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Modulation of NMDA receptor by huperzine A in rat cerebral cortex[1]

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KEY WORDS huperzine A; cholinesterase inhibitors; *N*-methyl-*D*-aspartate receptors; sodium glutamate; dizocilpine maleate; hippocampus; pyramidal cells; cerebral cortex; patch-clamp techniques; radioligand assay

ABSTRACT

AIM: To investigate the effects of huperzine A (Hup A) on NMDA receptors in rat cerebral cortex. **METHODS**: 1) The effect of hup A on NMDA-induced current was studied in acutely dissociated rat hippocampal pyramidal neurons using whole-cell recording. 2) The effect of Hup A on NMDA receptor binding was assessed using [^{3}H] dizocilpine (Diz) binding assay in synaptic membrane preparation of rat cerebral cortex. **RESULTS**: 1) Hup A reversibly inhibited NMDA-induced current in a concentration-dependent manner with IC_{50} of 45.4 $\mu mol \cdot L^{-1}$. 2) Hup A inhibited the specific binding of [^{3}H]MK-801 to extensively washed synaptic membrane of rat cerebral cortex in a concentration-dependent manner with IC_{50} of 0.5 (0.1 - 1.9) $\mu mol \cdot L^{-1}$ ($n = 4$). 3) *L*-Glutamate 10 $\mu mol \cdot L^{-1}$ markedly increased [^{3}H] MK-801 binding. In the presence of *L*-glutamate, Hup A 0.001 - 0.1 $\mu mol \cdot L^{-1}$ caused a further increase of the binding, whereas Hup A 1 - 300 $\mu mol \cdot L^{-1}$ inhibited the binding in a concentration-dependent manner with IC_{50} of 12.3 (5.8 - 26.3) $\mu mol \cdot L^{-1}$ ($n = 5$). **CONCLUSION**: Hup A acted as an antagonist of NMDA receptor in cerebral cortex in addition to its inhibitory effect on acetylcholinesterase.

INTRODUCTION

l-Huperzine A (Hup A), a novel alkaloid, was first isolated by Chinese scientists from a Chinese herb *Huperzia serrata* (Thunb) Trev.[1]. A series of pharmacological studies demonstrated that Hup A was a selective inhibitor of acetylcholinesterase (AChE)[2]. Based on the cholinergic hypothesis of Alzheimer's disease (AD), several cholinesterase inhibitors (ChEI), such as physostigmine, tacrine, E2020, and Hup A were found to improve memory in different animal models and in AD patients[3]. Hup A has been demonstrated as one of the most promising agent used to treat AD due to its high potency, high bioavailability, long duration of AChE inhibition and lower toxicity[4].

Excitotoxicity (neuronal cell death caused by overstimulation of glutamate receptors) has been proposed as the final common pathway for various neurodegenerative diseases such as AD, Parkinsonism, and Huntington disease[5]. Recent evidence showed that pretreatment of cultured brain neurons with Hup A reduced neuronal death caused by glutamate. Furthermore, Hup A also reduced glutamate-induced calcium mobilization, but did not affect the increase in intracellular free calcium induced by exposure to high KCl or a calcium channel activator Bay-K-8644[6]. These results suggested that Hup A might act on glutamate receptors to exert its neuroprotective

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effects. In the present study we investigated the effects of Hup A on NMDA receptor in rat cerebral cortex.

MATERIALS AND METHODS

Chemicals NMDA, *d*-dizocilpine maleate (Diz, MK-801), *L*-glutamic acid, and glycine (RBI, USA). Protease XXIII, trypsin inhibitor (type II-S, Sigma, USA). [^{3}H]Diz (832.5 TBq · mol^{-1}; DuPont NEN, USA). Hup A (colorless crystals, purity >98 %, prepared by Department of Phytochemistry in Shanghai Institute of Materia Medica).

Whole-cell recording Acutely disso-ciated hippocampal pyramidal neurons were prepared from 7-14-d-old Sprague-Dawley rats (Grade II, Certificate № 117), using a modified method of protease digestion[7]. In brief, rat brain was put in ice-cold, oxygenated external solution (ES) containing NaCl 140, KCl 3, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10, and HEPES 10 mmol·L^{-1} (pH 7.4 with NaOH). Hippocampus was cut into 500 μm-thick slices with a vibrator (Campden 753M Vibroslice), and incubated in ES at 20 – 25 ℃ for 1 h. The slices were treated with ES containing protease XXIII 1 g·L^{-1} at 32 ℃ for 4 min, and placed in fresh ES containing trypsin inhibitor (type II-S) 3 g·L^{-1} and BSA 3 g·L^{-1} for 20 min. Neurons were dissociated by triturating the slices through a series of fire-polished Pasteur pipettes. Dissociated cells were transferred to 35-mm culture dish and allowed to adhere to the bottom. Neurons with pyramidal shape and short dendrites and axon were used for study, and perfused with oxygenated ES during experiment.

Patch pipettes (tip resistance 3 – 4 MΩ) were pulled with Sutter P-97 puller and filled with a pipette solution containing KCl 140, $MgCl_2$ 1, $CaCl_2$ 1, egtazic acid 10, and HEPES 10 mmol·L^{-1} (pH 7.4 with KOH). NMDA and Hup A were dissolved in an oxygenated modified ES in which Mg^{2+} was omitted and glycine 2 μmol·L^{-1} was added (pH 7.4 with NaOH). Drug-containing ES was applied directly to the neuron through Rapid Solution Changer (Biologic RSC-100). NMDA-induced current was recorded under whole-cell voltage-clamp configuration using Axopatch 200A amplifier (Axon Instruments). Membrane potential of the neuron was held at – 60 mV. Signal was filtered at 5 kHz, then acquired on-line using a computer equipped with Axoscope 1.1 software through Axon DigiData-1200A interface.

Radioligand binding assay Extensively washed crude synaptic membrane was prepared from cerebral cortex of Sprague-Dawley rats (♂, 150 – 250 g)[8]. Cerebral cortex was homogenized in 10 volumes (vol/wt) of ice-cold sucrose solution 0.32 mol·L^{-1}. The homogenate was centrifuged at 1000 × *g* for 10 min and the resulting supernatant centrifuged at 10 000 × *g* for 20 min. The pellets were washed (resuspended, homogenized, and centrifuged at 48 000 × *g* for 20 min) twice in 50 volumes of Tris-HEPES buffer (Tris 4.5, HEPES 5 mmol·L^{-1}, pH 7.4) containing edetic acid 1 mmol·L^{-1}, and twice in Tris-HEPES buffer without edetic acid. The final pellets were stored at – 70 ℃ for at least 18 h. Before binding assay, the membrane pellets were washed 4 more times with Tris-HEPES buffer (Tris 4.5, HEPES 5 mmol·L^{-1}, pH 7.4) to remove the endogenous amino acids.

In binding assay, [^{3}H]Diz 100 μL (final concentration 3 nmmol · L^{-1}), membrane preparation 100 μL (50 – 300 μg protein), and Hup A 100 μL (0.001 – 300 μmol·L^{-1}) were mixed at 23 ℃ in the presence or absence of *L*-glutamate 100 μL (10 μmol·L^{-1}), and added with Tris-HEPES buffer (Tris 4.5, HEPES 5 mmol·L^{-1}, pH 7.4) to a final volume of 1 mL. Following incubation at 23 ℃ for 1 h, binding was terminated by filtration using Whatman GF/B

filters and a Brandel M-24 Cell Harvester. Radioactivity was measured using Beckman LS 6000LL liquid scintillometer. Nonspecific binding was determined in the presence of unlabeled *d*-Diz 100 $\mu mol \cdot L^{-1}$. All individual assays were carried out in replicates of three.

Data analysis The data were presented as $\bar{x} \pm s$. The IC_{50} values were calculated using computer software 'GraphPad InPlot'. Student's *t*-test was used to estimate the statistical significance.

RESULTS

Inhibitiory effect of Hup A on NMDA-induced current in acutely dissociated hippocampal pyramidal neurons Application of NMDA 100 $\mu mol \cdot L^{-1}$ to the recorded neuron induced large amplitude (0.5 – 1 nA) inward current with fast onset and slow decay. Hup A 0.1 – 300 $\mu mol \cdot L^{-1}$ inhibited NMDA-induced current in a concentration-dependent manner with IC_{50} of 45.4 $\mu mol \cdot L^{-1}$. The inhibitory effect caused by Hup A was reversible. NMDA-induced current recovered completely after washout for a few seconds. (Fig 1)

Modulation of [^{3}H]Diz binding by Hup A in synaptic membrane of cerebral cortex In extensively washed crude synaptic membrane, Hup A inhibited [^{3}H]Diz binding in a concentration-dependent manner with IC_{50} of 0.49 $\mu mol \cdot L^{-1}$ (Fig 2). We further investigated whether Hup A inhibited [^{3}H]Diz binding in the presence of *L*-glutamate. Addition of *L*-glutamate 10 $\mu mol \cdot L^{-1}$ markedly increased [^{3}H]Diz binding [from 160 to 370 $pmol \cdot g^{-1}$ (protein)]. Hup A 0.001 – 0.1 $\mu mol \cdot L^{-1}$ caused a further increase in the binding, whereas Hup A 1 – 300 $\mu mol \cdot L^{-1}$ inhibited the binding in a concentration-dependent manner with IC_{50} of 7.95 $\mu mol \cdot L^{-1}$.

The results of 4 – 5 separate experiments were summarized in Tab 1. It was clear that in

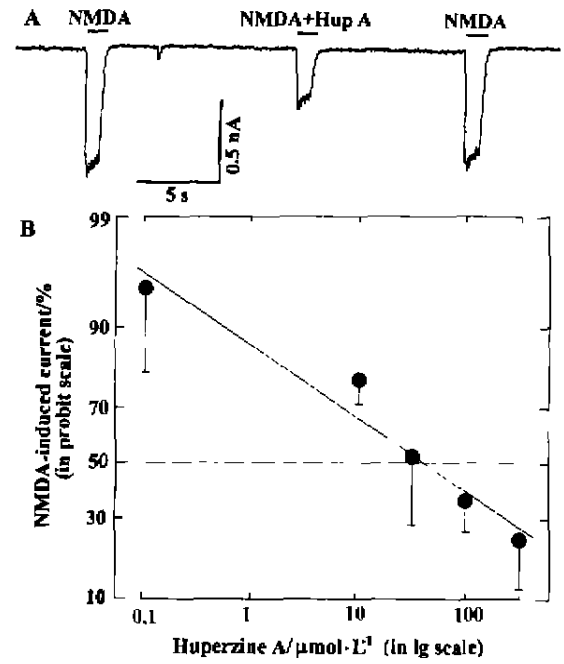

Fig 1. A) Hup A reversibly inhibited NMDA-induced current in acutely dissociated hippocampal pyramidal neurons. Both concentrations of NMDA and Hup A were 100 $\mu mol \cdot L^{-1}$. B) The concentration-response curve of Hup A inhibiting NMDA-induced current. $n = 2 - 5$ cells. $\bar{x} \pm s$.

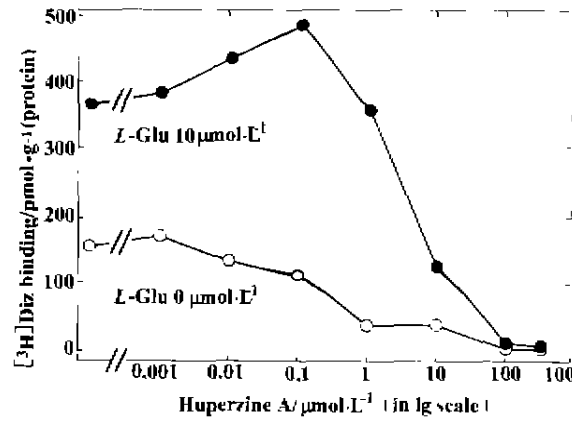

Fig 2. A representative experiment of modulation of specific binding of [^{3}H]Diz (3 $nmol \cdot L^{-1}$) by Hup A in the absence or presence of *L*-glutamate 10 $\mu mol \cdot L^{-1}$. The experiment was replicated at least 4 times with similar results.

the absence of *L*-glutamate Hup A caused concentration-dependent inhibition of [^{3}H]Diz

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binding. In the presence of *L*-glutamate Hup A 0.1 $\mu mol \cdot L^{-1}$ caused a small but statistically significant ($P < 0.05$) increase in the binding; whereas Hup A 1 – 300 $\mu mol \cdot L^{-1}$ markedly inhibited the binding. In the absence and presence of *L*-glutamate, the IC_{50} values were 0.5 (0.1 – 1.9) $\mu mol \cdot L^{-1}$ ($n = 4$) and 12.3 (5.8 – 26.3) $\mu mol \cdot L^{-1}$ ($n = 5$), respectively.

Tab 1. Effects of Hup A on [^{3}H]Diz (3 nmol · L^{-1}) binding in the absence and presence of *L*-glutamate. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Hup A / $\mu mol \cdot L^{-1}$	[^{3}H]Diz specific binding / $pmol \cdot g^{-1}$ (protein) Glutamate 0 $\mu mol \cdot L^{-1}$ ($n = 4$)	Glutamate 10 $\mu mol \cdot L^{-1}$ ($n = 5$)
Control	206 ± 36	438 ± 56
0.001	168 ± 26	377 ± 50
0.01	152 ± 24^b	462 ± 64
0.1	160 ± 61	619 ± 152^b
1	97 ± 43^c	507 ± 197
10	67 ± 37^c	297 ± 238
100	35 ± 25^c	118 ± 59^c
300	18 ± 36^c	98 ± 70^c

DISCUSSION

We investigated the effect of Hup A on NMDA receptor using electrophysiological method and radioligand binding assay. With whole-cell recording only the inhibitory effect of Hup A on NMDA-induced current was found, whereas both enhancement and inhibitory effect were observed in [^{3}H]Diz binding assay. The reason for this discrepancy in two systems remains to be elucidated. However, Mg^{2+} that blocks NMDA receptor-channels in electrophysiological study was found to exert similar dual effect on [^{3}H]Diz binding in brain synaptic membrane[9]. Although the preliminary results with whole-cell recording might be considered as an indirect effect of Hup A on NMDA receptor through some unidentified mechanisms, our results in [^{3}H]Diz binding assay clearly demonstrated that Hup A acted directly on NMDA receptor. But, these results do not imply that Hup A necessarily acted on Diz binding site within NMDA receptor-channel complex, since Diz binding site can be allosterically modulated by a variety of endogenous substances and drugs[9-11]. The action site of Hup A on NMDA receptor-channel complex now is under intensive investigation in our laboratory.

Our results are coincident with the neuroprotective action of Hup A reported by Ved *et al*[6]. Furthermore, the present study has revealed the mechanism by which Hup A protected the cultured neuronal cell from glutamate-induced excitotoxicity. Hup A indeed acted as a potent NMDA receptor antagonist to exert its neuroprotective action. We noted that the IC_{50} value of Hup A in [^{3}H]Diz binding assay (12.3 $\mu mol \cdot L^{-1}$) was about two orders of magnitude higher than the most effective concentration in neuroprotective action (100 $nmol \cdot L^{-1}$)[6]. On the other hand, the IC_{50} values in radioligand binding assay and electrophysiological study in the present study are comparatively close (12.3 *vs* 45.4 $\mu mol \cdot L^{-1}$). The main reason may be that NMDA receptor in cultured neurons possesses higher sensitivity to Hup A than that in naive neurons. Another explanation is that although the effect of Hup A measured in the two studies is closely related, but quite different. Cell survival was used to evaluate the neuroprotective action of Hup A[6]. This effect is obviously more complex than the effect measured at receptor level in the present study.

Hup A has been developed as a new drug to treat AD patients in China[4]. As a selective inhibitor of AChE, Hup A theoretically could only improve the symptoms of AD patients, but could not interfere with the process of pathogenesis in AD patient's brain. If excitotoxicity caused by overstimulation of glutamate receptor (particularly NMDA receptor) is indeed

involved in the pathogenesis in AD, Hup A, as potent NMDA receptor antagonist with less side effects, may be used as a preventative agent to slow down or block the pathogenesis process in early stage of AD.

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石杉碱甲对大鼠大脑皮层 NMDA 受体的调制作用[1]

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关键词 石杉碱甲; 胆碱酯酶抑制剂; *N*-甲基-*D*-天冬氨酸受体; 谷氨酸钠; 地佐环平马来酸盐; 海马; 锥体细胞; 大脑皮质; 膜片箝技术; 放射配位体测定

目的: 研究石杉碱甲(Hup A)对大脑皮层 NMDA 受体的影响. **方法**: 1) 用急性分离海马锥细胞全细胞记录研究Hup A对 NMDA 诱发电流的影响. 2) 用大脑皮层突触膜标本研究Hup A对[^{3}H]Diz 特异性结合的影响. **结果**: 1) Hup A 可逆地抑制 NMDA 诱发的电流反应(IC_{50} = 45.4 $\mu mol \cdot L^{-1}$). 2) 在突触膜标本, Hup A抑制[^{3}H]Diz 的结合量(IC_{50} = 0.5 (0.1 – 1.9) $\mu mol \cdot L^{-1}$, n = 4). 3) *L*-谷氨酸 10 $\mu mol \cdot L^{-1}$增加[^{3}H]Diz结合量. 加入 *L*-谷氨酸后, Hup A 0.001 – 0.1 $\mu mol \cdot L^{-1}$进一步增加结合量; Hup A 1 – 300 $\mu mol \cdot L^{-1}$则抑制结合量(IC_{50} = 12.3 (5.8 – 26.3) $\mu mol \cdot L^{-1}$, n = 5). **结论**: Hup A在大脑皮层除了抑制乙酰胆碱酯酶外, 还是 NMDA 受体拮抗剂.

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