Ginsenoside Rg1 Modulates Spontaneous Synchronous Ca^{2+} Oscillations of Cultured Hippocampal Neurons

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Abstract: Ginsenoside Rg1, the main active ingredient of Ginseng Radix which is a famous Chinese medicine, is widely used as an anti-stress, anti-aging and neurological performance improving agent. In this study, we used calcium imaging and whole-cell patch clamp techniques to investigate the effect of Ginsenoside Rg1 on spontaneous and synchronous Ca^{2+} oscillations and the possible mechanisms in primarily cultured hippocampal neuronal networks. We found that Ginsenoside Rg1 could decrease the frequencies of spontaneous and synchronous Ca^{2+} oscillations and inhibit the amplitude of high-voltage activated calcium channel currents. These results provided the experimental evidence for the clinical application of Rg1 as a neuroprotector on the cellular level, and enriched the theoretical system of Chinese medicine.

Keywords: Ginsenoside Rg1, Ca^{2+} oscillation, high-voltage gated Ca^{2+} channel, hippocampal neuron.

INTRODUCTION

Ginsenoside Rg1 is one of the most effective ingredients of Ginseng, Radix Ginseng from Korea, notoginseng and other traditional Chinese medicine. Although current studies have confirmed the main target organ of Rg1 is central nervous system (CNS) [1], its role in modulating CNS and the underlying mechanisms is still ambiguous.

Spontaneous and synchronous Ca^{2+} oscillations in the form of waves and spikes are found in many types of neural tissues *in vivo* and *in vitro*, ranging from the hippocampus to the visual system [2-4]. Synchronous spontaneous Ca^{2+} oscillations are often observed among neuronal networks without external stimuli *in vitro*, resulting from periodic burst firing of action potentials through excitatory synaptic transmission [5-7]. These spontaneous and synchronous activities are believed to play a pivotal role during neuronal development including neuronal migration, differentiation and connection patterning [8-12]. This process is modulated by many intrinsic and extrinsic factors. The voltage-gated and ligand-gated ion channels are also involved.

In this study, we used calcium imaging and whole-cell patch clamp techniques to investigate the effect of Ginsenoside Rg1 on spontaneous synchronous Ca^{2+} oscillations and the possible mechanisms in primarily cultured hippocampal neuronal networks.

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MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), equine serum, B27 supplements, 0.25% trypsin-EDTA and Hank's Balanced Salt Solution (HBSS) for cell culture were from Invitrogen (Carlsbad, CA, USA). L-glutamine was from Hyclone (Logan, UT, USA). Fluo-4-AM was from Molecular Probes (Eugene, OR, USA). Rg1 taken was from China National Institute for the Control of Pharmaceutical and Biological Products and dissolved in Krebs-Ringer's saline. Other reagents were purchased from Sigma (St Louis, MO, USA).

Cell Culture

Hippocampal neuron cultures were prepared as described previously [13]. Briefly, hippocampal tissues from 17-19d fetal rats were dissected and treated with 0.15% trypsin-EDTA at 37 ˚C for 15-20 min; then trypsin were removed with Pasteur pipette. The single cell suspension was diluted to a density of 120,000 cells/ml in high glucose DMEN containing 5% FBS, 5% equine serum, and 0.5 mM L-glutamine, and planted into 35 mm dishes with glass bottom for subsequent microscopy imaging or in 35 mm ordinary culture dishes for subsequent patch-clamp experiments. All dishes were pretreated with poly-D-lysine (20mg/L in deionized water) overnight. The cultures were incubated at 37 ˚C in a humidified incubator with 5% CO_2. After approximately 24 h, the medium was replaced by serum-free Neurobasal medium containing B27 supplement and 0.5 mM L-glutamine to inhibit the growth of glia cells. Then, half of the medium was replaced every three days. The cultures were used for experiments on 9-11 days after planting.

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Ca^{2+} Imaging

Hippocampal cells were loaded with 4μM Fluo-4-AM in Krebs-Ringer's saline (in mM: 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 HEPES, pH 7.4) [14] at 37 ˚C for 30 min, followed by three washes and a 15-min incubation period for de-esterification of Fluo-4-AM before imaging. Cells grown on the glass bottom in 35 mm dishes were directly imaged on a Nikon (Tokyo, Japan) inverted microscope (TE300) using a 40× numerical aperture 1.30 oil immersion Plan Fluor objective. A Lambda DG-4 high-speed wavelength switcher (Sutter Instrument, Novato, CA) was used for fluo-4 excitation at 480 nm and cooled CCD camera (CoolSnap FX; Roper Scientific, Princeton, NJ) was used for image acquisition. MetaFluor imaging software (Universal Imaging Corporation, Downingtown, PA) was used for hardware control, image acquisition, and image analysis. The experiments were performed at room temperature (25-30 ˚C). Typically, time-lapse recording of Ca^{2+} signals in hippocampal neurons was performed for 4 min (as control period) before and 18 min after the application of different chemicals; the sampling rate was one frame every 2 sec with exposure time of 80 msec with CCD binning of 4×4.

Quantitative Analysis of Frequencies of Synchronized Ca^{2+} Oscillation

Quantitative measurements of $[Ca^{2+}]_i$ oscillations were performed by obtaining average fluo-4 fluorescence intensity of a 3×3 $pixel^2$ analysis box placed at the center of the cell body; the intensity values were then subtracted of average background intensity measured in cell-free region. Changes of $[Ca^{2+}]_i$ in each cell were represented by the changes of relative fluo-4 fluorescence ($\Delta F/F_0$), where F_0 was the baseline intensity. Ca^{2+} spikes were defined as rapid elevation of $\Delta F/F_0 \geq 20\%$. Under our imaging settings, fields of 4-8 neurons were typically recorded and subsequently analyzed.

Statistical Analysis

Data from at least three dishes from different batches of cultures were pooled together and analyzed for statistical significant differences. We calculated the frequencies of Ca^{2+} oscillations by counting the number of spikes in 2 min of the records at a defined time point. The frequency values after the drug application were normalized to the control frequency value and expressed as percentages; a value of 100% indicated no change. Paired Student's t test was used to analyze data for statistical significance. Compiled data were expressed and graphed as mean ± SEM, with n denoting the number of neurons studied.

Electrophysiology

Voltage-gated calcium channel currents were recorded from the somatic region of pyramidal-like hippocampal neurons using the whole-cell patch clamp technique. Similar to the Ca^{2+} imaging, the culture medium was replaced with extracellular solution (in mM: 50 NaCL, 5 BaCL2, 90 TEA-Cl, 10 HEPES, 10 glucose, 5 4-AP, 0.001 TTX, pH 7.3) before recording. The patch electrodes (Fisherbrand, USA) were pulled on a PP-83 micropipette puller (Narishige, Japan). The typical resistance of glass electrodes was 3-8 MΩ when filled with intracellular pipette solution (in mM: 140 CsCl, 10 HEPES, 10 EGTA, 2 MgCl2, 2 Na2ATP, 1 CaCl2. pH 7.3). After the whole-cell recording configuration was formed, the cell was clamped at assumptive resting potential

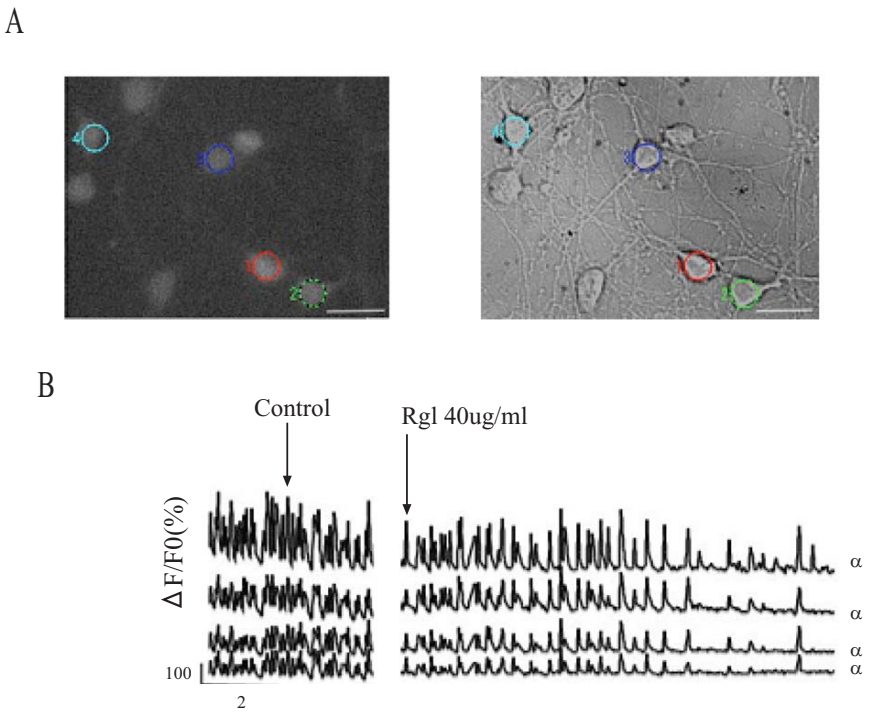

Fig. (1). A Hippocampal neurons 10 days in culture: light microscopic image on the right and the corresponding fluorescent microscopic image on the left. Scale bar: 25μm. **B** Traces show synchronized Ca^{2+} oscillations in three neurons randomly selected from a group of synchronically firing cells. The two parts represent Ca^{2+} oscillations under control condition and after bath application of 40μg/ml Ginsenoside Rg1.

(-70mV), and step depolarized to +60mV in 10mV increment. The experiments were performed at room temperature (22±2 ˚C) with the Axopatch-200B amplifier (Axon Instruments, Foster City, CA, USA). The signals were recorded with pClamp9 software (Axon Instruments) and analyzed with clampfit9 software (Axon Instruments). All data were analyzed for statistical significance using paired Student's t-test. The data were expressed as means ± SEM with n denoting the number of neurons studied.

RESULTS

Ginsenoside Rg1 Decreased the Frequency of Synchronized Ca^{2+} Oscillations

To examine the effects of Ginsenoside Rg1 on synchronized Ca^{2+} oscillations, we bath-applied Rg1 to hippocampal cultures and recorded Ca^{2+} imaging before and after application. Bath application of the control saline buffer (Krebs-Ringer saline) did not affect the frequency of Ca^{2+} oscillation indicating that no artifact was produced by the bath application method or by Krebs-Ringer saline (Fig. **2**). After the bath application of 20μg/ml Rg1,we found no change of Ca^{2+} oscillation frequencies in the 18 min recording period compared to the control group. After the bath application of 40μg/ml Rg1, there was no obvious change of the Ca^{2+} oscillation frequencies in the first 4 minutes, whereas it was remarkably decreased to 68.49±1.06%(mean±SEM, n=41) in 4-6 minutes period compared to the control period. The application of 100μg /ml and 200μg /ml Rg1 could immediately decrease it to 86.18±13.66%(n=8) and 54.13±1.96% (n=9) respectively. Moreover at the tenth minute, 40μg /ml. 100μg/ml and 200μg /ml Rg1 could decrease frequencies to 34.10±2.13% (n=41), 33.29±3.43%(n=8), 35.11±10.58% (n=9) respectively (Fig. **2**).

Ginsenoside Rg1 Inhibited High-Voltage Gated Calcium Channel Currents

It was reported that the influx of extracellular calcium shapes the synchronous oscillations and HVA(high-voltage activated) calcium channels are largely responsible for the regulation of calcium entry [15].We next investigated the effect of Ginsenoside Rg1 on HVA calcium channels. The cell was clamped at assumptive resting potential (-70mV), and step depolarized to +60mV in 10mV increment. Ba^{2+} was used as the charge carrier in the extracellular solution to avoid Ca^{2+}-induced inactivation. As different concentrations of Rgl had similar inhibitory function at 10 minutes, we recorded the calcium channel current at the tenth minute by using 40μg /ml Rg1 compared to the control group with the application of normal extracellular solution. The statistics indicated that the current amplitudes were significantly de-

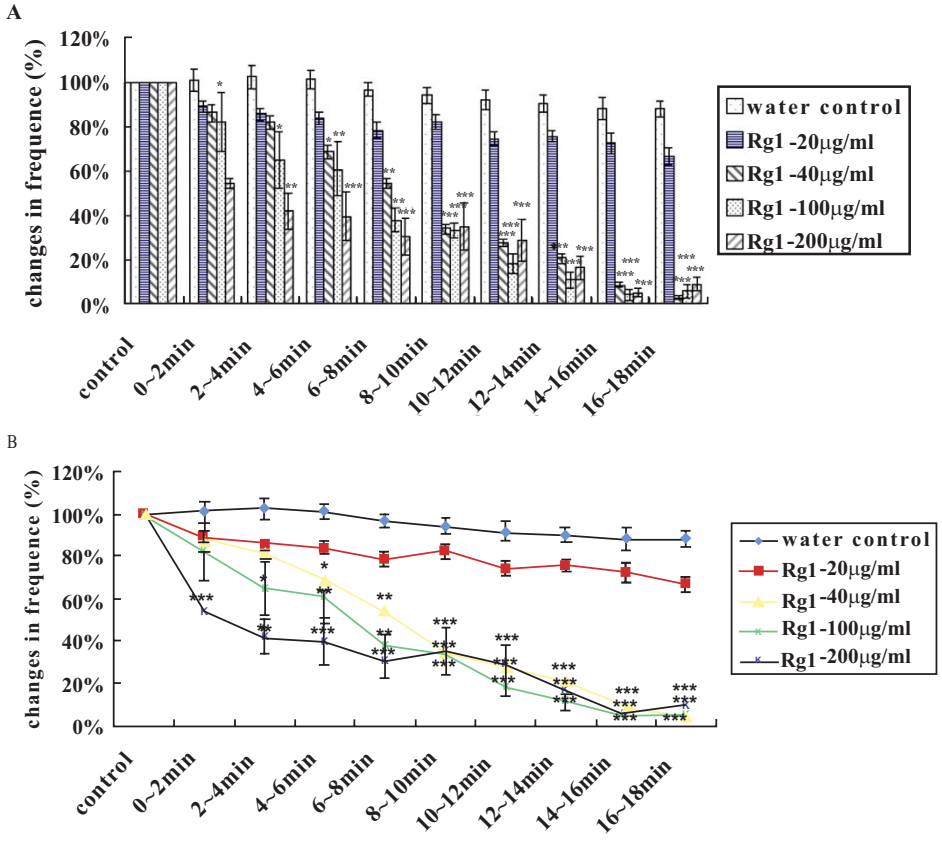

Fig. (2). The effect of Ginsenoside Rg1 on Ca^{2+} oscillations. **A**. Frequency changes of the spontaneous synchronized Ca^{2+} oscillations in every 2 minutes before and after the bath application of Krebs-Ringer 's saline (control) and Ginsenoside Rg1 with different concentration. **B**. Tendency of frequency changes of the spontaneous synchronized Ca^{2+} oscillations before and after the bath application of Krebs-Ringer 's saline (control) and Ginsenoside Rg1 with different concentration in the recording time. Data are presented as the mean±SEM(For Krebs-Ringer 's saline, n=21; for Rg1 20μg/ml, n=21; 40μg/ml, n=41;100μg/ml, n=8; 200μg/ml, n=9). Asterisks indicate significance against the control group ('*'p<0.05, '**'p<0.01, '***'p<0.001,t test).

creased at 10 min after the application of 40μg/ml Rg1 when membrane potential was depolarized from -10mV to + 60mV (Fig. **3**).

DISCUSSION

The pharmacological actions of Ginsenoside Rg1 has been widely studied, especially its effects on CNS. It can improve learning and memory, protect neurons and dilate cerebral blood vessel. However, the underlying mechanisms of these functions are still obscure. At present, there are some explanations for the underlying mechanisms of Rg1: 1. Rg1 can raise the level of cAMP in hippocampus which is a key crosspoint in cell metabolizm and an endogenous neuroprotective agent [16]. 2. Rg1 enhances the ability of cholinergic system by increasing the synthesis and release of acetylcholine and raising the density of M-receptors to improve learning and memory [17]. 3 Rg1 protects the nervous system by blocking calcium channels [18]. In this study, we focused on the effect of Rg1 on calcium oscillations and high-voltage-gated (HVA) Ca^{2+} channel currents.

It is reported that spontaneous Ca^{2+} oscillations are the direct result of membrane depolarization from action potentials among synaptically connected neurons [19-22]. The membrane depolarization can activate voltage-gated calcium channel(VGCC), which is the main way for the influx of extracellular calcium, thus resulting in the elevation of the intracellular calcium concentration. In central nervous system, calcium channels are broadly grouped into high voltage-activated (HVA) and low voltage-activated (LVA) families. HVA channels require strong membrane depolarization for gating and it has been previously reported that HVA calcium channels allow calcium influx from the extracellular medium and sustaining the long depolarization [7]. The inhibition of Rg1 on HVA might be one reason for its neuroprotective function. In addition, previous studies have confirmed that aging and AD are associated with the enhancement of VGCC [23-25]. Our finding that Rg1 can inhibit HVA is consistent with previous studies which offer further experimental evidence for its clinical application in aging and other degenerative diseases.

Synchronized spontaneous Ca^{2+} oscillation of neuronal networks is mediated by periodic burst firing of action potentials through excitatory synaptic transmission. Burst discharge initiates the influx of large number of Ca^{2+} ions which can then trigger signal transduction processes, induce changes in gene expression, and ultimately lead to important biological processes. But under ischemic and hypoxic conditions, the brain will produce excessive excitatory amino acids which can activate synaptic and non-synaptic glutamate receptors. A large number of extracellular calcium then will flow into cells which in turn destroy the calcium homeostasis and cause calcium to overload. Ultimately, this will cause neuronal death [26]. Therefore, in order to protect neurons, a number of researches have been done to decrease the excitability of neuronal networks as a treatment strategy. Methods such as depression of synaptic transmission, reduction of

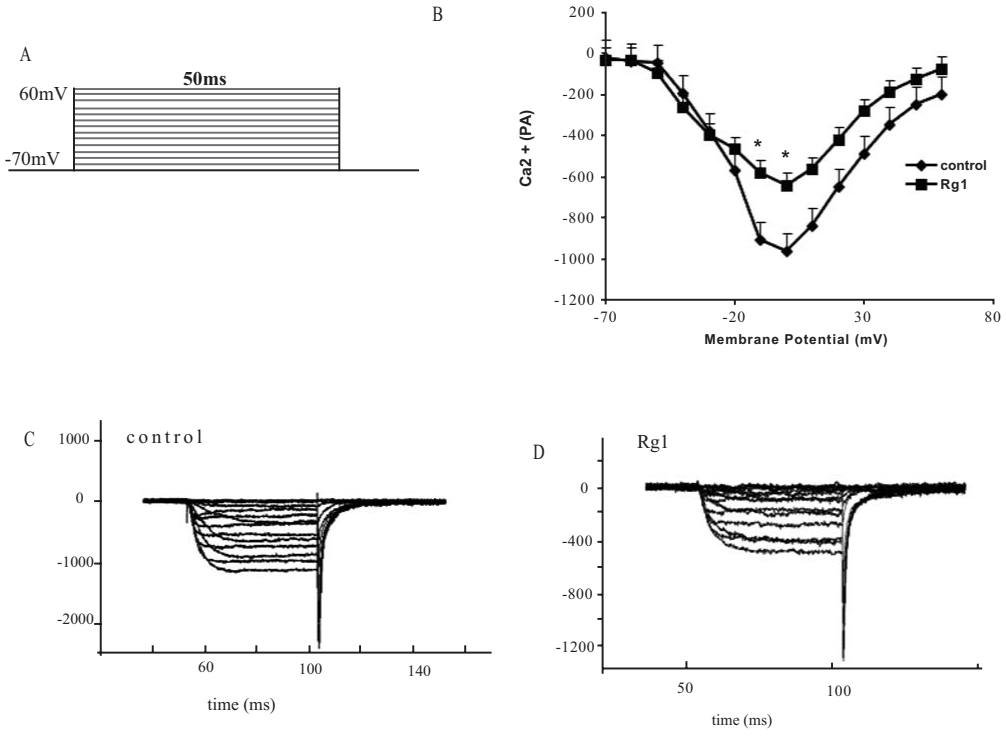

Fig. (3). Ginsenoside Rg1 inhibit high-voltage activated calcium current. **A**.Voltage protocol of a series of depolarizing steps to induce HVA calcium currents. C.D Representative recordings of HVA calcium currents induced by the voltage protocol with Krebs-Ringer's saline application or 10μg/ml Ginsenoside Rg1. **B**. I-V curve for HVA calcium currents 10 min after application of Krebs-Ringer's saline or 10μg/ml Ginsenoside Rg1 (for Rg1, n=12, for Krebs-Ringer's saline, n=5). Asterisks indicate significance against the control group (*'*'p<0.05 t test).

excitatory amino acids release and blockade of calcium influx, have been examined. Other methods to reduce the discharge of neurons are also constantly tested [27, 28]. In our study, we found that appropriate concentration of Ginsenoside Rg1 effectively decreased the frequency of Ca^{2+} oscillations in cultured rat hippocampal neurons and remarkably inhibited HVA Ca^{2+} channel currents amplitude which suggest Rg1 can act as a neuroprotective agent. We also found that the effect of Rg1 was not influenced dramatically by the dose, which indicates that it had a mild characteristic. To sum up, Rg1 is beneficial for CNS and can be applied widely for its stable characteristics.

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