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Effects of prolyl endopeptidase inhibitors and neuropeptides on delayed neuronal death in rats

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Abstract

We investigated the effects of the prolyl endopeptidase inhibitors 1-[1-(Benzyloxycarbonyl)-L-prolyl]prolinal (Z-Pro-Prolinal) and *N*-benzyloxycarbonyl-thioprolyl-thioprolinal-dimethylacetal (ZTTA) on delayed neuronal death induced by four-vessel-occlusion transient ischemia in rats. We also examined the effects of [pGlu^4, Cyt^6, Arg^8]vasopressin (vasopressin-(4–9)) and thyrotropin-releasing hormone (TRH) on the delayed neuronal death. Furthermore, we investigated the role of vasopressin receptors in the effects of vasopressin and prolyl endopeptidase inhibitors. Z-Pro-Prolinal, vasopressin-(4–9) and TRH protected pyramidal cells in the CA1 subfield of the rat hippocampus from delayed neuronal death after 10-min ischemia. The effect of vasopressin-(4–9) was abolished by vasopressin receptor antagonists. The effect of Z-Pro-Prolinal was also abrogated by the antagonists. These results suggest that the neuroprotective effect of prolyl endopeptidase inhibitors is mediated by neuropeptides such as [Arg^8]vasopressin and TRH, and indicate the involvement of vasopressin receptors in the neuroprotective effect of vasopressin-(4–9) and prolyl endopeptidase inhibitors.

Keywords: Prolyl endopeptidase inhibitor; Z-Pro-Prolinal; ZTTA (*N*-benzyloxycarbonyl-thioprolyl-thioprolinal-dimethylacetal); Ischemia; Pyramidal cell; Neuronal death, delayed; Vasopressin-(4–9)

1. Introduction

Brief and transient cerebral ischemia induces delayed neuronal death in the hippocampal CA1 subfield in Mongolian gerbils (Kirino, 1982) and rats (Pulsinelli et al., 1982a). Recent experiments have provided definitive evidence that an extracellular accumulation of synaptically released neurotransmitters, such as glutamate, is responsible for neuronal death in the hippocampus (Benveniste et al., 1984; Rothman and Olney, 1986). Some other mechanisms involving protein kinase C (Hara et al., 1990), cyclooxygenase (Nakagomi et al., 1989), γ-aminobutyric acid (Sternau et al., 1989), the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ (Schanne et al., 1979; Siesjö and Bengtsson, 1989), radical oxygen species (Flamm et al., 1978) and serotonin (Fujikura et al., 1989) have also been implicated in the development of delayed neuronal death.

We previously reported that a prolyl endopeptidase (EC.3.4.21.26) inhibitor, *N*-benzyloxycarbonyl-thioprolyl-thioprolinal-dimethylacetal (ZTTA), had a protective effect on cerebrovascular-type dementia caused by cerebral ischemia (Shishido et al., 1996). These results, observed in behavioral tests, prompted us to investigate in the present study whether prolyl endopeptidase inhibitors prevent neuronal damage, especially delayed neuronal death. We therefore studied the effects of the prolyl endopeptidase inhibitors 1-[1-(Benzyloxycarbonyl)-L-prolyl]prolinal (Z-Pro-Prolinal) (Wilk and Orlowski, 1983) and ZTTA (Shishido et al., 1996) on delayed neuronal death induced by four-vessel-occlusion transient ischemia.

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Since ZTTA is a prolyl endopeptidase inhibitor, prolyl endopeptiase itself may be involved in the ischemia-induced dysfunction in learning and memory. Several types of neuropeptides which have a proline residue in their molecule are suspected to play an important role in the neuroprotective effect of ZTTA on ischemia-induced learning and memory impairment. Of those neuropeptides, [Arg^8]vasopressin and TRH were reported to especially enhance memory consolidation (De Wied, 1976; Horita et al., 1986; Koob et al., 1989), and [Arg^8]vasopressin induces long-term potentiation in the hippocampus (Miura et al., 1997). It is reported that PEP inhibitor increased the [Arg^8]vasopressin- and TRH-like immunoreactivity in rat brain (Toide et al., 1995; Shinoda et al., 1996). Although many studies of animal behavior and clinical trials have suggested a memory-improving influence of [Arg^8]vasopressin and its analogue and we also observed that subcutaneous administration of vasopressin-(4–9) and its analogue facilitate or improve the passive avoidance response in rodents (Tanabe et al., 1997, 1999), little is known about their role in ischemic damage, especially in vivo. Thus, the present study was also conducted to clarify the involvement of vasopressin and its receptors in the effect of prolyl endopeptidase inhibitors.

2. Materials and methods

2.1. Animals

Eight-week-old male rats of the Wistar strain (Nippon SLC, Hamamatsu, Japan) were housed in groups of three per cage at a constant temperature (24 ± 1°C) and humidity (60 ± 5%) with a 12-h light–dark cycle (light period: 0830–2030 h). The rats were given food and water ad libitum. The animal experimentation guidelines of our institute were followed.

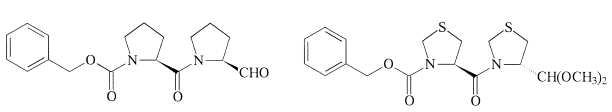
2.2. Surgery and experimental procedures

The rats underwent chronic cannula implantation for the microinjection of drugs into the 3rd cerebral ventricle. Each rat was anesthetized with sodium pentobarbital (Nembutal® Sodium solution; Abbott Laboratory, North Chicago, IL, USA; 40 mg/kg, intraperitoneally, i.p.) and was fixed in a stereotaxic apparatus. A stainless steel guide cannula (external diameter 0.5 mm, G-8, EiCom, Kyoto, Japan) was placed in the 3rd cerebral ventricle (4.2 mm posterior to the bregma, ± 0 mm lateral to the midline, 4.8 mm ventral to the surface of dura), according to the atlas of Paxinos and Watson (1982). The cannula was fixed to the skull with two screws and dental acrylic cement (Unifast Trad, GC, Tokyo, Japan). The rats were allowed at least 1 day to recover from surgery before the operation for ischemia. A stainless steel injection cannula (external diameter: 0.3 mm, Biomedica, Tokyo) was used to infuse the drugs. The injection cannula was connected to a 10-μl Hamilton® syringe via intramedic polyethylene tubing (extra diameter 1.09 mm, No. 7406 (PE20), Becton Dickinson, Lincoln Park, NJ). Two microliters of drug solution was injected into the 3rd cerebral ventricle through the injection cannula. The rate of injection was 1 μl/min. The injection cannula was left in place for 3 min after completion of the injection to facilitate the diffusion of the drug.

Transient forebrain ischemia was produced using the method of Pulsinelli and Brierley (1979). Briefly, the rats were anesthetized with sodium pentobarbital, and the vertebral arteries were cauterized bilaterally with a bipolar coagulator (MICRO-ID; Mizuho Ikakogyo, Tokyo). At the same time, threads were placed loosely around each common carotid artery, but carotid blood flow was not interrupted. On the following day, the rats were fixed ventral-side upwards on boards, and their common carotid arteries were exposed by pulling the threads. Forebrain ischemia was produced by occluding the bilateral carotid artery with Sugita's aneurysm clips (No. 52; Mizuho Ikakogyo) for 10 min. Body temperature was maintained at 37°C during the experiment by a heating mat (Animal Blanket Controller; Nihon Koden, Tokyo). The criteria for forebrain ischemia were bilateral loss of the righting reflex and paw extension. Only the animals which showed continuous loss of the righting reflex for over 15 min after recirculation were selected. The control (sham-operated) rats had their vertebral arteries cauterized, but did not have their carotid arteries occluded.

2.3. Drugs

The neuropeptides used in this study were [pGlu^4, Cyt^6, Arg^8]vasopressin (vasopressin-(4–9)) (Sigma, St. Louis, MO, USA) and thyrotropin-releasing hormone (TRH) (Peptide Institute, Osaka, Japan). These drugs were dissolved in saline and administered subcutaneously (s.c.) in a volume of 0.1 ml/100 g body weight 30 min prior to the carotid occlusion. The prolyl endopeptidase inhibitors used in this study were Z-Pro-Prolinal (MW 330; Yakult, Tokyo) (Fig. 1) and ZTTA (MW 412.53; Yakult, Tokyo) (Fig. 1). These were dissolved in saline containing 20% dimethylsulfoxide and administered i.p. in a volume of 0.1–0.4 ml/100 g body weight. The glutamate receptor antagonist MK-801 (Sigma) was used as a positive control neuroprotective agent. It was dissolved in saline and administered i.p. in a volume of 0.1 ml/100 g body weight. These drugs



Z-Pro-Prolinal ZTTA

Fig. 1. Chemical structures of Z-Pro-Prolinal and ZTTA.

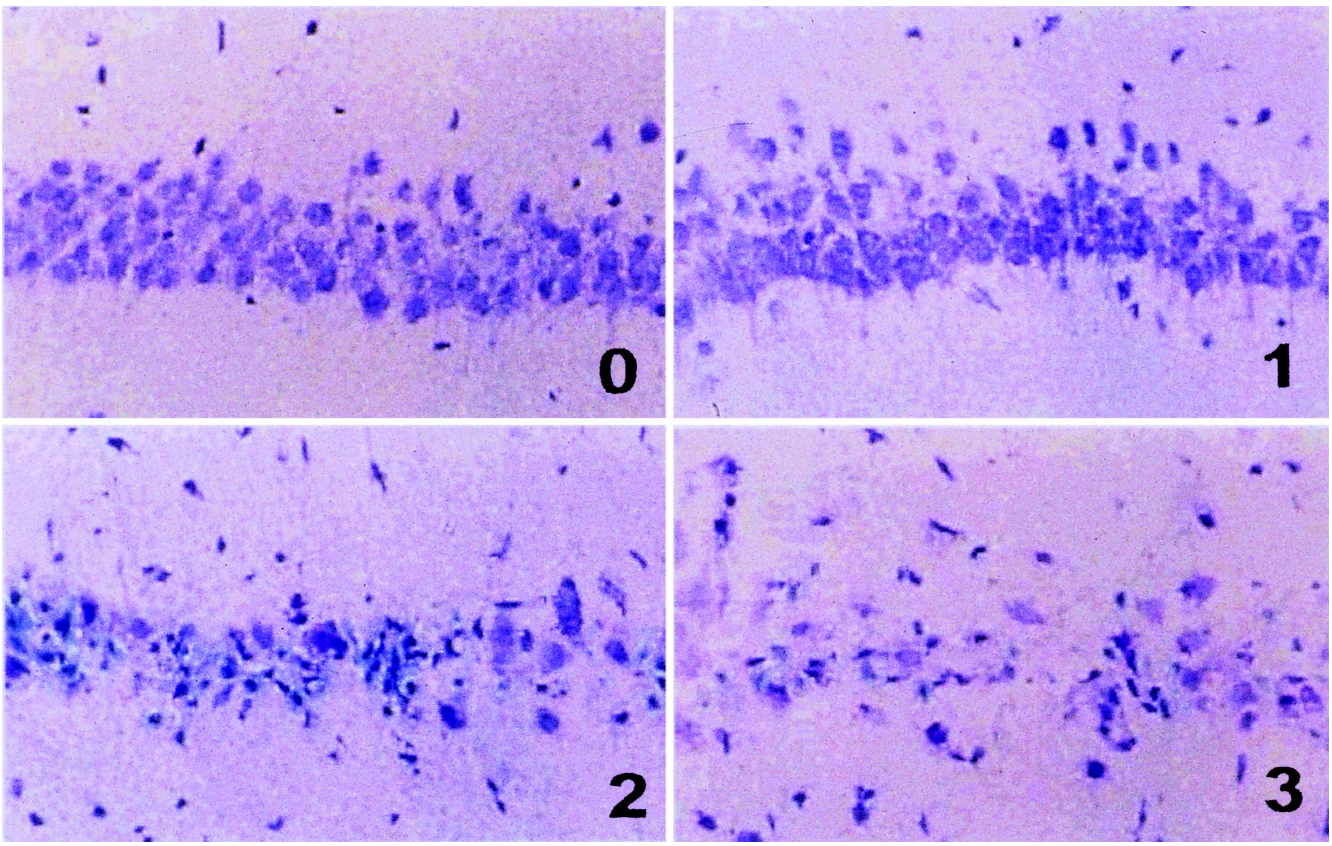


Fig. 2. Pulsinelli's criteria: (0) normal hippocampus; (1) < 10% of neurons damaged; (2) 10–50% of neurons damaged; (3) > 50% of neurons damaged.

were administered with the same schedule as that used for neuropeptides. The receptor antagonists used in this study were [β-mercapto-β, β-cyclopentamethylenepropionyl[1], *O*-Me-Tyr[2],Arg[8]]vasopressin (Peptide Institute) as a vasopressin V_1 receptor antagonist and [adamantaneacetyl[1], *O*-Et-D-Tyr[2],Val[4],aminobutyryl[6],Arg[8,9]]vasopressin (Sigma) as a vasopressin V_2 receptor antagonist. Rats were given these receptor antagonists dissolved in saline by microinjection just before the i.p. and s.c. injections. The microinjection procedure described in the previous section was used.

2.4. Evaluation of neuronal death

Serial hippocampal sections were examined to assess the brain damage by transient ischemia. In brief, 5 days after the ischemia, each rat was decapitated, and its brain was removed rapidly and embedded in Tissue Mount® (Shiraimatsu Kikai, Tokyo) using standard procedures. Coronal sections, 5 μm thick, were fixed with 4% paraformaldehyde and stained with Toluidine Blue (Sigma). Representative sections of the dorsal hippocampus were used for the quantification of neuronal degeneration. The criteria for hippocampus CA1 damage were based upon findings of Pulsinelli's group (Pulsinelli and Brierley, 1979; Pulsinelli et al., 1982a,b; Buchan and Pulsinelli, 1989, 1990), that is, 0: normal hippocampus, 1: < 10% of neurons damaged, 2: 10–50% of neurons damaged and 3: > 50% of neurons damaged (Fig. 2).

2.5. Data analysis

Data are expressed as means ± S.D. The significance of differences in neuronal damage between groups was determined using Wilcoxon's test and Dunnett's test.

3. Results

Brain sections from the anterior hippocampus of a non-ischemic control rat, a rat given 10 min of transient forebrain ischemia and a rat administered MK-801 30 min before the ischemia are shown in Fig. 3. In the control (sham-operated) rats, pyramidal cells were clear. In rats exposed to 10 min of transient forebrain ischemia (vehicle), extensive cell loss in the CA1 subfield was observed. In the MK-801-administered rats, little cell loss was observed. The severity of CA1 neuronal cell loss, as assessed by Pulsinelli's criteria, in each group is shown in Fig. 4. The difference between the 10-min ischemia (vehicle) group and the non-ischemia control (sham-operated) group was significant.

We then examined the effect of two prolyl endopeptidase inhibitors, ZTTA and Z-Pro-Prolinal, on the cell loss

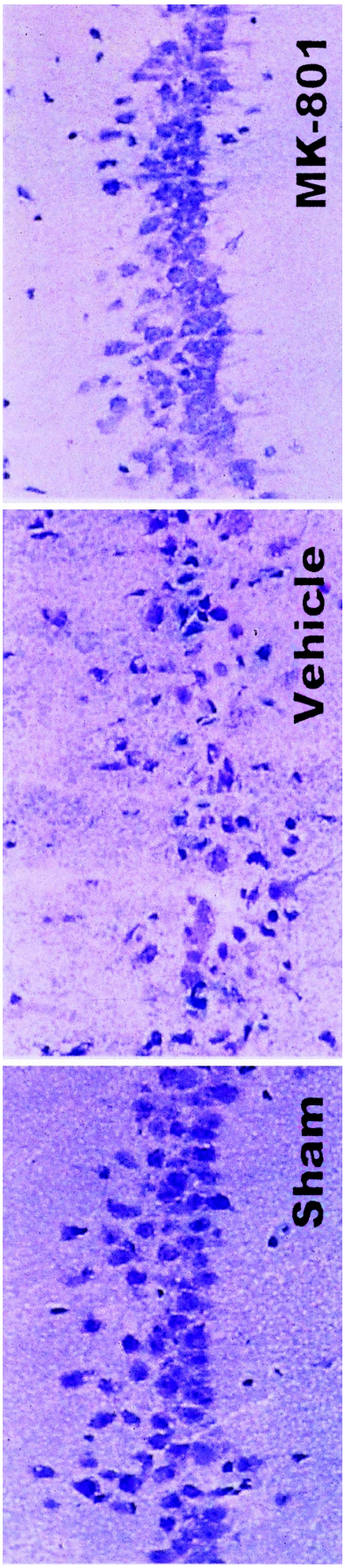


Fig. 3. Histological changes in pyramidal cells in the CA1 area of rat hippocampus at 5 days post-ischemia. Sham: sham-operated; Vehicle: ischemia + saline; MK-801: ischemia + MK-801 (1 mg/kg).

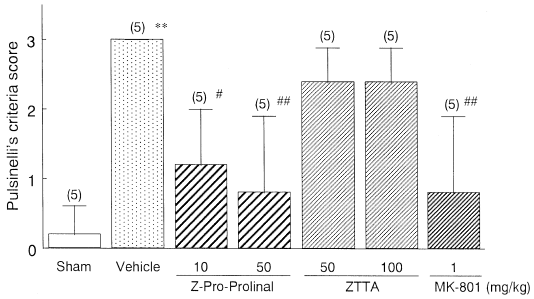


Fig. 4. Effects of ZTTA, Z-Pro-Prolinal and MK-801 on hippocampal CA1 neuronal cell loss evoked by 10 min global ischemia in rats. Numbers in parentheses indicate the number of animals. Data are the mean ± S.D. $^{**}P < 0.01$ vs. sham-operated group (Sham), $\#P < 0.05$, $\#\#P < 0.01$ vs. ischemia + saline group (Vehicle).

in the CA1 subfield. Treatment with Z-Pro-Prolinal or MK-801 as a positive control 30 min before ischemia significantly protected neurons from death 5 days after ischemia, although ZTTA had little effect on the delayed neuronal death up to the dose of 100 mg/kg (Fig. 4). The neuropeptides vasopressin-(4–9) and TRH also significantly protected neurons from delayed death, as shown in Fig. 5.

In the next experiments, we investigated the influence of vasopressin receptor antagonists on the neuroprotective effect of vasopressin-(4–9). It was observed that the co-administration of vasopressin V_1 receptor antagonist attenuated the neuroprotective effect of vasopressin-(4–9) (Fig. 6). The vasopressin V_2 receptor antagonist also attenuated the neuroprotective effect of vasopressin-(4–9) (Fig. 6). It was also demonstrated that the neuroprotective effect of Z-Pro-Prolinal was abolished by the antagonists (Fig. 6). We confirmed that the intracerebroventricular (i.c.v.) administration of saline as vehicle did not influence the neuroprotective effects of Z-Pro-Prolinal and vasopressin-(4–9) (data was not shown). The severity of CA1 neuronal cell loss, as assessed by Pulsinelli's criteria, in each group is shown in Fig. 7. The neuroprotective effects of Z-Pro-Prolinal or vasopressin-(4–9) were repressed by the antag-

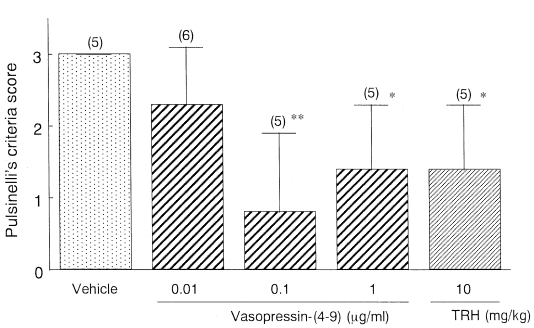


Fig. 5. Effects of vasopressin-(4–9) and TRH on hippocampal CA1 neuronal cell loss evoked by 10 min global ischemia in rats. Numbers in parentheses indicate the number of animals. Data are the mean ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. ischemia + saline group (Vehicle).

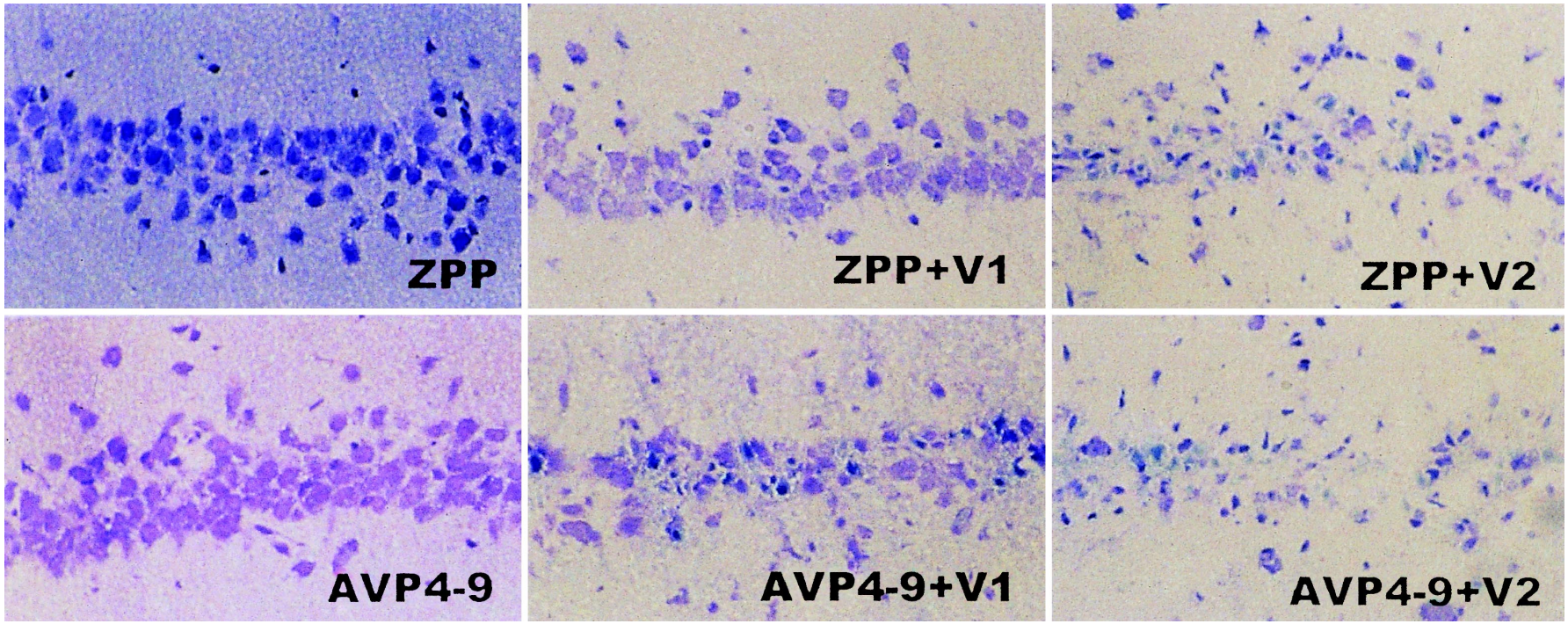


Fig. 6. Histological changes in pyramidal cells in the CA1 area of rat hippocampus at 5 days post-ischemia. ZPP: ischemia + Z-Pro-Prolinal (50 mg/kg); ZPP + V1: ischemia + Z-Pro-Prolinal + vasopressin V_1 receptor antagonist (2 μg); ZPP + V2: ischemia + Z-Pro-Prolinal + vasopressin V_2 receptor antagonist (2 μg); AVP4–9: ischemia + vasopressin-(4–9) (0.1 μg/kg); AVP4–9 + V1: ischemia + vasopressin-(4–9) + vasopressin V_1 receptor antagonist; AVP4–9 + V2: ischemia + vasopressin-(4–9) + vasopressin V_2 receptor antagonist.

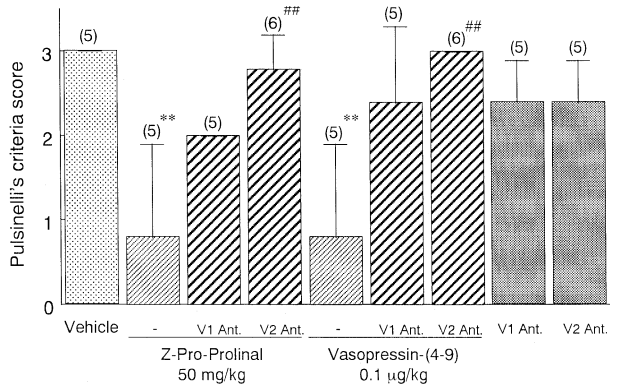


Fig. 7. Effects of the co-administration of vasopressin-(4–9) or Z-Pro-Prolinal with vasopressin V_1 receptor antagonist or vasopressin V_2 receptor antagonist on hippocampal CA1 neuronal cell loss evoked by 10 min global ischemia in rats. Numbers in parentheses indicate the number of animals. Data are the mean ± S.D. $^{**}P < 0.01$ vs. ischemia + saline group (Vehicle), $\#\#P < 0.01$ vs. ischemia + vasopressin-(4–9) or ischemia + Z-Pro-Prolinal group (corresponding Control).

onists. The microinjection of receptor antagonist alone had no effect on delayed neuronal death (Fig. 7).

4. Discussion

As we reported previously, ZTTA had an anti-amnesic effect on the memory impairment induced by cerebral ischemia, so we expected that ZTTA would have a neuroprotective effect on delayed neuronal death. However, ZTTA had no such effect in the present study. A more potent prolyl endopeptidase inhibitor, Z-Pro-Prolinal, did protect against delayed neuronal death. We speculated that this difference of bioavailability and efficacy might be a cause of the difference in the inhibition potencies of Z-Pro-Prolinal and ZTTA for prolyl endopeptidase, because it was reported that the anti-amnesic effects of prolyl endopeptidase inhibitors approximately paralleled their inhibition potencies for prolyl endopeptidase in vitro (Yoshimoto, 1984; Yoshimoto et al., 1987). In fact, the inhibition constants (K_i) of Z-Pro-Prolinal and ZTTA are 3.7 nM (Yoshimoto, 1991) and 2.9 μM (Shishido et al., 1996), respectively. In addition, the neuronal damage caused by 10 min-ischemia in the present study was much more severe than that caused by 5 min-ischemia in our previous behavioral study. This may be another reason why only Z-Pro-Prolinal was effective in protecting against delayed neuronal death.

The neuropeptides vasopressin-(4–9) and TRH also had neuroprotective effects on the delayed neuronal death after ischemia. These results suggest that the neuroprotective effect of Z-Pro-Prolinal is based on its prevention of the degradation of neuropeptides such as vasopressin-(4–9) and TRH.

Vasopressin-(4–9) and TRH can be categorized as excitatory neuropeptides in hippocampus (Joëls and Urban, 1982; Stocca and Nistri, 1996), because, for example, they play an important role in learning and memory by promoting the neurotransmission of acetylcholine (Horita et al., 1986; Maegawa et al., 1992; Miyamoto et al., 1993; Toide et al., 1993) and long-term potentiation (Ishihara et al., 1991; Miura et al., 1997). These findings led us to speculate that these neuropeptides exacerbate delayed neuronal death, as do excitatory amino acids (i.e., glutamate and aspartate). In fact, Tanaka et al. (1994) reported that [Arg^8]vasopressin potentiates the development of ischemic impairment of the CA1 presynaptic potential in rat hippocampal slices, and Liu et al. (1996) reported that [Arg^8]vasopressin exacerbates a delayed neuronal damage in the hippocampus of gerbils.

However, vasopressin-(4–9), one of the metabolites of [Arg^8]vasopressin, was found to be much more potent than [Arg^8]vasopressin in facilitating learning and memory in rats (Burbach et al., 1983; De Jong et al., 1985; Liu et al., 1990). It was also reported that vasopressin-(4–9) was devoid of peripheral effects on blood pressure, heart rate, urine flow and smooth muscle activity (De Wied et al., 1984; Lin et al., 1990). Although we did not investigate the effect of vasopressin-(4–9) on brain edema, these findings suggest that vasopressin-(4–9) hardly has any exacerbating effect on at least brain water content in contrast to [Arg^8]vasopressin. Further, Zhou et al. (1995, 1997) reported that a metabolite of [Arg^8]vasopressin, vasopressin-(4–8) enhances nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) expression in the cerebral cortex and hippocampus of the adult rat brain. These findings suggest that the [Arg^8]vasopressin metabolite vasopressin-(4–9) may have a more beneficial effect on ischemia than [Arg^8]vasopressin itself. In the present study, the body temperature of rats was maintained at 37°C to avoid hypothermia by a heating mat during ischemia and the first 15 min after reperfusion. But we did not monitor temperature until a few hours after the reperfusion. It is thus necessary to investigate the influence of the drugs including MK-801 on temperature, too.

The neuroprotective effects of vasopressin-(4–9) and Z-Pro-Prolinal were significantly attenuated by the vasopressin V_2 receptor antagonist, and the vasopressin V_1 receptor antagonist also tended to abolish the effects of vasopressin-(4–9) and Z-Pro-Prolinal. These results suggest that vasopressin-(4–9) acts neuroprotectively through the [Arg^8]vasopressin receptors. Since Albeck and Smock (1988) and Sakurai et al. (1998) reported that [Arg^8]vasopressin had an inhibitory effect in hippocampal slices, acting through [Arg^8]vasopressin receptors, our present results could be explained by the inhibitory action of [Arg^8]vasopressin. However, a type of specific binding site for vasopressin-(4–8) which differed from the known [Arg^8]vasopressin binding site and could not compete for [Arg^8]vasopressin is found in many regions of the rat

brain, especially in the limbic system (Du et al., 1994). The mechanisms underlying the effects of vasopressin-(4–9) and TRH observed in the present study remain a matter of debate. Further experiments must be conducted to elucidate the mechanisms of the neuroprotective action of neuropeptides in detail.

In conclusion, the prolyl endopeptidase inhibitor Z-Pro-Prolinal and the neuropeptides vasopressin-(4–9) and TRH were effective in protecting against delayed neuronal death, and their effects were abolished by [Arg^8]vasopressin receptor antagonists. These results indicate that prolyl endopeptidase inhibitors such as Z-Pro-Prolinal could be useful for the treatment of cerebrovascular disease, and that the neuroprotective effects of these inhibitors are mediated by at least [Arg^8]vasopressin receptors.

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