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ZTTA, a Postproline Cleaving Enzyme Inhibitor, Improves Cerebral Ischemia-Induced Deficits in a Three-Panel Runway Task in Rats

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SHISHIDO, Y., M. FURUSHIRO, S. TANABE, S. NISHIYAMA, S. HASHIMOTO, M. OHNO, T. YAMAMOTO AND S. WATANABE. *ZTTA, a postproline cleaving enzyme inhibitor, improves cerebral ischemia-induced deficits in a three-panel runway task in rats.* PHARMACOL BIOCHEM BEHAV **55**(3) 333–338, 1996.—We investigated the effect of N-benzyloxycarbonyl-thioprolyl-thioprolinal-dimethylacetal (ZTTA), a novel postproline cleaving enzyme (prolyl endopeptidase, PPCE) inhibitor, on the in vitro activity of rat brain PPCE and memory impairment induced by cerebral ischemia. ZTTA noncompetitively inhibited rat brain PPCE (k_i = 2.9 μM). Cerebral ischemia for 5 min increased the number of errors in a working memory task with a three-panel runway paradigm. ZTTA at 6 mg/kg, administered immediately after blood flow reperfusion, significantly reduced the increase in working memory errors expected to occur 24 h after 5 min of ischemia. The antiamnesic action of ZTTA may be ascribable to a neuroprotective effect on the central nervous system due to some neuropeptides that are substrates of PPCE in the brain. **Copyright © 1996 Elsevier Science Inc.**

ZTTA Postproline cleaving enzyme (PPCE) Prolyl endopeptidase Cerebral ischemia Runway task Working memory

POSTPROLINE cleaving enzyme (PPCE) is distributed in testis, liver, skeletal muscle, and the brain of rats or in human body fluids (48). And it may play some role in the metabolism of neuropeptides that has proline residue (18). But detail of its physical role still remains obscure. On the other hand, several kinds of neuropeptides that have proline residues are widely distributed within the mammalian central nervous system, and they play important roles as neurotransmitters and/or modulators for other neurotransmitters such as acetylcholine (ACh). For example, arginine vasopressin (AVP) enhances memory consolidation (6,9,23), and thyrotropin-releasing hormone (TRH) and its analogue reverse memory and learning impairment (14,46,47). Memory improvement with TRH has recently also been demonstrated for clinical conditions in Alzheimer's disease patients (28). Furthermore, neurotensin causes long-lasting excitation of nucleus basalis cholinergic neurons, which are thought to be involved in cortical learning and memory (8) and substance P exhibits protective activity against the neurodegenerative effect of β-amyloid (25).

Also, the activity of this enzyme may influence the memory process, because a PPCE inhibitory effect has been observed for nootropic aniracetam (26). Furthermore, *N*-benzyloxycarbonyl-proline (Z-Pro) and *N*-benzyloxycarbonyl-glycyl-proline (Z-Gly Pro), known PPCE inhibitors, showed antiamnesic properties in rodents (51). And a significant elevation of PPCE activity has recently been observed in the brain of patients with Alzheimer's disease (1).

The four-vessel occlusion rat model is relatively easy to produce and shows good reproducibility. Rats exposed to transient cerebral ischemia showed more marked impairment of

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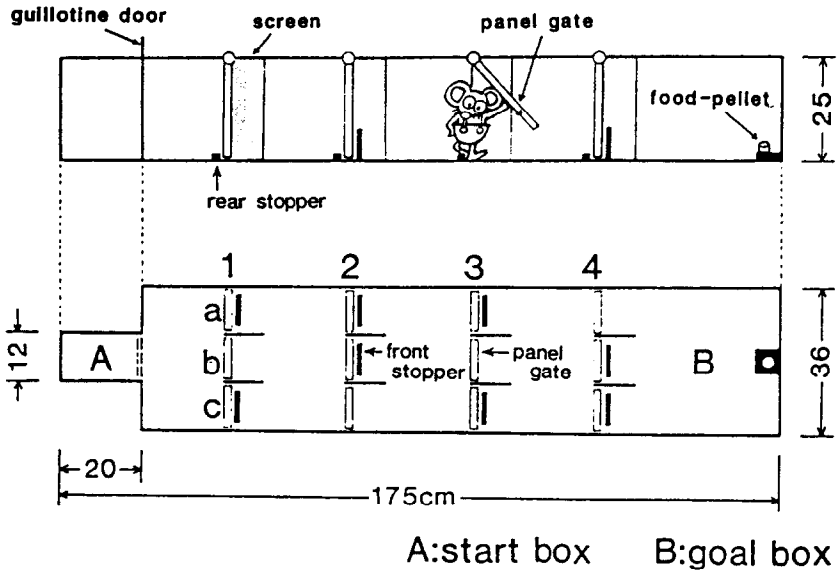


FIG. 1. Schematic drawing of the three-panel runway apparatus.

working memory than that of reference memory (33). On the other hand, some patients who have experienced cardiac arrest show deficiencies in their abilities to learn new and variable information (short-term memory), but they retain information learned prior to the ischemic brain insult (long-term memory) (5,52). In this way, the memory loss seen in ischemic rats is similar to the cardiac arrest amnesia seen in humans.

The effects of PPCE inhibitors on long-term memory have usually been investigated with relative easy tasks, such as passive avoidance (17,51). But there have not been any reports about the effects of these compounds on ischemia-induced impairment of short-term memory. Then, we studied the effect of *N*-benzyloxycarbonyl-thioprolyl-thioprolinal-dimethylacetal (ZTTA) a novel specific inhibitor of PPCE, using the four-vessel occlusion transient ischemia model, on working memory, that was assessed with a three-panel runway task.

METHOD

Animals

Eight-week-old male rats of the Wistar strain (Clea Japan Inc., Tokyo, Japan) were maintained at approximately 80% of their free feeding weight (200–230 g) prior to and during the experiment. The rats were housed in groups of three per cage at a constant temperature (24 ± 1°C) and humidity (60 ± 5%) with a 12 L:12 D cycle (light period: 08:30–20:30), and with water freely available.

Measurement of PPCE Inhibitory Activity of ZTTA

The PPCE solution was prepared from rat brain by a modification of the procedure described by Yoshimoto et al. (49,50), as follows. All procedures were carried out at 4°C. Rat brains, 34 g, were homogenized in 70 ml of 20 mM Tris-HCl buffer (pH 7.0), and the homogenate was centrifuged for 1 h at 1200 × *g*. The supernatant was fractionated by the ammonium sulfate precipitation method, 30–80% saturation. The precipitate was dissolved in 30 ml of 20 mM Tris-HCl buffer (pH 7.0), and desalted by dialysis against 20 mM Tris-HCl buffer (pH 7.0) containing 1 mM EDTA and 1 mM 2-mercaptoethanol. The dialysate was used as the enzyme solution for the enzyme kinetics study.

The in vitro activity of PPCE was assayed by the method of Yoshimoto et al. (51) with slight modifications, using Z-Gly Pro-β-naphthylamide (Z-Gly Pro-β-NA) as a substrate. Briefly, to 200 μl of 0.1 mM Tris-HCl buffer (pH 7.0) containing various concentrations of ZTTA was added 50 μl of the enzyme solution, and then the mixture was preincubated for 10 min at 37°C. Then, 50 μl of 1 mM Z-Gly Pro-β-NA in 40% dioxane was added to start the reaction. After 10 min incubation at 37°C, the reaction was stopped by adding 100 μl of 0.1 M acetate buffer (pH 4.0) containing 1 mg/ml Fast Garnet GBC salt (Sigma Chemical Co., St. Louis, MO) and 20% Triton X-100. The reaction mixture was left for 1 h at room temperature, and then centrifuged for 5 min at 1000 × *g* and 4°C. The activity of PPCE was determined by measuring the absorbance of the supernatant at 540 nm with a microplate spectrophotometer (NJ-2000; Nippon Inter Med, Tokyo, Japan). One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of β-naphthylamine per min at 37°C.

Apparatus

Working memory was assessed with a three-panel runway apparatus (10). In brief, the apparatus (175 × 36 × 25 cm) was composed of a start box and a goal box, with four consecutive choice points between them (Fig. 1). Each choice point consisted of a gate with three panels (a–c, 12 × 25 cm). The rats were prevented from passing through two of the three panels in the gate by means of front stoppers and from returning to the start box or to a previous choice point by rear stoppers affixed to each of the panels of the gates. When the rats reached the goal box, they received one food pellet (about 55 mg; Muromachi Kikai Inc., Tokyo, Japan) as a positive reinforcement.

Acquisition Training

The rats were subjected to six consecutive trials (defined as one session) per day, with removal of the front stopper of

one of the three panels of the gate (the correct panel) at each choice point. Trials were performed at 2-min intervals, and water was freely available between trials in the home cage. The locations of the correct panels were kept constant within one session, but were changed pseudo randomly from one session to the next (working memory). Twelve different patterns of correct panel locations were used in this experiment, as described previously (Table 1) (10,33). The number of attempts to pass through an incorrect panel (defined as errors) and the time required to obtain the food pellet (defined as latency) were recorded for each rat during the trials of one session. The criterion for learning was fewer than eight errors for the second to sixth trials of a session. Rats were used in the experiment if they satisfied this criterion throughout three consecutive sessions.

Production of Ischemia

Transient forebrain ischemia was produced by the method of Pulsinelli and Brierley (36). Briefly, the rats were anesthetized with sodium pentobarbital (Nembutal® Sodium solution; Abbott Laboratory, N. Chicago, IL; 35 mg/kg, i.p.), and the vertebral arteries were cauterized bilaterally with a bipolar coagulator (MICRO-ID; Mizuho Ikakogyo Inc., Tokyo, Japan). At the same time, threads were placed loosely around each common carotid artery, but carotid blood flow was not interrupted. On the following day, rats that behaved normally were used for the final phase of ischemia production. After a runway trial, the rats, under light anesthesia with ether, were fixed ventral side upwards on boards, and their common carotid arteries were exposed by pulling the threads. After recovery from anesthesia, forebrain ischemia was produced by occluding the bilateral carotid artery with aneurysm clips (No. 52; Sugita Inc., Tokyo, Japan) for 5 min (35). The criteria for forebrain ischemia were determined as bilateral loss of the righting reflex and paw extension. Furthermore, only those animals that showed continuous loss of the righting reflex for over 15 min after reperfusion were selected. The control rats also had their vertebral arteries cauterized, and they, under light anesthesia with ether, were fixed ventral side upwards on boards, and their common carotid arteries were exposed by pulling the thread. But after recovery from anesthesia, they did not have their carotid arteries occluded. The runway test was given for rats behaved normally 24 h after blood flow reperfusion.

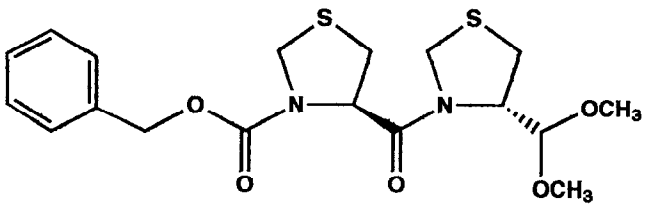
Drugs

The drug used in this study was *N*-benzyloxycarbonyl-thioprolyl-thioprolynal-dimethylacetal (ZTTA, MW 412.53; Yakult Inc.; Fig. 2). The drug was suspended in a 4% arabic gum solution and administered orally in a volume of 0.5 ml per 100 g body weight, immediately after blood flow reperfusion. Four percent arabic gum solution was administered as vehicle. The number of errors and latency for the second to sixth trials of a session were used to evaluate the ability of the rats to remember new correct panel locations, and are presented separately from those observed in the first trial.

TABLE 1

TWELVE TYPES OF SEQUENCES OF CORRECT PANEL POSITION IN THE THREE-PANEL RUNWAY APPARATUS

	Choice Point					Choice Point			
	1	2	3	4		1	2	3	4
1.	a	b	a	c	7.	c	a	b	a
2.	c	b	c	a	8.	b	a	c	b
3.	b	c	b	a	9.	b	c	a	c
4.	c	a	c	b	10.	a	b	c	b
5.	c	b	a	c	11.	b	a	c	a
6.	a	c	b	c	12.	a	c	b	a



Chemical structure of ZTTA

FIG. 2. Chemical structure of ZTTA, *N*-benzyloxycarbonyl-thioprolyl-thioprolinal-dimethylacetal.

Data Analysis

The significance of the differences between the groups was determined by one-way analysis of variance (ANOVA), followed by Williams' test when the *F* ratios reached significance ($p < 0.05$).

RESULTS

ZTTA exhibited inhibitory activity, with an IC_{50} value of 0.12 nM, toward PPCE of a rat brain crude extract (Fig. 3a). Figure 3b shows the kinetic characteristics of ZTTA. The PPCE solution used in this experiment had the following kinetic parameters: a K_m value of 0.21 mM and a V_{max} of 3.7 nmol NA/min/mg protein. A Dixon plot (7) (Fig. 3b) demonstrates that ZTTA noncompetitively inhibited rat brain PPCE, the K_i value being 2.9 μM.

In the three-panel runway task, the random performance level was four errors per trial, for instance, 24 errors per session. With repetitive training, the number of errors made from the second to the sixth trial (working memory errors) markedly decreased, while errors in the first trial remained constant at approximately four. About 17 training sessions were required for the rats to reach the criterion, and almost every rat reached the criterion after the 26th session. The number of errors made from the second to the sixth trial increased from 2.2 ± 0.9 ($n = 5$) to 14.0 ± 1.4 ($p < 0.01$, $n = 5$), 16.3 ± 1.8 ($p < 0.01$, $n = 4$), and 17.6 ± 1.4 ($p < 0.01$, $n = 7$) after 5 min, 10 min, and 15 min of cerebral ischemia, respectively. Ten-minute and 15-min ischemia was so severe that most of the animals in 10-min and 15-min duration four-vessel occlusion groups had motor dysfunction; they were not able to pass the first gate in the first trial in 600 s even if they chose the correct panel. It prevented accomplishment of the task by the animals. There were no sensory disorder or disappearance of motivation with the exception of motor dysfunction in 5 min of ischemia group. So we investigated the effect of ZTTA on 5 min cerebral ischemia-induced impairment of working memory. There was no significant difference in the number of errors and latency in the first trial among every group. The administration of ZTTA immediately after blood flow reperfusion significantly reduced the increase in working errors that would have been expected 24 h after the 5-min ischemia in a dose-dependent

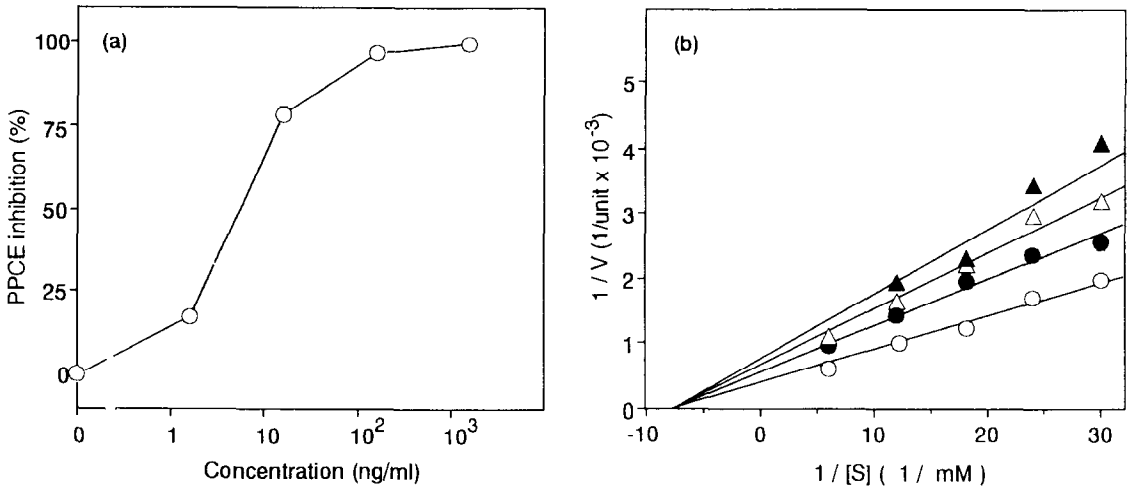


FIG. 3. (a) Concentration-dependent inhibition of rat brain PPCE by ZTTA. Activity was determined with Z-Gly Pro-β-naphthylamide as the substrate as described in the Method section. (b) Noncompetitive inhibition of rat brain PPCE by ZTTA. The results are presented in the form of a Lineweaver-Burk plot in the absence (○) and presence of ZTTA at concentrations of 1.33 μM (●), 2.00 μM (△), and 2.66 μM (▲).

manner (Fig. 4a). ZTTA did not affect the prolonged latency in the ischemic rats (Fig. 4b).

DISCUSSION

Our present results show that the PPCE inhibitor, ZTTA, prevents the impairment of accomplishment of three-panel runway task following transient forebrain ischemia. The working memory impairment in this model is not attributable to hippocampal delayed neuronal death, but may be ascribable to reversible types of cell damage to the central nervous system following postischemic disturbance of the brain blood flow and/or energy metabolism (15,21), because Kirino et al. reported that there were no morphological changes in the hippocampal CA1 pyramidal cells 24 h after 5 min of ischemia (20). On the other hand, previous reports show that some kinds of drugs have neuroprotective effect on the ischemia-induced cell damage or neuronal death. They are calcium channel blockers (2,45), glutamate receptor antagonists (35), protein kinase C inhibitors (34), protein synthesis inhibitors (12), 5-hydroxytryptamine (5-HT) receptor agonists and antagonists (41), cerebral vasodilators and metabolic enhancers (16,19), and cholinergic drugs (11,32,39). Cholinergic drugs, cerebral vasodilators and metabolic enhancers, and 5-HT_2 receptor antagonist among them are especially considered to show the neuroprotective effect through the retainment of ACh level in central cholinergic system (22,31,40,42).

The mechanism of the cerebral protection observed in the present study is still a matter of debate. Because ZTTA is a potent PPCE inhibitor, several kinds of neuropeptide that has a proline residue in its molecule may play an important role in the neuroprotective effect of ZTTA on ischemia-induced learning and memory impairment. It is, therefore, proposed that this protection may be mediated by the retainment of ACh level due to several kinds of neuropeptide. The relationships between neuropeptides and central cholinergic neurons have been elucidated. These studies have shown that TRH accelerates the neurotransmission of ACh in the brain (13,29,38,44), or AVP fragment 4–9 stimulates the ACh release in hippocampus of rats (27). Furthermore, a nonpeptide PPCE inhibitor was reported to potentiate the effect of TRH on the release of ACh in the rat hippocampus (30). The activation of TRH was potentiated due to the inhibition of inactivation of TRH through the PPCE inhibitor, because purified TRH-deamidase from bovine brain has been identified as PPCE (43). We observed that ZTTA improved the deficiencies in learning and memory functions produced by scopolamine or bilateral electrolytic lesioning of the basal forebrain in passive avoidance and three-panel runway tasks (unpublished data). These findings suggest that ZTTA has some positive effect on the metabolism of reduced ACh in the central nervous system. The activation of cholinergic neurotransmission induced by some neuropeptides may, therefore, also be involved in the neuroprotective effect of ZTTA.

TRH selectively antagonizes neuronal excitation evoked by glutamate in rat cerebral cortex (37), and an excessive

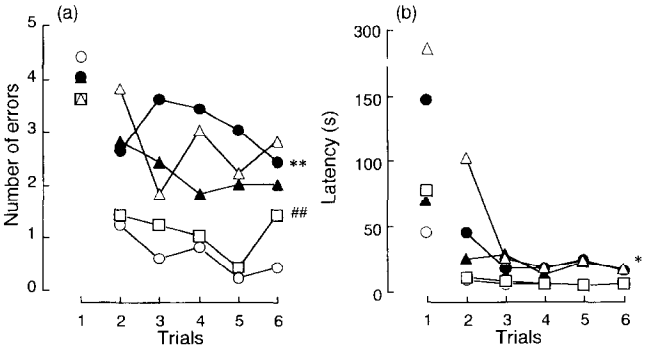


FIG. 4. Effects of ZTTA on the cerebral ischemia-induced increases in working memory errors (a) and latency (b) in the three-panel runway task. The runway task was performed 24 h after carotid artery occlusion (○, control; ●, 5 min of ischemia). ZTTA was administered po immediately after blood flow reperfusion (△, 1 mg/kg; ▲, 3 mg/kg; □, 6 mg/kg). Each point represents the mean ± SEM of errors for five animals recorded in each trial of a session. Statistical significance: $*p < 0.05$, $**p < 0.01$ vs. control; $\#\#p < 0.01$ vs. ischemia.

release of glutamate during ischemia results in glutamate neurotoxicity in cells. Taken together, ZTTA may potentiates the antagonistic effect of TRH on glutamate-evoked neuronal excitation through the PPCE inhibition. Although ZTTA was first introduced as a PPCE inhibitor, ZTTA may have another beneficial effect on ischemia-induced neurotransmitter abnormalities. To elucidate the mechanism of action of ZTTA, it is essential to examine the effect of ZTTA on the PPCE activity, neuropeptide contents and ACh release in the brain.

In conclusion, ZTTA has protective effect in cerebrovascular type dementia caused by cerebral ischemia. This effect may be ascribable to the retainment of ACh level in central nervous system mediated by the actions of several kinds of neuropeptides. The present results suggest that ZTTA may prevent brain damage due to recurrent ischemia, and that may be useful as a treatment of cerebrovascular disease.

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