Effects of ginkgolide B on neuronal discharges in paraventricular nucleus of rat hypothalamic slices

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Abstract: Objective To study the central role of ginkgolide B (BN52021) in regulating cardiovascular function of nerve center by examining the effects of ginkgolide B on the electrical activity of rat paraventricular nucleus (PVN) neurons in hypothalamic slice preparation and to elucidate the mechanism involved. **Methods** Extracellular single-unit discharge recording technique. **Results** (1) In response to the application of ginkgolide B (0.1, 1, 10 $\mu\text{mol/L}$; $n = 27$) into the perfusate for 2 min, the spontaneous discharge rates (SDR) of 26 (26/27, 96.30%) neurons were significantly decreased in a dose-dependent manner. (2) Pretreatment with *L*-glutamate (*L*-Glu, 0.2 mmol/L) led to a marked increase in the SDR of all 8 (100%) neurons in an epileptiform pattern. The increased discharges were suppressed significantly after ginkgolide B (1 $\mu\text{mol/L}$) was applied into the perfusate for 2 min. (3) In 8 neurons, perfusion of the selective L-type calcium channel agonist, Bay K 8644 (0.1 $\mu\text{mol/L}$), induced a significant increase in the discharge rates of 8 (8/8, 100%) neurons, while ginkgolide B (1 $\mu\text{mol/L}$) applied into the perfusate, could inhibit the discharges of 8 (100%) neurons. (4) In 8 neurons, the broad potassium channels blocker, tetraethylammonium (TEA, 1 mmol/L) completely blocked the inhibitory effect of ginkgolide B (1 $\mu\text{mol/L}$). **Conclusion** These results suggest that ginkgolide B can inhibit the electrical activity of paraventricular neurons. The inhibitory effect may be related to the blockade of *L*-type voltage-activated calcium channel and potentially concerned with delayed rectifier potassium channel (K_{DR}).

Keywords: paraventricular hypothalamic nucleus; ginkgolide B; *L*-glutamate; Bay K 8644; TEA

1 Introduction

Ginkgo biloba, as an important Chinese medicine, has been cultivated and held sacred for its health-promoting properties. Since the development of *Ginkgo biloba* extract (*GbE*) in 1964, its effects on an extensive range of disorders and diseases have been investigated. *GbE* has antioxidative, vascular, and neuroprotective effects and is widely prescribed in Europe for the treatment of cerebrovascular and neurodegenerative disorders^[1,2]. It contains multiple compounds, e.g., flavonoids and terpenoids, which are thought to con-

tribute to its beneficial effects. *GbE* has been becoming increasingly popular as a treatment that is claimed to reduce memory loss and the symptoms of mild cognitive disorders including Alzheimer's disease (AD). Ginkgolide B is one of the major constituents of the terpenoid fraction of *GbE*. Numerous studies demonstrate that ginkgolide B, as a specific antagonist of platelet-activating factor (PAF), presents beneficial effects on various PAF-related diseases and specially has neuroprotective effects in the CNS^[3-6]. Recent evidence suggests that *GbE* protects against neuronal death in global brain ischemia and in glutamate-induced excitotoxicity^[7]. Moreover, PAF is involved in glutamate neurotoxicity in rat neuronal cultures^[8]. And calcium influx constitutes the ionic basis for the maintenance of glutamate-induced extended neuronal depolarization associated with neuronal death^[9]. The hypothalamic paraventricular nucleus (PVN) contains spinally-projecting neurons implicated in fine-tuning the car-

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Article ID: 1673-7067(2008)-06-0345-06
CLC: Q426; R338
Document code: A
Received date: 2008-07-16

diovascular system, and it is possible that ginkgolide B may exert its cardiovascular action through this area. The effect of ginkgolide B on spontaneous discharge of paraventricular nucleus neurons has not been reported. Therefore, to examine the effects of ginkgolide B on the spontaneous discharge of PVN neurons will contribute to understanding the effects of ginkgolide B in CNS. This study was designed to examine the effect of ginkgolide B on the electrical activity of rat paraventricular neurons in hypothalamic slice preparation using extracellular recording technique and to elucidate the mechanisms involved.

2 Materials and methods

2.1 Slice preparation All experiments were performed on paraventricular neurons of hypothalamic slices prepared from 18- to 24-day-old Sprague-Dawley rats (Grade II, Certificate No.802110, Hebei Laboratory Animal Center). Rats were rapidly decapitated, and the forebrains were removed and placed into a dish with ice-cold oxygenated artificial cerebrospinal fluid (ACSF). The composition of ACSF was (in mmol/L) 124 NaCl, 3.5 KCl, 1.24 KH_2PO_4 , 1.3 MgSO_4 , 1.5 CaCl_2 , 18 NaHCO_3 and 20 glucose; pH 7.40-7.45. The hypothalamus was dissected according to anatomic signs (Fig. 1) and then sliced (thickness of 350-500 μm). The slices were preincubated in ACSF aerated with 95% O_2 and 5% CO_2 for 60-90 min at room temperature before recording. A single slice was then transferred and placed on nylon net in a chamber (1 mL) and immobilized with a nylon net on the upper surface of the slice. The preparation was completely submerged in the perfusate which

maintained at 33-35 $^{\circ}\text{C}$ and perfused constantly at a rate of 2 mL/min.

2.2 Extracellular single-unit recording Extracellular single-unit recordings from paraventricular neurons of hypothalamic slices were obtained with glass microelectrodes filled with 0.5 mol/L sodium acetate containing 2% pontamine sky blue. Microelectrodes were pulled from 1 mm micropipettes. DC resistance of the microelectrode was 5-10 $\text{M}\Omega$. Electrical signals were amplified by a microelectrode amplifier (MEZ-8201, Nihon Kohden), displayed on a dual-beam oscilloscope (VC-9, Nihon Kohden), and analyzed by a program for histogram (exploited by our department) on a computer.

2.3 Experimental protocols After a stable spontaneous discharge had been obtained for at least 10 min and recorded 3-5 min as control, a drug infusion was started. Neurons were classified as having been excited or inhibited if their firing rate after application of drug was increased or decreased by more than 20%. The protocols were divided into the following groups: (1) Ginkgolide B ($n=27$): Superfused with ginkgolide B (0.1, 1, 10 $\mu\text{mol/L}$) for 2 min, and recorded its effect on discharge rate; (2) *L*-Glu + Ginkgolide B ($n=8$): After pretreatment with *L*-Glu (0.2 mmol/L), ginkgolide B (1 $\mu\text{mol/L}$) was perfused for 2 min; (3) Bay K 8644 + Ginkgolide B ($n=8$): After pretreatment with Bay K 8644 (0.1 $\mu\text{mol/L}$), ginkgolide B (1 $\mu\text{mol/L}$) was administered for 2 min; (4) Tetraethylammonium (TEA) + Ginkgolide B ($n=8$): Perfuse ginkgolide B (1 $\mu\text{mol/L}$) into the perfusate for 2 min, then pretreat the slices with TEA (1 mmol/L) after recovering from the effects of ginkgolide B; After pretreatment with TEA, ginkgolide B (1 $\mu\text{mol/L}$) was perfused for 2 min.

2.4 Reagents Ginkgolide B, TEA, and Bay K 8644 were purchased from Sigma. *L*-glutamate was purchased from Institute of Shanghai Zheng-Xiang. For each experiment, all drugs and solutions were freshly prepared. TEA and *L*-glutamate were dissolved by ACSF. Ginkgolide B was first dissolved in dimethyl sulfoxide (DMSO) and diluted in the ACSF, and the final concentration of DMSO was less than 0.004%. Bay K8644 was first dissolved in 99% ethylalcohol and diluted in the ACSF, and the final concentration of ethylalcohol was less than 0.5%.

2.5 Data analysis All data were expressed as mean $\pm$ SD. Statistical differences were evaluated by one-way ANOVA followed by *q* test. Statistical significances were determined at level of $P < 0.05$.

Fig. 1 Schematic diagram showing the coronal hypothalamus slice with paraventricular nuclei. F: fornix; 3V: third ventricle; PVN: paraventricular nucleus; P: parvocellular portion; m: magnocellular portion; ot: optic tract.

3 Results

Twenty-seven spontaneously active single-units were recorded in paraventricular nucleus neurons from 27 slice preparations. The patterns of discharge were irregular. Mean discharge rate of paraventricular neurons was (1.75 ± 0.19) Hz ($n = 27$).

3.1 Effects of ginkgolide B on discharges in paraventricular neurons In 11 neurons, after application of ginkgolide B ($0.1 \mu\text{mol/L}$) into the perfusate for 2 min, the SDR of 10 (10/11, 90.91%) neurons was decreased significantly from (1.71 ± 0.20) Hz to (1.37 ± 0.16) Hz ($n = 11$, $P < 0.01$), while only 1 (9.09%) neuron was not affected (Fig. 2A). After application of ginkgolide B ($1 \mu\text{mol/L}$) into the perfusate for 2 min, the SDR of 8

(8/8, 100%) neurons was decreased significantly from (1.80 ± 0.18) Hz to (1.01 ± 0.14) Hz ($n = 8$, $P < 0.01$) (Fig. 2B). After application of ginkgolide B ($10 \mu\text{mol/L}$) into the perfusate for 2 min, the SDR of 8 (8/8, 100%) neurons was decreased significantly from (1.74 ± 0.20) Hz to (0.67 ± 0.17) Hz ($n = 8$, $P < 0.01$) (Fig. 2C). The inhibitory effects of ginkgolide B on SDR of paraventricular neurons were dose-dependent manner (Fig. 3).

Fig. 2 Inhibitory effects of different concentrations of ginkgolide B (0.1 , 1 , $10 \mu\text{mol/L}$) on spontaneous discharges of neurons in paraventricular slices. Horizontal bar denotes the time of drug application.

Fig. 3 Inhibitory effects of ginkgolide B (0.1 , 1 , $10 \mu\text{mol/L}$) on spontaneous discharges of neurons in paraventricular slices. $*P < 0.01$, vs control, respectively.

3.2 Effects of ginkgolide B on *L*-Glu-induced discharges of neurons in paraventricular slices In 8 single-units of paraventricular slices, pretreatment with *L*-glutamate (0.2 mmol/L) led to a marked increase in the SDR of all 8 neurons from (1.98 ± 0.15) Hz to (3.99 ± 0.35) Hz ($n = 8$, $P < 0.01$) in an epileptiform pattern (high frequent and repeated discharge, fulminant discharge with disconnected outbreak or a pack of various discharge). Ginkgolide B ($1 \mu\text{mol/L}$) can significantly attenuate the increased SDR induced by *L*-glutamate of all 8 neurons from (3.99 ± 0.35) Hz to (1.94 ± 0.14) Hz ($n = 8$, $P < 0.01$) (Fig. 4).

Fig. 4 Inhibitory effect of ginkgolide B ($1 \mu\text{mol/L}$) on *L*-Glu-induced spontaneous discharges in paraventricular neurons

3.3 Effects of ginkgolide B on Bay K 8644-induced discharges of neurons in paraventricular slices In 8 single-units of paraventricular slices, perfusion of the selective L-type calcium channel agonist, Bay K 8644 (0.1 $\mu\text{mol/L}$), induced a significant increase in the discharge rate of 8 (8/8, 100%) neurons from (2.01 ± 0.38) Hz to (3.35 ± 0.44) Hz ($n = 8$, $P < 0.01$). The increased discharges of 8 (100%) neurons were significantly declined by application of ginkgolide B (1 $\mu\text{mol/L}$) from (3.35 ± 0.44) Hz to (1.93 ± 0.39) Hz ($n = 8$, $P < 0.01$) (Fig. 5).

Fig. 5 Inhibitory effect of ginkgolide B (1 $\mu\text{mol/L}$) on Bay K 8644-induced spontaneous discharges in paraventricular neurons.

3.4 Effects of ginkgolide B on TEA-induced discharges in paraventricular neurons After administering ginkgolide B (1 $\mu\text{mol/L}$) into the perfusate for 2 min, the SDR of 8 (100%) paraventricular neurons were significantly decreased; After recovering from the effects of ginkgolide B, pretreatment with TEA (1 mmol/L) led to an increase in the SDR of all 8 neurons from (1.93 ± 0.29) Hz to (2.96 ± 0.22) Hz ($n = 8$, $P < 0.01$), and can block the inhibitory effect of ginkgolide B (1 $\mu\text{mol/L}$) in all 8 neurons (Fig. 6).

Fig. 6 Effect of ginkgolide B (1 $\mu\text{mol/L}$) on TEA-induced spontaneous discharges in paraventricular neurons.

4 Discussion

The present study demonstrates that ginkgolide B can inhibit SDR of paraventricular neurons in a dose-dependent

manner, and also attenuate *L*-glutamate, Bay K 8644-induced discharges in paraventricular slice preparations. This inhibitory effect of ginkgolide B can be blocked by TEA.

L-glutamate is a major excitatory neurotransmitter in CNS and its receptors have *N*-methyl-D-aspartate (NMDA) and non-NMDA subtypes. It has effects on each subtype but mainly activates NMDA receptor and increases $[\text{Ca}^{2+}]_i$. Calcium ion passing NMDA receptor was toxic^[10] and the NMDA receptor-mediated elevations in $[\text{Ca}^{2+}]_i$ were shown to induce “epilepsy” in cultured neurons^[11,12]. Just as we observed in our previous experiments, *L*-glutamate could induce the epileptiform discharges of neurons in hippocampal and paraventricular slices, an effect which was related to increased influx of calcium^[13,14]. Zablocka *et al.* suggested that NMDA receptor activation might stimulate the release of PAF and, in turn, PAF could amplify the Ca^{2+} signal generated by NMDA receptor activation^[15]. Ginkgolide B may prevent *L*-glutamate-mediated neurotoxic levels of Ca^{2+} from entering neurons.

While ginkgolide B has been shown to scavenge free radicals and non-specifically inhibit seryl and aspartyl proteases, its most potent and extensively studied action is its antagonist action at the PAF receptor. PAF is a potent proinflammatory phospholipid with diverse pathological and physiological effects. The action of PAF is mediated by the PAF receptor, a G protein-coupled membrane-spanning molecule that can engage multiple signaling pathways in various cell types. Inappropriate activation of this signaling pathway is associated with many diseases. PAF receptor mRNA has been detected in the hypothalamus, medulla-pons, olfactory bulb, hippocampus, cerebral cortex, spinal cord, thalamus and cerebellum in rat brain. Furthermore, It has been demonstrated that the highly expressed mRNAs of PAF receptor in apoptosis cells can be detected during the glutamate-induced neuronal apoptosis^[16]. Bito *et al.* also found that on hippocampal neurons some PAF receptors co-localised with NMDA receptors and PAF could induce a massive influx of Ca^{2+} through glutamate-receptors^[17]. All above intensively indicate that PAF may be involved in neuronal apoptosis induced by glutamate. And ginkgolide B possesses specifically strong inhibition to the effects of PAF via competitive combination with PAF receptor^[18]. The addition of ginkgolide B resulted in a concentration-dependent inhibition of the PAF-induced Ca^{2+} overload^[19]. Obviously the glutamate excitotoxicity has been clearly attributed to a massive influx

of Ca^{2+} through NMDA channels^[20] and PAF receptor. The fact that ginkgolide B can significantly reduce epileptiform discharges induced by *L*-glutamate in our study suggests that the inhibitory effects of ginkgolide B might be due to the blockade of calcium influx.

Recent study indicates that ginkgolide B can inhibit abnormal calcium influx caused by β -amyloid peptide (1-40), which leads to an intracellular calcium overload by increasing high voltage-activated (HVA) calcium channel current via AC-cAMP-PKA^[21]. The HVA calcium channels can be divided into different types such as L-, N-, P/Q- and R-type, with L- and N- type being the major components of HVA channels. Considering the fact that ginkgolide B can reverse the increased discharges induced by Bay K 8644 and Bay K 8644 is a selective L-type voltage-activated calcium channel agonist, this inhibitory effect might be mainly due to the blockade of L-type voltage-activated calcium channel and subsequently inhibit calcium influx. These results are compatible with prior reports that ginkgolide B decreased L-type calcium current ($I_{\text{Ca,L}}$) significantly in a concentration-dependent manner in guinea pig's ventricular myocytes^[22]. It is possible that $I_{\text{Ca,L}}$ is essential to the ginkgolide B-mediated protection of central nervous system. However the precise mechanism of ginkgolide B on Ca^{2+} influx still needs to be further investigated.

Delayed rectifier potassium channels (K_{DR}) have been described in nearly all excitable membranes including neurons. And K_{DR} can be inhibited by high concentrations of TEA. There is evidence suggesting that ginkgolide B can increase delayed rectifier potassium current (I_{K}) in normal guinea pig ventricular myocytes^[23]. TEA (1 mmol/L), a broad potassium channels inhibitor, was employed to investigate whether K_{DR} was involved in the effect of ginkgolide B on paraventricular neurons. The fact that increased discharges induced by TEA can not be decreased by ginkgolide B indicates that the effect of ginkgolide B on hippocampus may associate with K_{DR} . Since potassium channels are the main determinant of resting membrane potential, their activation causes hyperpolarization and inhibition of voltage-gated calcium channels. Moreover, I_{K} is shown to be modulated by various intracellular and extracellular signals, which include increase in $[\text{Ca}^{2+}]_{\text{i}}$ ^[24,25]. All of these appears to be involved in ginkgolide B-induced I_{K} enhancement and $I_{\text{Ca,L}}$ inhibition.

Above all, numerous studies demonstrate that ginkgolide

B, as a specific antagonist of PAF, presents beneficial effects on various PAF-related diseases. And current research suggests that PAF may play an important role in the damage of the central nervous system. Our studies have revealed that ginkgolide B can inhibit the spontaneous discharges of paraventricular neurons and protect central neurons by modulating the effect of glutamate on $[\text{Ca}^{2+}]_{\text{i}}$. The inhibitory effect is likely due to its properties of blockade of L-type voltage-activated calcium channel and may be related to the delayed rectifier potassium channel. Ginkgolide B might be a crucial step in the neuroprotective effects of the PAF receptor antagonist, and further study is required to evaluate the benefits of it.

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银杏内酯 B 对大鼠下丘脑室旁核神经元自发放电的影响

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摘要: 目的 研究银杏内酯B (Ginkgolide B, BN52021) 对静息状态下的下丘脑室旁核神经元自发放电活动的影响。方法 应用细胞外记录单位放电技术。结果 (1) 在 27 个下丘脑室旁核神经元放电单位给予银杏内酯 B (0.1, 1, 10 $\mu\text{mol/L}$) 2 分钟, 有 26 个放电单位 (96.30%) 放电频率明显降低, 且呈剂量依赖性; (2) 预先用 0.2 mmol/L 的 *L*-glutamate (*L*-Glu) 灌流下丘脑片, 8 个放电单位放电频率明显增加, 表现为癫痫样放电, 在此基础上灌流银杏内酯 B (1 $\mu\text{mol/L}$) 2 分钟, 其癫痫样放电全部被抑制; (3) 预先用 L 型钙通道开放剂 Bay K 8644 灌流 8 个下丘脑片, 8 个放电单位 (100%) 全部放电增加, 在此基础上灌流银杏内酯 B (1 $\mu\text{mol/L}$) 2 分钟, 8 个放电单位 (100%) 放电频率明显减低; (4) 在 8 个下丘脑室旁核神经元放电单位上, 银杏内酯 B (1 $\mu\text{mol/L}$) 的抑制效应可被广泛钾通道阻断剂 (tetraethylammonium, TEA) 1 mmol/L 完全阻断。结论 银杏内酯 B (Ginkgolide B, BN52021) 可抑制下丘脑室旁核神经元自发放电, 并可抑制由 *L*-glutamate 诱发的神经元放电。提示银杏内酯 B 对心血管中枢神经元通过降低其活动而具有一定程度的保护作用, 这种作用可能与银杏内酯 B 抑制 L 型钙通道有关, 而且可能与延迟整流型钾通道 (delayed rectifier potassium channel, K_{DR}) 有关。

关键词: 下丘脑室旁核; 银杏内酯 B; *L*-glutamate; Bay K 8644; TEA