Original Article

Ginsenoside Rb1 selectively inhibits the activity of L-type voltage-gated calcium channels in cultured rat hippocampal neurons

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Aim: To investigate the effect of ginsenoside Rb1 on voltage-gated calcium currents in cultured rat hippocampal neurons and the modulatory mechanism.

Methods: Cultured hippocampal neurons were prepared from Sprague Dawley rat embryos. Whole-cell configuration of the patch-clamp technique was used to record the voltage-gated calcium currents (VGCCs) from the hippocampal neurons, and the effect of Rb1 was examined.

Results: Rb1 (2–100 $\mu\text{mol/L}$) inhibited VGCCs in a concentration-dependent manner, and the current was mostly recovered upon wash-out. The specific L-type Ca^{2+} channel inhibitor nifedipine (10 $\mu\text{mol/L}$) occluded Rb1-induced inhibition on VGCCs. Neither the selective N-type Ca^{2+} channel blocker ω -conotoxin-GVIA (1 $\mu\text{mol/L}$), nor the selective P/Q-type Ca^{2+} channel blocker ω -agatoxin IVA (30 nmol/L) diminished Rb1-sensitive VGCCs. Rb1 induced a leftward shift of the steady-state inactivation curve of I_{Ca} to a negative potential without affecting its activation kinetics or reversal potential in the I – V curve. The inhibitory effect of Rb1 was neither abolished by the adenyl cyclase activator forskolin (10 $\mu\text{mol/L}$), nor by the PKA inhibitor H-89 (10 $\mu\text{mol/L}$).

Conclusion: Ginsenoside Rb1 selectively inhibits the activity of L-type voltage-gated calcium channels, without affecting the N-type or P/Q-type Ca^{2+} channels in hippocampal neurons. cAMP-PKA signaling pathway is not involved in this effect.

Keywords: ginsenoside Rb1; L-type Ca^{2+} channel; nifedipine; ω -conotoxin-GVIA; ω -agatoxin IVA; patch-clamp technique; hippocampus; cAMP-PKA signaling pathway

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Introduction

Ca^{2+} , an important regulator of neuronal activity, controls membrane excitability, triggers the release of neurotransmitters and mediates activity-dependent changes in gene expression. Voltage-gated Ca^{2+} channels play a key role in the control of free cytosolic Ca^{2+} [1]. According to their pharmacological and electrophysiological properties, at least 5 distinct types of voltage-gated Ca^{2+} channels have been identified and designated as L, N, P/Q, R, and T^[2, 3]. L-, N-, and P/Q-type Ca^{2+} channels are pharmacologically identified and characterized by their specific blockers. Nifedipine is an L-type Ca^{2+} channel inhibitor, ω -conotoxin GVIA is an N-type Ca^{2+} channel inhibitor, and ω -agatoxin IVA is a P/Q-type Ca^{2+} channel

inhibitor^[4–9]. Because the elevation of intracellular Ca^{2+} levels ($[\text{Ca}^{2+}]_i$) caused by the excessive stimulation of Ca^{2+} channels plays a key role in the excitotoxic damage of neurons, agents blocking the elevation of $[\text{Ca}^{2+}]_i$ by regulating Ca^{2+} channels might have neuroprotective effects^[10–12].

Ginseng, the root of *Panax ginseng* CA Meyer, has been used worldwide as an herbal medicine for the alleviation of many ailments, particularly those associated with aging and memory deterioration. Ginsenoside Rb1, a protopanaxadiol type saponin, is one of the most important active compounds of ginseng. Recently, ginsenoside Rb1 has been reported to effectively protect neurons from glutamate-induced toxicity and β -amyloid ($\text{A}\beta$)-induced toxicity by reducing intracellular Ca^{2+} levels^[13–18]. However, how Rb1 decreases $[\text{Ca}^{2+}]_i$ by regulating Ca^{2+} channels remains unclear. Although studies have found that Rb1 inhibits voltage-gated Ca^{2+} channels in other cell types and that its mechanisms of action vary by cell type, there have been no research studies to date that have inves-

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tigated the action of ginsenoside Rb1 on voltage-gated Ca^{2+} channels in hippocampal neurons. In this paper, we analyzed the effect of ginsenoside Rb1 on voltage-gated Ca^{2+} channels in hippocampal neurons and the possible mechanism for this modulation.

Materials and methods

Materials

Ginsenoside Rb1 was obtained from the Department of Organic Chemistry at Jilin University (Changchun, China) with a purity >98%. Stock solutions of the Ca^{2+} channel antagonists, ω -conotoxin GVIA (Alomone Labs, UK), nifedipine (Sigma, UK) ω -agatoxin IVA (Alomone Labs, UK) and adenylyl cyclase agonist Forskolin (Sigma, UK), were prepared with the appropriate amounts of deionized water or dimethyl sulfoxide (DMSO) and frozen at -20°C before appropriate dilution in the recording medium. H-89 was dissolved in the pipette solution (described below) and stored at -20°C . After the whole-cell configuration was obtained, H-89 was dialyzed into the cell through the pipette.

Hippocampal neuron cultures

Chemical media and culture media were obtained from Sigma unless otherwise noted. The care and use of animals followed the guidelines of the Shanghai Institutes for Biological Sciences Animal Research Advisory Committee. The hippocampal neuron cultures were prepared as described previously^[19] with some modifications. Briefly, whole brains were isolated from 18-day-old SD rat embryos, and the hippocampi were dissected and treated with 0.125% trypsin at 37°C for 12 min. The cells were suspended with Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO) containing 10% fetal bovine serum (HyClone, Logan, UT, USA) and 10% F-12 (GIBCO) and were plated at a density of 60 000 cells/mL on poly D-lysine-coated 35 mm dishes (Costar). Twenty-four hours after plating, half of the medium was changed to serum-free Neurobasal (NB) medium with 2% B27 supplement (GIBCO) and 1% glutamine. Thereafter, half of the changed medium was replaced twice a week with NB medium containing 2% B27 supplement and 0.25% glutamine. After 7 d *in vitro*, glial cell proliferation was inhibited by exposure to 2–4 mmol/L cytosine arabinoside. All the recordings were made with cells between d 6 and 8.

Electrophysiological recordings

Single patch recordings of Ca^{2+} channels from cultured hippocampal neurons at 6–8 d *in vitro* were made at room temperature using an EPC-9 patch-clamp amplifier and its corresponding Patchmaster software (Heka Electronics, Germany) or an Axopatch-200B amplifier (Axon Instruments) with pCLAMP acquisition software. The gain was set to 1, filtered at 1 kHz, stored on videotape after digitization with a PCM processor, and displayed with a thermal pen recorder. The membrane capacitance and series resistance compensation were optimized.

Patch pipettes were fabricated from borosilicate glass capillaries (outer diameter 1.2 mm, inner diameter 0.69 mm, length

7.5 cm; B-120-69-15, Sutter Instruments) on a horizontal puller (Sutter Instruments). The microelectrodes had tip diameters of 2–3 μm and resistances of 3–6 M Ω . The pipettes were filled with an intracellular solution containing 80 mmol/L Cs-methanesulfonate, 20 mmol/L tetraethylammonium chloride (TEA-Cl), 1 mmol/L CaCl_2 , 5 mmol/L MgCl_2 , 11 mmol/L ethylene glycol-bis-(2-aminoethyl)-tetraacetic acid (EGTA), 10 mmol/L N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), and 10 mmol/L Na_2ATP . The chemicals were obtained from Sigma. CsOH was used to adjust the pH to 7.2–7.3. The osmolality of the pipette solution was adjusted to 300 mOsm with sucrose. As suggested by Smirnov^[20], the replacement of 1.5 mmol/L Ca^{2+} with 5 mmol/L Ba^{2+} was used to augment the amplitude of the inward current through Ca^{2+} channels. The potential dependency of activation and inactivation with 5 mmol/L Ba^{2+} was very similar to the results observed in 1.5 mmol/L Ca^{2+} . To isolate the Ba^{2+} current (I_{Ba}), the following reagents were used for the external solution: 115 mmol/L choline-Cl, 25 mmol/L TEA-Cl, 5 mmol/L 4-aminopyridine (4-AP), 5 mmol/L BaCl_2 , 10 mmol/L glucose, 10 mmol/L HEPES, and 0.0005 mmol/L tetrodotoxin (TTX). Tris was used to adjust the pH to 7.4. The osmolality of the extracellular solution was adjusted to 300 mOsm with sucrose. 4-AP and TEA were used to eliminate outward K^{+} currents. TTX was used to eliminate inward Na^{+} currents.

We recorded the voltage-gated calcium channel Ba^{2+} current (I_{Ba}) and used the following stimulating programs: an activation procedure and a drug application program. For the activation procedure, the cells were held at a potential of -60 mV and depolarized to potentials ranging from -70 mV to $+70$ mV with 10 mV as a step for a duration of 150 ms, and the steps were repeated every 10 s. For the drug application program, the cells were held at a potential of -60 mV and depolarized to 0 mV (0 – $+20$ mV) for a duration of 200 ms, and the steps were repeated every 10 s.

Experimental drug application and treatment

The "U-tube" solution exchange method^[21, 22] was used to apply the drugs. The whole cell measurements were initiated 5 min after break-in. Little run-down was observed during the 15 min necessary to collect the data. The current amplitudes of the cell before and after the experiment and the current densities of the cells of the different groups were compared.

Analysis of the electrophysiological recordings

The current recordings were analyzed using Clampfit 8.0 software (Axon, USA). Further analyses were performed using Microsoft Excel 2003 and Microcal Origin 8.0. All the data were described as the mean $\pm$ the standard error of the mean. All the current recordings were normalized according to the whole cell capacitance to give the current density. A repeated measures ANOVA with Tukey-Kramer's post-test was used to compare the differences among entire current-voltage (*I*-*V*) relationships, and an unpaired Student's *t*-test was used to compare points on different curves that were activated by stepping to the same potential. *P* values less than 0.05 were

considered significant. The peak current was measured as the maximal current observed during the depolarizing step.

The calcium current steady-state activation curve was fitted to a Boltzmann equation of the following form:

$$I/I_{\max} = 1 / \{1 + \exp[(V - V_{1/2})/k]\}$$

where I is the voltage-dependent current amplitude, V is the membrane potential for activation, $V_{1/2}$ is the voltage at which activation is half maximal, and k is the slope factor.

Results

Voltage-gated calcium channel currents in hippocampal neurons

We recorded the whole-cell membrane currents from the somatic region of the neurons (Figure 1A) and identified a potent Ca^{2+} channel antagonist by its sensitivity to cadmium. We successively discriminated N-, P/Q-, and L-type Ca^{2+} channels by their specific blockers. ω -conotoxin GVIA and ω -agatoxin IVA showed irreversible blocking effects, while

nifedipine exerted a partially reversible blocking effect (Figure 1B).

Effect of ginsenoside Rb1 on VGCCs in hippocampal neurons

The method of extracellular micro-perfusion was used to study the effects of Rb1 on VGCCs. In these experiments with Rb1 treatment, only the peak currents were selected for comparison, and the current was evoked by a pulse of 200 ms duration from -60 mV to 0 mV (0–+20 mV). The inhibition rate of the I_{Ba} peak current was calculated as follows: [(maximum current value before administration – maximum current value after administration)/maximum current value before administration $\times 100\%$]. The control group was treated with the extracellular solution, and the experimental groups were treated with 1, 2, 5, 10, and 100 $\mu\text{mol/L}$ Rb1. Both the control group and the 1 $\mu\text{mol/L}$ Rb1 group showed no inhibitory effects on the I_{Ba} (Figure 2A, 2B). However, the other experimental groups with 2, 5, 10, and 100 $\mu\text{mol/L}$ Rb1 demonstrated inhibitory effects on the I_{Ba} . The inhibition rates were $2\% \pm 0.87\%$ ($n=6$), $5\% \pm 1.78\%$ ($n=4$), $20\% \pm 3.96\%$ ($n=9$), and $40\% \pm 6.71\%$ ($n=5$), respectively. The I_{Ba} peak current inhibition rate of each group was significantly higher than that of the previous dose group ($P < 0.01$) (Table 1). The effects of Rb1 on the I_{Ba} were partially reversible after wash-out with the bathing solution (Figure 2C, 2D).

Figure 1. (A) Phase-contrast image showing a single patch recording from 7-d cultured hippocampal neurons for the recording of the VGCCs. Scale bar, 10 μm . (B) Pharmacological separation of the VGCC subtypes in hippocampal neurons. Upper panel, inward Ca^{2+} channel Ba^{2+} currents evoked by pulses from -60 mV to 0 mV at the times indicated in the lower panel. Lower panel, time course of effects of ω -conotoxin GVIA (1 $\mu\text{mol/L}$), ω -agatoxin IVA (30 nmol/L) and nifedipine (10 $\mu\text{mol/L}$) on the Ba^{2+} current amplitude.

Table 1. Effects of Ginsenoside Rb1 at different concentrations (1, 2, 5, 10, and 100 $\mu\text{mol/L}$) on the amplitude of I_{Ba} . Mean $\pm$ SD. $^{\circ}P < 0.01$ compared with the previous group.

Groups	I_{Ba} inhibition (%)
Control	0 ± 0.74
1 $\mu\text{mol/L}$ Rb1	0 ± 1.12
2 $\mu\text{mol/L}$ Rb1	$2 \pm 0.87^{\circ}$
5 $\mu\text{mol/L}$ Rb1	$5 \pm 1.78^{\circ}$
10 $\mu\text{mol/L}$ Rb1	$20 \pm 3.96^{\circ}$
100 $\mu\text{mol/L}$ Rb1	$40 \pm 6.71^{\circ}$

Mechanism of action of ginsenoside Rb1 on the VGCCs in hippocampal neurons

The I_{Ba} was still elicited by depolarizing from -60 mV to 0 mV (0–+20 mV) and recorded continuously every 10 s. Under the maximum activated voltage, the currents achieved stability after recording 5 to 6 times. As shown in Figure 3A, 10 $\mu\text{mol/L}$ ginsenoside Rb1 inhibited the I_{Ba} by $21.53\% \pm 2.81\%$ ($n=5$). The inhibitory effect was eliminated after the application of nifedipine, a selective blocker of L-type Ca^{2+} channels.

As shown in Figure 3B, 10 $\mu\text{mol/L}$ Rb1 inhibited the I_{Ba} by $20.19\% \pm 2.98\%$ ($n=5$) before ω -conotoxin-GVIA treatment and inhibited the I_{Ba} by $20.51\% \pm 3.15\%$ ($n=5$) in the presence of ω -conotoxin-GVIA ($P > 0.05$ compared to Rb1 treatment alone). As shown in Figure 3C, 10 $\mu\text{mol/L}$ Rb1 inhibited the I_{Ba} by $19.80\% \pm 3.21\%$ ($n=5$) before ω -agatoxin IVA treatment and inhibited the I_{Ba} by $20.34\% \pm 2.58\%$ ($n=5$) in the presence

Figure 2. Effect of the extracellular solution, Rb1 (1 $\mu\text{mol/L}$) and Rb1 (10 $\mu\text{mol/L}$), on the I_{Ba} (A, B, and C, respectively). (1) Before drug application; (2) drug application; (3) washout. (D) represents the time course of the experiment corresponding to (C).

Figure 3. Rb1 inhibited the I_{Ba} in hippocampal neurons, and this inhibitory effect was eliminated after the application of nifedipine (A). Neither $\omega\text{-conotoxin-GVIA}$ nor $\omega\text{-agatoxin IVA}$ diminished the Rb1-sensitive I_{Ba} (B and C, respectively). Upper panel, pairs of the inward currents evoked by pulses from -60 to $+0$ mV (0 – $+20$ mV) at the times indicated in the lower panel. Lower panel, time course of the effects of 10 $\mu\text{mol/L}$ Rb1 on the I_{Ba} amplitude before and after application of the Ca^{2+} channel antagonists (10 $\mu\text{mol/L}$ nifedipine, 1 $\mu\text{mol/L}$ $\omega\text{-conotoxin GVIA}$ and 30 nmol/L $\omega\text{-agatoxin IVA}$). The bar graphs for Rb1 inhibition (mean $\pm$ SEM, $n=5$ for Rb1) on the I_{Ba} in cells untreated or treated with Ca^{2+} channel antagonists. $^{\circ}P<0.01$ compared with Rb1 treatment alone.

of $\omega\text{-agatoxin IVA}$ ($P>0.05$ compared to Rb1 treatment alone). Thus, neither $\omega\text{-conotoxin-GVIA}$ nor $\omega\text{-agatoxin IVA}$ could diminish the Rb1-sensitive VGCCs.

To gain a better understanding of the action of Rb1 on the I_{Ba} , we explored its action on the I-V curve and the steady-state inactivation curve of the I_{Ba} . A dose of 10 $\mu\text{mol/L}$ ginsenoside Rb1 inhibited the I_{Ba} at the maximum amplitude (control: $I_{\text{Ba}}=752.56\pm48.42$ pA, Rb1: $I_{\text{Ba}}=600\pm40.70$ pA, $P<0.01$, $n=6$), but had no effect on the activation threshold potential or the reversal potential of the I_{Ba} in the I-V relationship (Figure 4). Furthermore, 10 $\mu\text{mol/L}$ Rb1 shifted the steady-state inactivation curve of the I_{Ba} to a hyperpolarizing voltage (Figure 5) (control: $V_{1/2}=-17.70\pm0.40$ mV, $k=6.26\pm0.41$; Rb1: $V_{1/2}=-25.53\pm0.53$ mV, $k=8.24\pm0.47$; $P<0.05$, $n=6$).

A continuous recording with 10 s intervals was used after

the application of Rb1, and the inhibitory effects of Rb1 on the I_{Ba} were observed during the first 10 s interval in hippocampal neurons. The results indicated that Rb1 inhibited the I_{Ba} within 10 s. To determine if phosphorylation was involved in the inhibition of the I_{Ba} by ginsenoside Rb1, the adenylyl cyclase (AC) agonist forskolin and the protein kinase A (PKA) antagonist H-89 were used. The percentage of I_{Ba} inhibitory action by Rb1 was $20.15\pm3.96\%$ ($n=9$), while that with the bath application of forskolin (10 $\mu\text{mol/L}$) and Rb1 was $22.5\pm2.95\%$ ($n=11$). Forskolin did not offset the inhibitory effect of Rb1. In the presence of H-89 (10 $\mu\text{mol/L}$), the percent inhibitory action by Rb1 was reduced to $20.85\pm3.78\%$ ($n=12$), a value with no statistical significance compared with that of Rb1 alone ($P>0.05$), demonstrating that H-89 did not affect the inhibition of the I_{Ba} caused by Rb1 (Figure 6).

Figure 4. The I-V relationships of the I_{Ba} showed the inhibitory effects of 10 $\mu\text{mol/L}$ Rb1 on the VGCCs ($n=6$). The holding potential was -60 mV, and the test potentials ranged from -70 mV to +70 mV in 10 mV increments.

Figure 5. The effects of 10 $\mu\text{mol/L}$ Rb1 on the voltage-dependence of the steady-state of I_{Ba} inactivation ($n=6$). The data were fitted to the Boltzmann equation.

Discussion

Ginsenosides, which are the pharmacologically active ingredients of *Panax ginseng*, produce reversible and selective inhibitory effects on voltage-dependent and ligand-gated ion channels^[23–26]. Studies have also found that the mechanisms of action of saponins vary due to their types or the cell types they act on. For example, ginsenosides activate $G_{\alpha_{q/11}}$, a protein coupled to PLC, leading to IP_3 -dependent endoplasmic reticulum calcium release in *Xenopus oocytes*. However, this effect does not occur in neurons^[27]. Although previous studies have reported the diversity of voltage-dependent Ca^{2+} channels in hippocampal neurons, and this diversity was also confirmed in the present experiment, none of the research literature mentions the action of the ginsenoside Rb1 on VGCCs in hippocampal neurons. We found that Rb1 at a concentration range of 1 to 100 $\mu\text{mol/L}$ inhibited the calcium channel currents of hippocampal neurons in a dose-dependent manner.

Figure 6. (A) The percentage of inhibitory action by 10 $\mu\text{mol/L}$ Rb1 and Rb1 co-administered with forskolin. (B) The percentage of inhibitory action by 10 $\mu\text{mol/L}$ Rb1 in the presence and absence of H-89.

The I_{Ca} peak current inhibition rate paralleled the increase of Rb1 concentration, and the inhibitory effect was mostly reversible.

Most studies support the effect of Rb1 in preventing neuronal death linked to neurodegenerative diseases^[13, 15, 28, 29]. Its perturbed neuronal Ca^{2+} homeostasis is implicated in aging and age-related cognitive impairments^[12]. Chen also showed that it could decrease the $\text{A}\beta$ -induced elevation of intracellular calcium and stabilize microtubule integrity^[18]. It is widely known that postsynaptic $[\text{Ca}^{2+}]_i$ and L-type voltage-gated calcium channel currents are upregulated in the hippocampus during aging, despite a significant decrease of cell density^[30]. Elevated postsynaptic $[\text{Ca}^{2+}]_i$ and L-type voltage-gated calcium channel activity contribute to impaired synaptic plasticity^[31] and working memory^[32] in aged hippocampal neurons. The increase of L-type voltage-gated calcium channel currents also enhances the susceptibility of aging neurons for apoptosis. Fu has shown that the cholinesterase inhibitor tacrine can reduce $\text{A}\beta$ -induced neuronal apoptosis by regulating L-type voltage-gated calcium channel activity^[33]. Our results show that the ginsenoside Rb1 selectively targets L-type calcium channels by inhibiting voltage-gated calcium channels. Different calcium channels have distinct electrophysiological characteristics and are closely related to different cell functions. For example, ginsenoside selectively acts on the non-L-type calcium channels of chromaffin cells, which are related to the regulation of the secretion of catecholamines^[34]. Additionally, ginsenoside Rf selectively acts on the N-type calcium channels of sensory neurons, which are related to the inhibition of neurotransmitter release following painful stimuli^[35]. Therefore, we infer that the selective action of Rb1 on the L-type calcium channels of hippocampal neurons may be the cellular basis of its pharmacological effects in preventing neuronal death linked to neurodegenerative diseases.

In this study, Rb1 induced a leftward shift of the steady-

state inactivation curves of the I_{Ba} to a negative potential without affecting its activation kinetics or reversal potential in the I-V curve, indicating that Rb1 may alter the biophysical nature of the calcium channel and inhibit channel activity by accelerating channel activity access to the inactivation state without affecting its activation characteristics. These results suggest that Rb1 regulates the activity of calcium channels by altering their time dependence.

Protein phosphorylation modulates the function of VGCCs, and the AC-cAMP-PKA system plays a key role in this phosphorylation^[36, 37]. Therefore, we used forskolin and H-89 to investigate whether the action of Rb1 on the I_{Ca} is involved in this mechanism. We found that co-application of forskolin and Rb1 did not affect the reduction caused by Rb1. Additionally, the action of Rb1 was not affected by H-89, indicating that the cAMP-PKA system might not be involved in the mechanism by which Rb1 reduces the I_{Ca} . These results confirmed our previous speculation that it is difficult to achieve Ca^{2+} channels phosphorylation within 10 s.

In summary, this study provides electrophysiological evidence that Rb1 induces calcium current inhibition by inhibiting the activity of the L-type Ca^{2+} channels in hippocampal neurons. This finding raises the possibilities that Rb1 may be useful and potentially therapeutic choices in the treatment of neurological disorders.

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Author contribution

Xiao-chun CHEN designed the research; Zhi-ying LIN, Li-min CHEN, Jing ZHANG, and Xiao-dong PAN performed the experiments; Yuan-gui ZHU, Qin-yong YE, and Hua-pin HUANG contributed new analytical tools and performed the data analysis; Zhi-ying LIN wrote the paper.

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