ZTTA, a Prolyl Endopeptidase Inhibitor, Potentiates the Arginine-Vasopressin-Induced Incorporation of [^{14}C]Leucine in Rat Amygdaloid and Cortical Slices

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INTRODUCTION

Arginine-vasopressin (AVP), a nonapeptide, is widely distributed within the mammalian central nervous system (CNS) and has an effect on CNS neurons. Relatively high levels of AVP binding sites have been detected in the rat amygdala and cerebral cortex. AVP produces an excitation in hippocampal neuronal activity. It is well documented that systemically and centrally administered AVP prolongs extinction and improves consolidation in avoidance tasks in rats (1) and enhances cognition in humans. Old rats and humans exhibit selective deficits in short-term memory, related to an age-associated decline in the availability of AVP (2). Thus, the regulatory role of AVP in the CNS is revealed in the influence of AVP on learning and memory.

Protein synthesis is an important brain function. Previous studies have indicated that a brief period of forebrain ischemia leads to a suppression of the central protein synthesis rate in the gerbil hippocampus (3); the protein synthesis rate was attenuated in energy failure states, and a protein synthesis inhibitor, cycloheximide, induced deficits in CNS functions such as learning and memory (4), hypothermia, feeding and sleeping behavior. Leucine incorporation (protein synthesis) was therefore measured as a marker of neuronal function (5). It was indicated that neuronal activity regulates the local protein synthesis, because leucine incorporation into the dendritic region was augmented by treatments with afferent nerve stimulation and cholinergic drugs (6). The contribution from glia was thus minimized.

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Prolyl endopeptidase (PEP, EC 3.4.21.26) specifically cleaves the peptide bond(s) at the C-terminal side of the proline residue(s) of oligopeptides. It is highly active in the brain and readily degrades proline-containing biologically active peptides such as vasopressin. An inhibitor which specifically blocks PEP may thus potentiate an AVP-induced effect. In the first experiment of the present study, we examined the effect of AVP on protein synthesis by determining the [^{14}C]leucine incorporation in rat amygdaloid and cortical slices. Although we observed that an oral administration of N-benzyloxycarbonyl-thioprolyl-thioprolinal-dimethylacetal (ZTTA) (7), an inhibitor of PEP, improved the impairment of learning and memory in rats (7,8), the detailed mechanism of the effect was not revealed. In the second experiment, therefore, we investigated whether the facilitation of protein synthesis by AVP was potentiated by the oral administration of ZTTA.

MATERIALS AND METHODS

Animals

Male Wistar rats (7 weeks old, Seiwa Experimental Animals, Fukuoka, Japan) were housed under a 12 h light/dark cycle and maintained in temperature (23 ± 2°C)- and humidity (60 ± 1%)-controlled animal quarters, with food and water ad libitum. The animal experiments were performed in accordance with the Kyushu University Guidelines for Animal Experiments.

Measurement of Leucine Incorporation

The procedure used here for monitoring leucine incorporation was that reported by Banker and Cotman (9). The composition of the control Krebs-Ringer solution, equilibrated with 95% O_2/5% CO_2, was (in mM): NaCl 129, $MgSO_4$ 1.3, $NaHCO_3$ 22.4, KH_2PO_4 1.2, KCl 4.2, glucose 10.0 and $CaCl_2$ 1.5. The buffer was maintained at pH 7.3–7.4. Rats were decapitated, and each brain was quickly removed from the skull and cooled in ice-cold Krebs-Ringer solution. Parasagittal brain slices (450 μm thickness), including those of the amygdala and cerebral cortex (temporal lobe), were prepared using a tissue chopper. The slices were maintained between two disks made of stainless steel. The preparations were preincubated in normal Krebs-Ringer solution for 30 min in a recirculation chamber. The incubation chamber was arranged so as to recirculate 14 ml of buffer at 4.4 ml/min with continuous bubbling of humidified air through the buffer as it entered the chamber, as described in a previous paper (5). The incubation medium contained 4.6 kBq/ml of L-[1-^{14}C]-leucine (L-leucine, specific activity, 1.85–2.2 GBq/mmol; Muromachi Chemical Inc., Tokyo, Japan). Incubations were carried out for 45 min at 37 ± 0.2°C. The incubations were terminated by removing the disks from the incubation chamber and rinsing them with 20 ml of warm preincubated buffer, but before terminating the incubations the soluble radioactive leucine was "chased" by means of a 30-min washout with 0.1 mM unlabeled leucine. This procedure preferentially removed the soluble [^{14}C]leucine and reduced the level of soluble radioactive leucine to <10% of that in slices which were incubated with [^{14}C]leucine but not chased (10). At the end of the washout period, the slices were immediately

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fixed in iced cold 4% paraformaldehyde and left for 24 h. After the wet weight of a fixed tissue was determined, the tissue was solubilized in 300 μl of Solubable® (DuPont, Wilmington, DE, USA), and then the radioactivity was measured with a liquid scintillation counter.

Drugs

The PEP inhibitor used in this study was N-benzyloxycarbonyl-thioprolylthioprolinal-dimethylacetal (ZTTA; MW 412.53; Yakult Inc., Tokyo; Fig. 1) (7). It was suspended in a 4% arabic gum solution, and was administered per os (p.o.) in a volume of 0.5 ml per 100g body weight. Administration of the vehicle (4% arabic gum solution) only was used as the control. For the *ex vivo* experiment, rats were decapitated 1h after the oral treatment with ZTTA. Arginine-vasopressin (AVP) was purchased from the Peptide Institute (Osaka, Japan), and dissolved in distilled water. Eserine sulfate (physostigmine sulfate, PHY) was purchased from Sigma Inc. (St. Louis, MO). The PHY was dissolved in saline and administered intraperitoneally (i.p.) at the dose of 0.1 mg/kg/day for 3 consecutive days in the *ex vivo* experiment.

Data Analysis

Data are expressed as means ± S.D. The significance of differences between groups was determined using Dunnett's test and Student's t-test.

RESULTS

Banker and Cotman reported the metabolism of carboxy-labeled leucine, tyrosine and valine, and the incorporation of these isotopes into proteins (9). In experiments preliminary to the present study, the incorporation of leucine into proteins was linear for up to 60 min (data not shown).

To test whether exogenous vasopressin affects leucine incorporation in the amygdala or cerebral cortex, some of the slices described above were incubated with [^{14}C]leucine in the presence or absence of AVP. The leucine incorporation in the normal Krebs-Ringer solution was 1152 ± 71.8 (n = 19) dpm/mg wet weight per 45 min in the amygdala and 747 ± 41.1 (n = 20) in the cerebral cortex. These results demonstrated that vasopressin significantly elevated the leucine incorporation in the amygdala and cortex. The leucine incorporation in the control vehicle-treated slices was regarded as 100%. This effect was concentration-dependent, and the maximal increases (140% by amygdala and 132% by cerebral cortex) occurred with 1 μM of peptide (Fig. 2). For the determination of whether the PEP inhibitor ZTTA affects leucine incorporation, 1 h after the administration of ZTTA *in vivo*, rats were decapitated and slices were prepared for the *in vitro* experiment. The treatment with

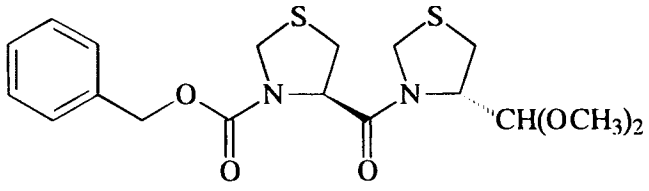

Fig. 1. Chemical structure of N-benzyloxycarbonyl-thioprolylthioprolinal-dimethylacetal (ZTTA).

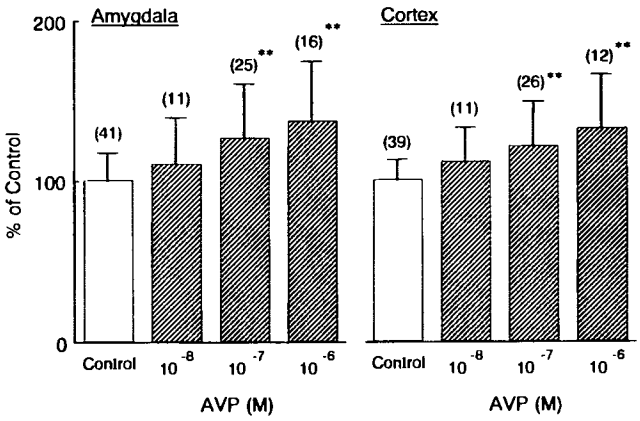

Fig. 2. Effects of various concentrations of AVP on leucine incorporation in rat amygdaloid and cortical slices. Leucine incorporation by control slices (AVP 0 μM) was regarded as 100%. The columns and bars represent means ± S.D. of the numbers of slices given in parentheses. ** $p < 0.01$ vs. control (AVP 0 μM).

ZTTA *in vivo* did not elevate the leucine incorporation in amygdaloid and cortical slices (Fig. 3). In contrast, the treatment with the cholinesterase inhibitor PHY as a positive control *in vivo* for 3 consecutive days elevated the leucine incorporation in cortical slices (Fig. 3). To determine whether ZTTA potentiates the AVP-induced elevation of leucine incorporation, we examined the effect of AVP on the leucine incorporation in amygdaloid and cortical slices prepared from rats treated with ZTTA *in vivo*. Although ZTTA (10 mg/kg) alone had no effect on leucine incorporation, the same ZTTA treatment significantly potentiated the 10^{-8} M of AVP-induced elevation of leucine incorporation in the amygdala and cerebral cortex (Fig. 4).

DISCUSSION

The present results demonstrated that treatment with AVP increased the leucine incorporation in the rat amygdaloid and

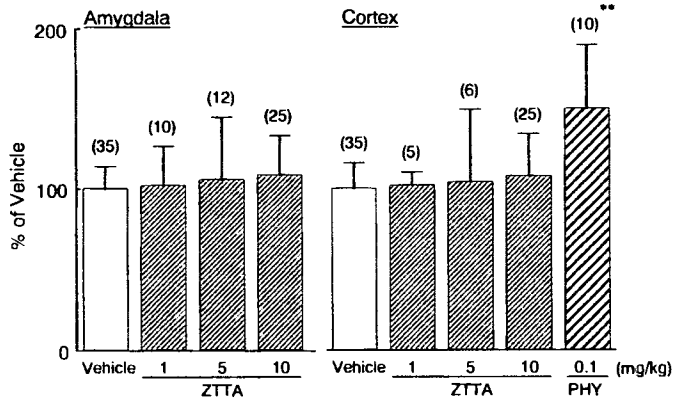

Fig. 3. Leucine incorporation in amygdaloid and cortical slices of rats treated with ZTTA (p.o.) or PHY (i.p.). Leucine incorporation was measured 1 h after the administration of ZTTA or PHY. Leucine incorporation by control slices (vehicle) was regarded as 100%. The columns and bars represent means ± S.D. of the numbers of slices given in parentheses. Open columns, vehicle group; striped columns, ZTTA groups; bold-striped column, PHY group. ** $p < 0.01$ vs. vehicle group.

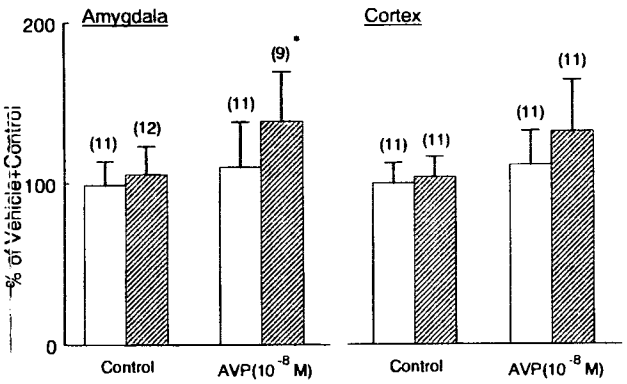

Fig. 4. Effect of ZTTA on the AVP-induced increase of leucine incorporation in rat amygdaloid and cortical slices. Leucine incorporation was measured 1 h after the administration of ZTTA. Leucine incorporation by control slices (AVP 0 μM and vehicle) was regarded as 100%. The columns and bars represent means ± S.D. of the numbers of slices given in parentheses. Open columns, vehicle; striped columns, ZTTA 10 mg/kg. * $p < 0.05$ vs. corresponding vehicle.

cerebral cortical slices. AVP has been shown to play an excitatory role in the hippocampus and cerebral cortex at the electrophysiological (11) and neurochemical (12) levels of analysis. Based on the findings of the present experiments, we suggest that the increase of [^{14}C]leucine incorporation reflects the neuronal excitation.

PEP specifically cleaves the peptide bond(s) at the C-terminal side of the proline residue(s) of oligopeptides. It is highly active in the brain and readily degrades proline-containing (especially short-chained) biologically active peptides such as vasopressin and its metabolites, thyrotropin-releasing hormone (TRH), luteinizing hormone-releasing hormone (LHRH), angiotensin, bradykinin, substance P and neurotensin (13). We investigated here whether the AVP-induced facilitation was potentiated by treatment with ZTTA *in vivo*. The treatment with ZTTA *in vivo* alone did not elevate the leucine incorporation. In contrast, the treatment with ZTTA *in vivo* significantly potentiated the AVP-induced effect. Although only a single concentration of AVP was used in the present experiments (10^{-8} M), these results indicate that the *in vivo* ZTTA treatment specifically blocked PEP and potentiated the AVP-induced facilitation. We could not determine whether and by what mechanism ZTTA potentiates pituitary-derived intraneuronal AVP or exogenous AVP perfused in the medium. There was no distinction between these two types of AVP in our experimental method. However, it is likely that ZTTA potentiated the effect of AVP by elevating the intracellular AVP level. It is thus necessary to evaluate the effects of ZTTA on AVP metabolism in detail *in vivo* and *in vitro*.

Protein synthesis is an important brain function. In the present study, treatment with the cholinesterase inhibitor PHY elevated the leucine incorporation. This is because PHY may activate the functional neuronal activity of cholinergic neurons in the cerebral cortex. The treatment with AVP facilitated the protein synthesis rate in rat cortical slices. The protein synthesis inhibitor cycloheximide can induce amnesia in rats (4). The facilitative effects of AVP and ZTTA on the protein synthesis rate indicate that neuropeptides and PEP inhibitors have excitatory effects on neuronal functions and may be able to improve impairments of learning and memory. Indeed, AVP facilitates memory through its receptors in the brain (14). Several types of PEP inhibitors were found to have anti-amnesic properties in various forms of learning and memory impairment (15).

In conclusion, AVP and the PEP inhibitor ZTTA cooperatively had a facilitatory role in leucine incorporation in the rat amygdaloid and cortical area. These results may support the beneficial effects of ZTTA on learning and memory impairment (7,8,15), and suggest the possibility that AVP and ZTTA have a promotive effect on cognitive functions.

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