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CHLORPROMAZINE AGAINST THE DAMAGES OF CEREBRAL ISCHEMIA RELATED TO THE INHIBITORY EFFECTS OF VOLTAGE-GATED SODIUM CHANNEL

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Abstract: The purpose of the study was to investigate the effects of chlorpromazine (CPZ) on cerebral ischemia in rats and their relative ion channel mechanism. Effects of CPZ were tested *in vitro* on voltage-dependent sodium channel (VGSC) using patch-clamp in freshly dissociated rat hippocampal neurons and *in vivo* using a rat model of experimental stroke caused by transient middle cerebral artery occlusion (MCAO). The results showed that CPZ at 10 mg/kg given 1 h after the initiation of MCAO was effective in reducing cerebral infarct volumes measured 24 h later. CPZ at 30 μmol/L reversibly reduced the amplitudes of Na^+ current and activation process. In conclusion, CPZ is neuroprotective when given as a single administration after initiation of MCAO. These data indicate that CPZ may be a useful neuroprotectant in stroke therapy; its neuroprotective potential may come from the inhibitory effects of CPZ on Na^+ channel.

Key Words: Na^+ current; Patch clamp; Neurons; Cerebral ischemia; Chlorpromazine; Neuroprotective

0 Introduction

At present, cerebral ischemia is still one of the main causes for death and disability. Phenothiazines (PTZ) are commonly used in treatment of psychiatric disorders such as schizophrenia. However, it is controversial whether using PTZ alone plays a neuroprotective role in cerebral ischemia.

Ion channels in cell membrane are targets for many toxins and drugs. Much damage on the central nervous system (CNS) is caused by interrupting the function of ion channels [1]. The voltage-gated sodium channel (VGSC) is a fundamental element in the central and peripheral nervous systems. It is general accepted that the damage of neurons is induced partly by the changes of VGSC currents in cerebral ischemia. The inhibitory effect is an important mechanism of many kinds of neuroprotective drugs. In the first step of the study, we investigated weather CPZ had neuroprotective effects in brain ischemia with transient middle cerebral artery occlusion (MCAO) model of rats. The changes of VGSC kinetic properties induced by CPZ are examined that seems to be necessary for understanding the underlied mechanisms, since the changes of VGSC currents play an important role in cerebral ischemia.

1 Materials and Methods

1.1 Transient Middle Cerebral Artery Occlusion (MCAO)

Male Sprague-Dawley (SD, Chinese academy

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of medical sciences) rats weighing 280~300 g were housed on a 12-hour day-night cycle with free access to food and water. Anesthesia was induced with 10% Chloral Hydrate i.p. Body temperature was maintained at 37℃ using a heating pad during the experiment. One hour after the MCAO induction, induced by an intraluminal method described by Koizumi *et al*[2], CPZ (10 mg/kg) was given i.p. followed by reperfusion. Normal saline was given to control rats under the same protocol. 24 h later, the brain was removed and sectioned coronally into seven slices of 2 mm thickness starting from the frontal pole. Slices were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) for 30 min at room temperature. Areas ipsilateral to the occlusion, which were not stained, were recorded as infarcted. Infarct volume was measured by using an imaging system.

1.2 Cell isolation

Animals were provided by the experimental animal center of Tianjin academy of medicine. Hippocampal CA1 neurons were acutely isolated by enzymatic digestion and mechanical dispersion from 7 to 10-d-old Wistar rats as described in our previous works[3], with a few modifications. Rats were decapitated under ether anesthesia. The hippocampi were removed and coronary slices were cut at a thickness of approximately 500 μm in ice-cold oxygenated artificial cerebrospinal fluid (ACSF) containing (mmol/L): NaCl 134, KCl 5, NaH_2PO_4 1.5, $MgSO_4$ 2, $CaCl_2$ 2, $NaHCO_3$ 25, Glucose 10, Hepes 10 (pH 7.25 with NaOH) within 30 s. The slices were incubated in an ACSF saturated with pure O_2 at 37℃ for 1 h, treated with Pronase E 6.0~7.0 kU/L for 25 min in the oxygenated ACSF at 37℃. After digestion the slices were washed six times with ACSF and incubated in the same solution saturated with pure O_2 at room temperature. CA1 regions were dissected out and transferred into centrifuge tubes. Hippocampal neurons were dispersed by gentle pipetting using fine glass tubes. After 5 min, the cell suspension was transferred into the recording chamber with a glass coverslip filled with external solution. The cells were left for approximately 30 min before beginning the experiments.

1.3 Electrophysiology

The neurons were placed in a recording chamber mounted on the stage of positive direction microscope (Olympus, Japan) and super fused with extra cellular solution at room temperature (21~22℃). Extracellular solution for recording whole cell currents was composed of (mmol/L): NaCl 134，KCl 5, $CaCl_2$ 2, HEPES 10, glucose 10, NaH_2PO_4 1.5, $MgSO_4$ 2, $NaHCO_3$ 25, and the pH was adjusted to 7.2 with NaOH. Extracellular application of drugs was carried out by perfusing cells with extracellular solution containing the drugs. Whole-cell patch experiments were carried out using an EPC10 (HEKA Electronics, Lambrecht, Germany) amplifier driven by Pulse software (HEKA, German). In the voltage-clamp experiments, the cells were stepped from −80 mV (20 ms) to −10 mV (40 ms). Inward currents were evoked by depolarizing pulses, and reversibly abolished by application of 0.5 μmol/L tetrodotoxin (TTX) and thus considered as I_{Na}. The protocol was applied every 1 min. Glass pipettes were used with a resistance of about 3~ 5 MΩ, when filled with a pipette solution composed of (mmol/L): 120 CsCl, 20 tetraethylammonium chloride (TEA-Cl), 2 $MgCl_2$, 10 EGTA, 2 Na_2-ATP, 10 HEPES, and the pH was adjusted to 7.20 with CsOH. Data were acquired at a sampling rate of 5 kHz, filtered at 2 kHz, stored on hard disk and analyzed off-line using the Pulsefit analysis software package (HEKA, German). Graphical and statistical data analyses were carried out using Origin 7.0.

1.4 Data analysis

Data are presented as mean±SEM. Statistical significance was assessed using a Student's paired or unpaired *t* test as appropriate, and $P<0.05$ was considered significant. All data analyses were performed using the software SPSS 11.5.

2 Results

2.1 Effects of CPZ on ischemic brain injury *in vivo*

24 hours after MCAO surgery, control rats exhibited visible intracerebral damage 〔(32.75 ± 7.09)%, Fig.1A〕. In rats treated with CPZ, the infarct volume was significantly reduced to (24.24±3.68)% (Fig.1B. P<0.05 vs model group).

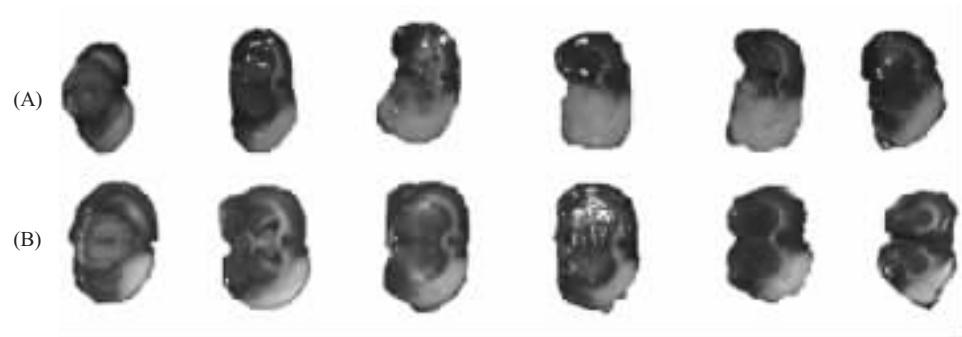

Fig.1 Ischemic area by TTC staining. (A) Model group; (B) CPZ administration group

2.2 Effects of CPZ on sodium currents and current-voltage relationship

Figure 2A showed I_{Na} elicited by a depolarizing command pulse from a holding potential of −100 to 60 mV for 40 ms in a hippocampal CA1 neuron. CPZ was applied in the external medium once I_{Na} were stable after membrane rupture. CPZ at 30 μmol/L decreased the current peak to (82.5± 7.9)% (n=8, P<0.05) (Fig.2B).

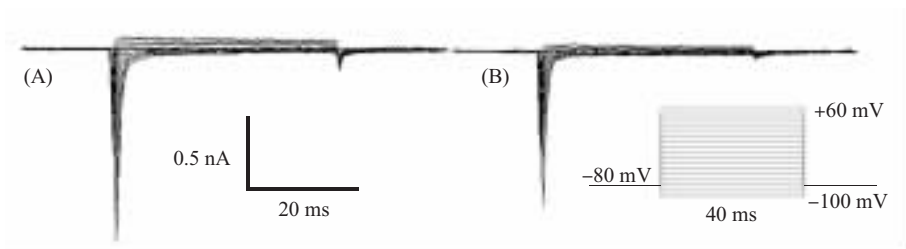

Fig.2 Effect of CPZ on I_{Na}. (A) Before application of CPZ; (B) Current traces obtained after treatment with CPZ

For analysis of the current-voltage (I-V) relationship, neurons were held at −80 mV, I_{Na} was obtained by depolarizing steps from a command potential of −100 to +60 mV at 10 mV steps. Upon the application of 30 μmol/L CPZ, the amplitudes of I_{Na} were decreased differently at different membrane potential, which indicated that CPZ decreased the amplitudes of I_{Na} in a voltage-dependent manner (Fig.3).

2.3 Effects of CPZ on activation and inactivation kinetics of sodium current

The steady-state activation curves for I_{Na} under control and after the application of 30 μmol/L

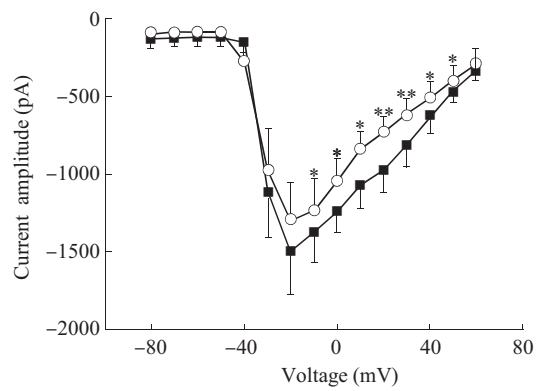

Fig.3 Effect of 30 μmol/L CPZ on the I-V curve of I_{Na}. *P<0.05 **P<0.01 versus control. ■: Control; ○: CPZ

CPZ are exhibited in Fig.4. Peak amplitude for I_{Na} evoked by the step pulses from −80 to +20 mV was converted into conductance by use of the equation $G=I/(V-V_{Na})$, where G is the conductance, I the current, V the membrane potential, and V_{Na} the reversal potential. With the least squares fit procedure, the normalized conductance was well fitted with a Boltzmann equation: $G/G_{max}=1/\{1+\exp[(V-V_h)/k]\}$, where G_{max} the maximal conductance, V_h the membrane potential at half-activation, and k the slope factor. Before and after application of 30 μmol/L CPZ, the values of V_h were (−31.9±8.25) mV and (−25.2±8.04) mV, respectively (n=10, P<0.05), with k of (2.48±0.71) and (1.02±0.68) (n=10, P>0.05), indicated that the activation curve was shifted toward more negative potential and the activation process was reduced.

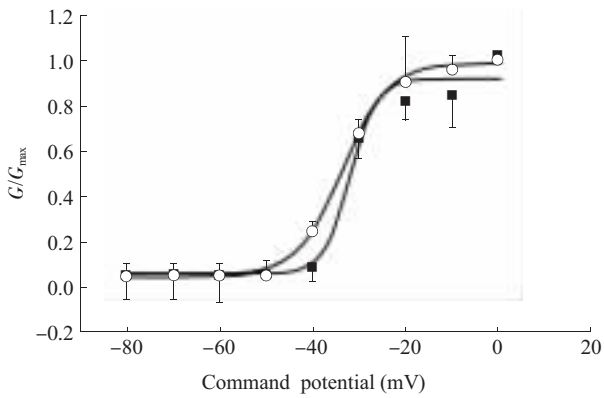

Fig.4 Effect of CPZ on steady-state activation kinetics of sodium current. Steady-state activation curves of I_{Na} before and after application of 30 μmol/L CPZ. ■: Control; ○: CPZ

Fig.5 presented the steady-state inactivation curves for I_{Na} under control and after the application of 30 μmol/L CPZ. The steady-state inactivation was studied using the protocols below: neurons were held at −80 mV and currents were elicited with a 40 ms test pulse to −10 mV preceded by 100 ms prepulses to potentials between −80 and +20 mV. Peak amplitudes for I_{Na} were normalized and plotted versus prepulse potentials. With the least square fit method, the curves were well fitted with Boltzmann equation: $I/I_{max}=1/\{1+\exp[(V-V_h)/k]\}$, where I_{max} the maximal

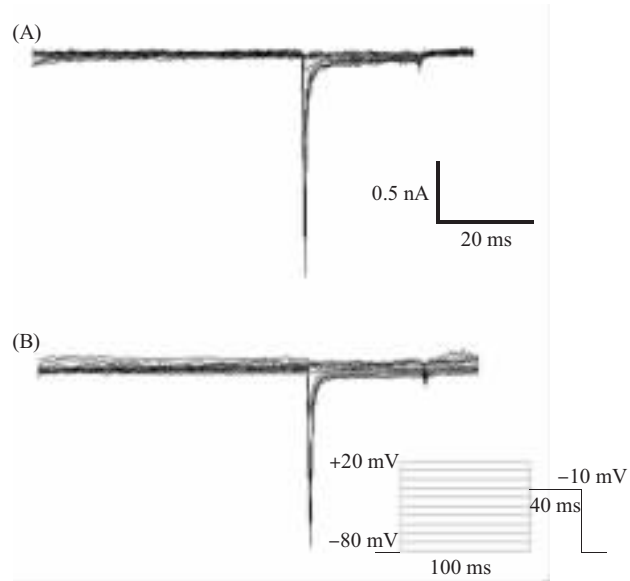

Fig.5 Effect of CPZ on inactivation I_{Na}. (A) Before application of CPZ; (B) Current traces obtained after treatment with CPZ

current, V_h the membrane potential at half- activation, and k slope factor. Before and after application of 30 μmol/L CPZ, the values of V_h were (−43.90±3.74) mV and (−46.08±3.69) mV (n=6, P>0.05), with k of (6.32±1.18) mV and (6.32±1.18) mV (n=6, P>0.05), indicated that CPZ had no effect on the inactivation progress of I_{Na}. (Fig.6).

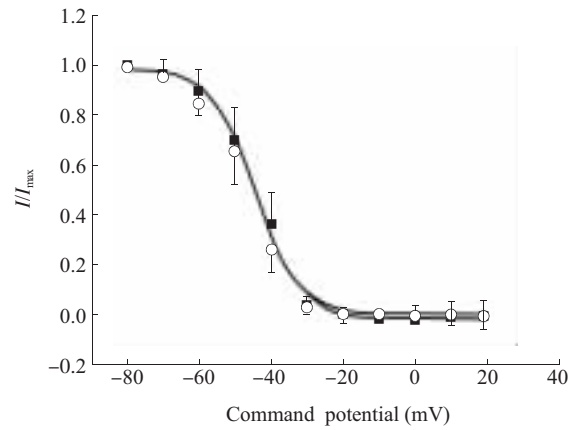

Fig.6 Effect of CPZ on steady-state inactivation kinetics of I_{Na}. Steady-state inactivation curves of I_{Na} before and after application of 30 μmol/L CPZ. ■: Control; ○: CPZ

2.4 Effects of CPZ on the recovery from inactivation of sodium channels

The time course of recovery of sodium

channels from inactivation was studied using the protocols below: a 7-ms conditioning depolarizing pulse to −10 mV was employed to fully inactivate the sodium channels, and then a test pulse of −10 mV was applied after a series of −80 mV intervals varying from 2.0 to 24.0 ms (Fig.7). The peak value of I_{Na} evoked by the conditioning pulse was designated I_1, while the peak value of the I_{Na}

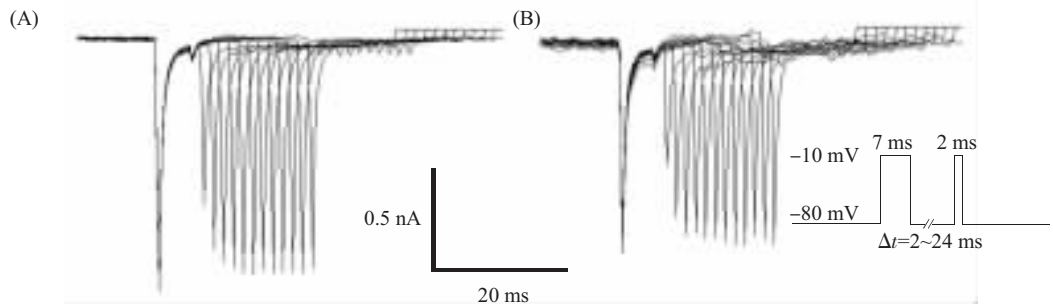

Fig.7 Effect of CPZ on recovery form inactivation I_{Na}. (A) Before application of CPZ; (B) Current traces obtained after treatment with CPZ

evoked by the test pulse was designated I_2. The ratio of I_2 to I_1 represents the recovery of I_{Na} from inactivation. The plot of I_2/I_1 vs. the duration of the −80-mV intervals was well fitted with a monoexponential function ($I/I_{max}=A+B_{exp}(-t/\tau)$, and the time constant was calculated. Before and after application of 30 μmol/L CPZ, the rates of I_{Na} recovery were (2.04±0.75) ms and (2.44±1.31) ms (n=6, P>0.05). Application of 30 μmol/L CPZ had no obvious effect on the rate of I_{Na} recovery from inactivation and delayed the channel recovery course (Fig.8).

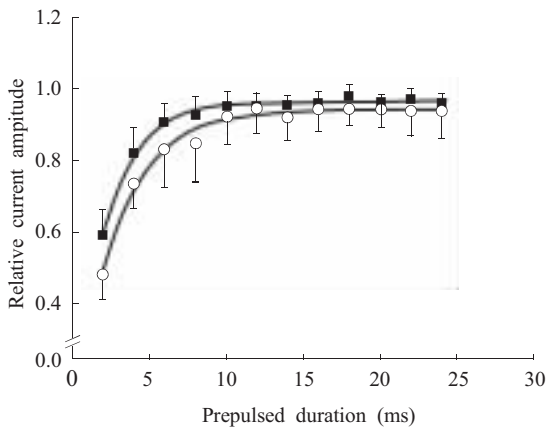

Fig.8 Effect of CPZ on recovery kinetics of I_{Na}. Recovery kinetics curves of sodium currents before and after application of 30 μmol/L CPZ. ■: Control; ○: CPZ

3 Discussion

MCAO model has been used widely, and it is considered to be a model of severe ischemia. In the present study, CPZ showed a neuroprotective effect according to the results of TTC staining.

It has been reported that cerebral ischemia or severe anoxia could induce sustained Na^+ influx. The present results indicated that CPZ inhibited I_{Na} currents of rat hippocampal neurons. Na^+ overload reversed the action of the Na^+/K^+-ATPase and stimulated ATP turnover, facilitating energy expenditure [4]. In addition, the excessive Na^+ also reversed Na^+/Ca^{2+} exchange, resulting in an increase of intracellular Ca^{2+} [5], which triggered a cascade of harmful events via activation of protease, phospholipases and endonucleases [6]. Therefore blockade of Na^+ channel is considered to be a target for cerebral protection [7]. Many studies have demonstrated that Na^+ channel blockers such as lidocaine, lifarizine and magnesium, prevent ischemic and anoxic brain damages[8~11]. The neuroprotective effect of CPZ on cerebral ischemia may be related to the inhibitory action on the amplitudes of I_{Na}.

A negative shift of inactivation curve means

a lower membrane potential threshold for closing these channels [12]. In this study, CPZ had no effects on the steady-state inactivation of I_{Na}. On the other hand, CPZ produced a hyperpolarizing shift in the activation-voltage curve. The neuronal excitability was partly maintained by I_{Na}, the action of CPZ on the activation of I_{Na}, which might be related to the neuroprotective effects. However, the exact mechanisms remain undefined.

The effects of CPZ on I_{Na} may be related to the channel phosphorylation, because the VGSC of brain is a primary target for PKA and PKC. Activation of PKA and PKC reduces alterable kinetics of VGSC currents in CA1 neurons. Therefore, CPZ might affect the I_{Na} through the action of protein kinase.

In conclusion, the changes of I_{Na} currents induced by CPZ are likely related to its neuroprotective effects in cerebral ischemia. A further study seems to be necessary for understanding the mechanisms underlying CPZ inhibitory effects on the I_{Na} currents.

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氯丙嗪抑制电压门控钠通道电流拮抗脑缺血损伤

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摘要：为探讨氯丙嗪对脑缺血的保护作用及其可能的离子通道机制，采用全细胞膜片钳技术，在急性分离的新生大鼠海马锥体细胞上研究氯丙嗪对电压门控钠通道电流（I_{Na}）的影响，利用线栓法建立大鼠脑缺血再灌注动物模型，研究氯丙嗪对脑缺血的保护作用。结果显示，大鼠缺血 1 h 后腹腔注射氯丙嗪（10 mg/kg），24 h 后梗塞面积明显减小。30 μmol/L 氯丙嗪可以减小钠电流幅值及使 I_{Na} 激活曲线左移。实验结果提示氯丙嗪可能通过抑制 I_{Na} 而拮抗大鼠脑缺血所引起的损伤。

关键词：钠电流；膜片钳；神经元；缺血再灌注；氯丙嗪；神经保护
中图分类号：R338.8

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第九次全国暨海内外生物膜学术研讨会通知

由中国生物物理学会、中国生物化学与分子生物学会和中国细胞生物学会联合主办，中国生物物理学会＜膜与细胞生物物理专业委员会＞、中科院生物物理研究所“生物大分子国家重点实验室”、国家科技部“生物膜与膜蛋白的结构与功能研究”<973>项目和“生物膜与膜工程国家重点实验室”等联合承办的《第九次全国暨海内外生物膜学术研讨会》（简称 2007“生物膜学术研讨会”） 定于 2007 年 10 月 12~15 日在湖北宜昌召开。这次会议的主题有：(1)膜与膜蛋白的结构; (2)信号跨膜转导与细胞功能调控; (3)膜运送（Membrane traffic and transport）; (4)离子通道; (5)膜与能量转换; (6)生物膜与细胞凋亡; (7)膜与疾病和药物靶点; (8)膜蛋白组学; (9)生物膜研究的新技术和新方法（如 image,labels and probes, SiRNA and gene knockout, etc.）。

这是在国内召开的生物膜研究领域内的最高水准的学术会议。参加会议的除有来自中国科学院、中国医学科学院、高等院校和军队科研院校在内的全国从事生物膜及其相关研究和教学的教授、学者和研究生等外，我们还特别邀请了海外从事生物膜相关领域研究的中国学者蒞会。会上将有多位中科院院士和教授以及十余名海外中国学者做大会特邀报告。大会还将安排一般学术报告和研究生专题交流并进行评选，以及科学墙报和公司先进仪器和新技术、新方法介绍等多种学术交流形式。我们希望通过每三年届时举行的生物膜学术研讨会，共同交流和讨论当前国际、国内生物膜研究的现状、发展和动向，明确问题，找出差距。通过深入交流，认真切磋，热烈而无拘束的讨论，抓住热点，瞄准前沿，以利创新，推动我国生物膜领域中的理论和实际相结合的研究，使我国生物膜研究更加适应创新的形势要求，为进一步推动我国生命科学研究的发展做出贡献。有关详细信息请见会议的第一轮通知。

《第九次全国暨海内外生物膜学术研讨会》组委会
2007年 2 月