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Effects of tetrandrine on changes of NMDA receptor channel in cortical neurons of rat induced by anoxia

WANG Zhong-Feng[1], XUE Chun-Sheng, ZHOU Qi-Xin, WAN Zi-Bing[2], LUO Quan-Sheng[2]
(*Department of Pharmacology, Chongqing University of Medical Sciences, Chongqing 400046, [2]Department of Physiology, Third Military Medical University, Chongqing 400038, China*)

KEY WORDS tetrandrine; *N*-methyl-*D*-aspartate receptors; ion channels; patch-clamp techniques; cerebral cortex; anoxia; dizocilpine maleate

ABSTRACT

AIM: To study the effects of tetrandrine (Tet) on the changes of NMDA receptor channels in cortical neurons induced by anoxia. **METHODS**: Cell-attached configuration of patch-clamp techniques. Anoxia was produced by perfused cells with 95 % N_2 + 5 % CO_2 gassed bath solution. **RESULTS**: During anoxia, the open time constant (τ_2), open probability (P_o) of 35-pS and 100-pS channels increased. Tet 7.5 μmol·L^{-1} reduced the P_o of 35-pS and 100-pS channels. 15 and 30 μmol·L^{-1} inhibited open of 100-pS channel fully, and changed the open time constant of 35-pS from two to single exponential distribution. **CONCLUSION**: Tet inhibition of the open of NMDA receptor channels induced by anoxia was one of its protective mechanisms.

INTRODUCTION

N-methyl-*D*-aspartate (NMDA) receptor was one of the most important receptors which played an important role in physiologic and pathologic processes. NMDA receptor subtypes formed cation selective ion channel, NMDA receptor channel. The channel was activated due to increased release of excitatory amino acids (EAA) in brain induced by anoxia, which resulted in Ca^{2+} inflow across the cell membrane, intracellular Ca^{2+} concentration raising and cell injury[1,2]. Previous study in our lab demonstrated that tetrandrine (Tet) inhibited the release of EAA from ischemic brain, and decreased the intracellular Ca^{2+} concentration in rat brain synaptosome induced by EAA[3]. In this study, we investigated the effects of Tet on the changes of NMDA receptor channel induced by anoxia in rat cortical neurons.

MATERIALS AND METHODS

Drugs and reagents Tet, a product of Jinhua Pharmaceutical Factory (Jinhua, Zhejiang Province, China), with a purity of 98 %. Trypsin, NMDA, HEPES, and *L*-glycine were purchased from Sigma Chemical Co (St Louis Mo, USA). Tetrodotoxin (TTX) was produced by Aquatic Institute of Hebei Province.

Isolation of rat cerebral cortical neurons[4] Wistar rats (2 - 4 wk old, Certificate № 24301050) supplied by Experimental Animal Center, Third Military Medical University, were decapitated. The cerebrum was cut into 400 - 500 μm thick coronal slices, then transferred to artificial cerebrospinal fluid (ACSF) containing NaCl 126, KCl 5, NaH_2PO_4 1.25, $NaHCO_3$ 26, $CaCl_2$ 2, $MgSO_4$ 2, glucose 10 mmol·L^{-1}, pH 7.2, gassed continuously with 95 % O_2 + 5 % CO_2. After 30 min, two slices were transferred to 2 mL ACSF containing trypsin 2 mg to be digested for 30 min at (23 ± 2) ℃. The slices were rinsed with ACSF containing HEPES 10 mmol·L^{-1}, then cut into 1 - 2 mm^3 blocks, and the cells were dissociated using fire-polished Pasteur pipette with tip diameter of 0.2 - 0.5 mm. The suspension of dissociated cells was dropped onto poly-*L*-lysine coated coverslips to allow neurons settled on.

[1] Correspondence to Dr WANG Zhong-Feng, now in *Shanghai Institute of Physiology, Chinese Academy of Sciences, Shanghai 200031, China*. Phn 86-21-6437-0080. Fax 86-21-6433-2445. E-mail zfwang@sunm.shcnc.ac.cn
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Single channel recording Single channel recording was performed at (23 ± 2) ℃ using the cell-attached configuration of the patch-clamp techniques [5]. The electrode was pulled at two stages by puller (PP-83, Narishige, Japan) with a tip diameter 1 – 1.5 μm and resistance 4 – 5 MΩ. Single channel currents were detected by a patch-clamp amplifier (CEZ-2200, Nihon Kohden, Japan) and gap-free recorded by Fetchex program in the pClamp software (Ver 6.0.2, Axon Instrument, USA) at a frequency 20 kHz. The cell holding potential was set at −80 mV.

Solution Bath solution contained NaCl 140, CsCl 5, $CaCl_2$ 1.8, HEPES 10, TTX 0.001, and glucose 10 mmol·L^{-1} (pH 7.2). The patch electrode solution contained NMDA 20 μmol·L^{-1} and *L*-glycine 1 μmol·L^{-1}.

The anoxia bath solution was the same as the nonnal bath solution except glucose, and gassed with 95 % N_2 + 5 % CO_2 for 1 h before use.

Groups There were 6 groups: control, anoxia, anoxia + Tet 7.5, 15, 30 μmol·L^{-1} and anoxia + dizocilpine maleate 0.05 g·L^{-1}.

Anoxia was obtained by adding anoxic bath solution to cells[6,7].

Tet was made up as concentrated stocks in HCl 1 mmol·L^{-1}. After the anoxic currents were recorded, Tet was added cumulatively into bath solution at an interval of 15 min, then the currents in the same patch were recorded. There was no effect on pH when Tet was added into bath solution. In anoxia + dizocilpine maleate group, dizocilpine maleate was preadded into bath solution before recorded.

Data analysis The open and close times were exponential fitting. Current amplitudes were fitted by Gaussian distributions. Open probability (P_o) was calculated from the sum of open time for each sweep divided by sweep duration. The conductance of channel was calculated[8]. The data were expressed as $\bar{x} \pm s$ and treated with ANOVA.

RESULTS

NMDA-receptor channel in rat cerebral neurons has two conductance levels, (35.15 ± 0.18) pS and (100.13 ± 0.06) pS. The current amplitudes were (2.79 ± 0.14) pA and (7.79 ± 0.05) pA, respectively. The open time of 35 pS and 100 pS channel accorded with two exponential distributions, and close time with single exponential distribution (n = 8 cell patches in 8 rats, Tab 1, Fig 1).

During anoxia, the open time constant τ_2 and P_o increased in 35 pS channel, open time constant τ_1 and τ_2 of 100 pS channel had no significant change in comparison with control group, but P_o increased. In some cell patches which contained 35 pS and 100 pS channel, the P_o reached 0.90 ± 0.03 (n = 7). The close time constant τ decreased.

We chose the cell patches which contained two level channels for experiment. After the anoxia channel current was recorded, Tet was added cumulatively to bath solution at an interval of 15 min. The current in the same patch was recorded again. Tet 7.5 μmol·L^{-1} decreased the P_o of 35 pS and 100 pS channel, the open time constant τ_1 of 35 pS channel and τ_2 of 100 pS channel decreased, the close time constant τ increased when compared with that in anoxia. Tet at the concentration of 15 and 30 μmol·L^{-1} inhibited the open of the 100 pS channel completely, and changed

Tab 1. Effect of tetrandrine (Tet, μmol·L^{-1}) on changes of NMDA receptor channel of rat cortical neurons induced by anoxia. $\bar{x} \pm s$, n = 7. $^aP > 0.05$, $^cP < 0.01$ *vs* control. $^dP > 0.05$, $^eP < 0.05$, $^fP < 0.01$ *vs* anoxia.

	10^2 × Open time ms				Close time/ms	10^3 × Open probability	
	35 pS		100 pS				
	τ_1	τ_2	τ_1	τ_2		35 pS	100 pS
Control	56 ± 10	417 ± 38	50 ± 38	780 ± 174	75 ± 14	79 ± 6	67 ± 4
Anoxia	69 ± 15^a	854 ± 205^c	58 ± 12^a	791 ± 253^a	11 ± 4^c	308 ± 156^c	488 ± 126^c
Tet (7.5)	15 ± 2^e	893 ± 137^d	51 ± 23^d	541 ± 87^e	80 ± 10^f	62 ± 10^f	32 ± 7^f
Tet (15)	273 ± 25				373 ± 36^f	9 ± 2^f	
Tet (30)	139 ± 21				800 ± 80^f	1 ± 1^f	

the open time constant of 35 pS channel to single exponential distribution, P_o of 35 pS channel decreased ($n = 7$ cell patches from 7 rats, Tab 1, Fig 1).

Dizocilpine maleate, an NMDA receptor antagonist, also inhibited the open of NMDA receptor channel induced by anoxia ($n = 5$ cell patches in 5 rats, Fig 1).

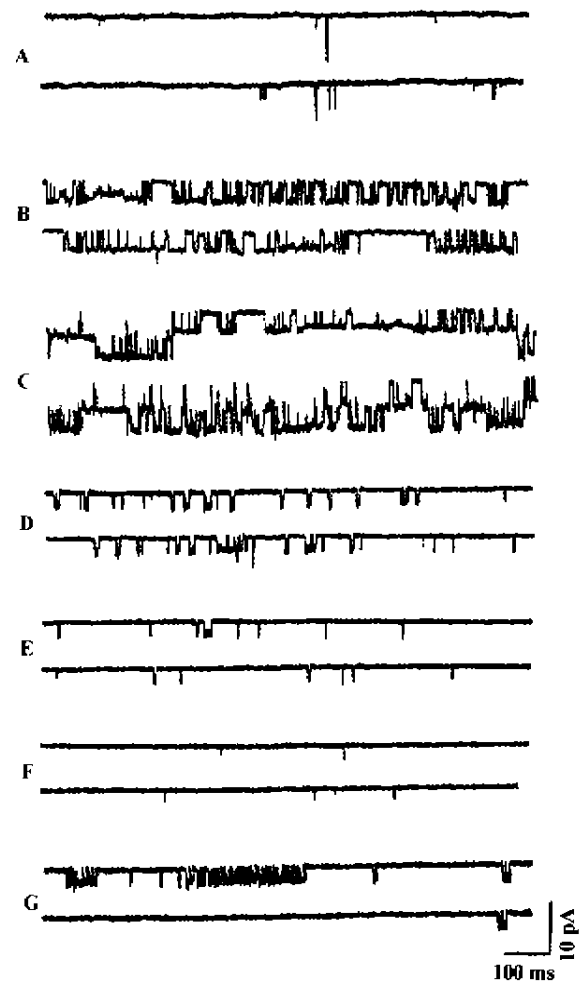

Fig 1. Effects of tetrandrine (Tet, μmol·L^{-1}) on changes of NMDA receptor channels. Holding potential −80 mV. A) single channel current in normal condition, B) 35 pS channel current in anoxia, C) 35 and 100 pS channel currents in anoxia, D) − G) Tet 7.5, 15, 30 μmol·L^{-1} and dizocilpine maleate 0.05 g·L^{-1} on channel current in anoxia, respectively.

DISCUSSION

Many researchers have identified the NMDA receptor channel properties. The high Ca^{2+} permeability, non-selective permeability to Na^+ and K^+ and not Cl^-determined Ca^{2+} inflow when NMDA receptor channel was activated[9-11]. The results presented here show that those properties of NMDA receptor channels are the same as others reported, and suggest that the traces in our test are the definite NMDA receptor channels.

During anoxia, NMDA receptor channels were activated, which induced an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The elevated $[Ca^{2+}]_i$ triggers a cascade of intracellular events, leading to acute neuronal injury. When Tet was added to the outside of the cells, the NMDA receptor channel openings induced by anoxia were dramatically inhibited in a concentration-dependent manner. Tet 7.5 μmol·L^{-1} affected open channel parameters including open frequency and probability, 15 and 30 μmol·L^{-1} inhibited fully the 100 pS channel opening, which suggest that Tet have stronger effects on large conductance subtype channel. Furthermore, Tet changed the open time constant distribution, which indicate that it may inhibit the NMDA receptor channels induced by anoxia through influencing the open model and kinetics. Our results suggest that Tet protect the brain from anoxic injury through inhibiting the open of NMDA receptor channels, and reducing the $[Ca^{2+}]_i$.

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粉防己碱对缺氧诱导的大鼠大脑皮层神经元 NMDA 受体通道变化的影响

王中峰[1]，薛春生，周歧新，万子兵[2]，罗全生[2]
(重庆医科大学药理教研室，重庆 400046；
[2]第三军医大学生理教研室，重庆 400038，中国)

关键词 粉防己碱；*N*-甲基-*D*-天冬氨酸受体；离子通道；膜片箝技术；大脑皮质；低氧；地佐环平马来酸盐

目的：研究 Tet 对缺氧大鼠大脑皮层神经元 NMDA 受体通道变化的影响。**方法**：细胞贴附膜片箝法，用 95 %N_2 + 5 %CO_2 饱合无糖浴液产生细胞缺氧模型。**结果**：在缺氧状态下，35 pS 通道开放时间常数 τ_2、35 和 100 pS 通道开放概率增加，Tet 7.5 $\mu mol \cdot L^{-1}$降低缺血所致 35 和 100 pS 通道开放概率，15 和 30 $\mu mol \cdot L^{-1}$完全抑制 100 pS 通道，改变 35 pS 的开放时间常数为单指数分布。**结论**：抑制缺氧所致 NMDA 受体通道开放是 Tet 对受缺氧损伤大脑保护作用的机制之一。

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Corrigendum

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In **Methods**: The tablets of Hup were manufactured by Joysun Aggregation/ Henan Zhulin Pharmaceutical Company Ltd, the trade mark is Haboyin, while the capsules of Hup were provided by Ningbo Lihua Pharmaceutical Company Ltd.