mM Ca^{2+} (B) or under the calcium-free condition (C) at 37°C for 60 min. The ACh released in the incubation supernatant was quantified with HPLC-ECD as described in the Method section. Each column presents the mean ± SEM of four or five separate experiments. *$p < 0.05$ vs. control in each experiment.

significantly inhibited by the V_1-selective receptor antagonist, but not by the V_2-selective receptor antagonist.

Effects of Ca^{2+} and Temperature on the AVP_{4-9}-Stimulated ACh Release From Rat Hippocampal Slices

In the presence of 1.3 mM Ca^{2+} at 4°C, 0.1 μM AVP_{4-9} showed no significant effects on the basal ACh release from the slices compared with the control slices (control; 10.1 ± 1.5 pmol/mg protein) (Fig. 3A). The high K^+-evoked ACh release (control; 689.9 ± 49.6 pmol/mg) was significantly elevated by 0.1 μM AVP_{4-9} (828.6 ± 59.0 pmol/mg protein, 120.0 ± 8.5% of control) in the presence of 1.3 mM Ca^{2+} at 37°C (Fig. 3B). Under the Ca^{2+}-free condition at 37°C, 0.1 μM AVP_{4-9} showed no significant effect on the basal ACh release compared with the control (control; 39.5 ± 1.6 pmol/mg protein) (Fig. 3C).

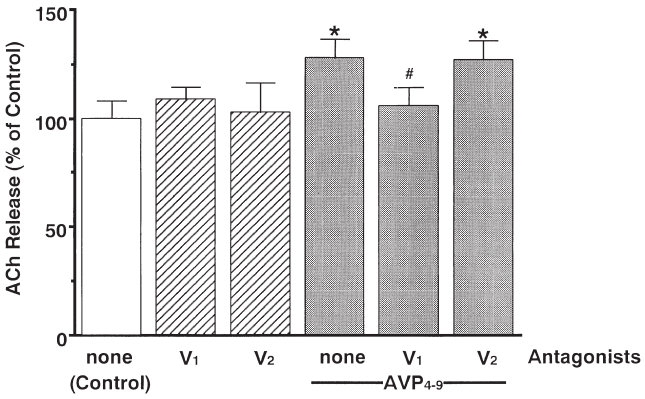

FIG. 2. Effects of V_1 and V_2-selective receptor antagonists on AVP_{4-9}-stimulated ACh release from rat hippocampal slices. Rat dorsal hippocampal slices were incubated with a V_1 or V_2-selective receptor antagonist (20 μM) with or without 0.1 μM AVP_{4-9} in the presence of 1.3 mM Ca^{2+} at 37°C for 60 min. The ACh released in the incubation supernatant was quantified with HPLC-ECD as described in the Method section. Each column presents the mean ± SEM of five separate experiments. *$p < 0.05$ vs. control. #$p < 0.05$ vs. AVP_{4-9} treatment.

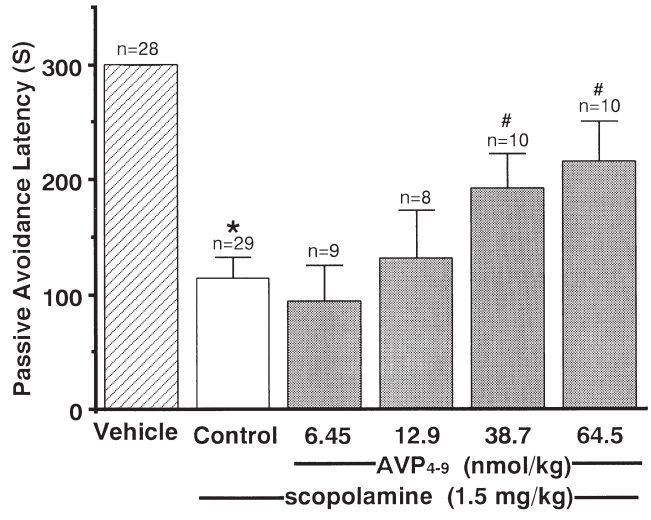

FIG. 4. Effects of AVP_{4-9} on the passive-avoidance response of scopolamine-induced memory-deficient mice. The mice were sc-administered the indicated dose of AVP_{4-9} and ip-administered 1.5 mg/kg scopolamine at 60 and 30 min prior to the learning trial, respectively. In the vehicle-treated mice, saline was substituted for scopolamine and AVP_{4-9}. As a control, saline was substituted for AVP_{4-9}. Twenty-four hours after the learning trial, the passive-avoidance latency was recorded. Each column presents the mean ± SEM. The number of mice is expressed above each column. *$p < 0.01$ vs. vehicle. #$p < 0.05$ vs. control.

Behavioral Study

As shown in Fig. 4, the passive-avoidance latencies of the control mice were shortened significantly compared with the vehicle-treated mice. The mice treated with AVP_{4-9} at 38.7 and 64.5 nmol/kg showed significantly elongated passive-avoidance latencies compared with the control group.

DISCUSSION

Although AVP_{4-9} is known as one of the most potent neuropeptides, the mechanisms of its facilitative effects on learning and memory are poorly understood. To clarify these mechanisms, we examined the effect of AVP_{4-9} on ACh release using slices of the rat hippocampus, an important region for learning and memory (19,20), and we evaluated the contribution of AVP_{4-9}-stimulated ACh release to the facilitative effects of AVP_{4-9} on learning and memory in a behavioral study.

As shown in Fig. 1, AVP_{4-9} enhanced the basal ACh release from rat hippocampal slices in our *in vitro* system, consistent with the results of *in vivo* microdialysis studies by Fujiwara *et al*. (13) and Maegawa *et al*. (18). We then investigated the details of the mechanisms underlying the AVP_{4-9}-stimulated ACh release at the hippocampus, using our *in vitro* system. The expressions of V_1-like vasopressin receptors (V_{1a} and V_{1b}) in the CNS were recently observed at the molecular level (17,27,31). It was also previously demonstrated that AVP_{4-9} inhibits the binding of radiolabeled AVP to the rat hippocampal synaptic membrane (6). Several other studies previously suggested that AVP_{4-9} and AVP fragment 4–8 [another neuroactive AVP metabolite (8)] bind to the receptors differ from that of AVP (3,11,12). In light of these evidences, we speculated that AVP_{4-9} would bind to several types of binding sites containing V_1-like vasopressin receptors in the CNS. In the present study, the V_1-selective receptor antagonist inhibited the AVP_{4-9}-stimulated basal ACh release (Fig. 3), suggesting at least that AVP_{4-9} binds to the V_1-like vasopressin receptors at several types of binding sites and that the V_1-like vasopressin receptors mediate the AVP_{4-9}-stimulated basal ACh release in the rat hippocampus.

Although AVP_{4-9} enhanced the ACh release in the presence of both the physiological level (1.3 mM) of Ca^{2+} at 37°C (Fig. 1) and the increased intracellular Ca^{2+} by depolarization with high K^+ stimulation at 37°C (Fig. 3B), AVP_{4-9} showed no effect on the basal ACh release in the Ca^{2+}-free condition at 37°C (Fig. 3C), as in previously reported NGF and TRH experiments (16,24,26). AVP_{4-9} also had no effect on the basal ACh release in the presence of the physiological level of Ca^{2+} at 4°C (Fig. 3A). In addition, it was recently demonstrated that AVP enhances intracellular Ca^{2+} in several types of cells via V_1-like vasopressin receptors (10,30). Therefore, we presumed that the AVP_{4-9}-stimulated ACh release is due to the temperature-dependent Ca^{2+} transport via mediation by V_1-like vasopressin receptors. The effects of AVP_{4-9} on the intracellular Ca^{2+} concentration in the hippocampus are presently under investigation.

Finally, we attempted to determine the contribution of the presently observed AVP_{4-9}-stimulated ACh release to the facilitative effects of AVP_{4-9} on leaning and memory. If the facilitative effect of AVP_{4-9} consists of only the stimulation of ACh release by AVP_{4-9}, AVP_{4-9} would show no effect on the passive-avoidance response of scopolamine-treated mice, because scopolamine blocks cholinergic receptors. In the present study, AVP_{4-9} significantly facilitated the passive-avoidance response of scopolamine-induced memory-deficient mice (Fig. 4). Fujiwara *et al*. (13) also demonstrated that AVP_{4-9} improves the scopolamine-induced memory impairment of rats in an eight-arm radial maze task. Thus, these behavioral studies suggest that the facilitative effects of AVP_{4-9} on learning and memory are controlled not only by the AVP_{4-9}-stimulated ACh release, but also by other parallel pathways, overcoming the cholinergic blocking by scopolamine. To characterize the pathways that overcome cholinergic blocking, studies of the effects of AVP_{4-9} on other neurotransmitter systems are in progress.

In conclusion, we suggest that AVP_{4-9} stimulates the ACh release from the rat hippocampus via V_1-like vasopressin receptors, and that this stimulation is dependent on Ca^{2+} and temperature. The present results also suggest that AVP_{4-9} facilitates learning and memory in rodents via the stimulation of ACh release and other parallel pathways that overcome cholinergic blocking.

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