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 μmol/L) inhibited VGCCs in a concentration-dependent manner, and the current was mostly recovered upon wash-out. The specific L-type Ca2+ channel inhibitor nifedipine (10 μmol/L) occluded Rb1-induced inhibition on VGCCs. Neither the selective N-type Ca2+ channel blocker ω-conotoxin-GVIA (1 μmol/L), nor the selective P/Q-type Ca2+ 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 μmol/L), nor by the PKA inhibitor H-89 (10 μmol/L).

Conclusion: Ginsenoside Rb1 selectively inhibits the activity of L-type voltage-gated calcium channels, without affecting the N-type or P/Q-type Ca2+ channels in hippocampal neurons. cAMP-PKA signaling pathway is not involved in this effect.

Keywords: ginsenoside Rb1; L-type Ca2+ channel; nifedipine; ω-conotoxin-GVIA; ω-agatoxin IVA; patch-clamp technique; hippocampus; cAMP-PKA signaling pathway
tigated the action of ginsenoside Rb1 on voltage-gated Ca2+ channels in hippocampal neurons. In this paper, we analyzed the effect of ginsenoside Rb1 on voltage-gated Ca2+ 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 Ca2+ channel antagonists, α-conotoxin GVIA (Alomone Labs, UK), nifedipine (Sigma, UK) α-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.

**Electrophysiological recordings**

Single patch recordings of Ca2+ 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 Electroniks, 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 MΩ. The pipettes were filled with an intracellular solution containing 80 mmol/L Cs-methanesulfonate, 20 mmol/L tetraethylammonium chloride (TEA-Cl), 1 mmol/L CaCl2, 5 mmol/L MgCl2, 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 Na2ATP. The chemicals were obtained from Sigma. CsOH was used to adjust the pH to 7.2–7.3. The osmolarity of the pipette solution was adjusted to 300 mOsm with sucrose. As suggested by Smirnov [20], the replacement of 1.5 mmol/L Ca2+ with 5 mmol/L Ba2+ was used to augment the amplitude of the inward current through Ca2+ channels. The potential dependency of activation and inactivation with 5 mmol/L Ba2+ was very similar to the results observed in 1.5 mmol/L Ca2+. To isolate the Ba2+ 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 BaCl2, 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 osmolarity of the extracellular solution was adjusted to 300 mOsm with sucrose. 4-AP and TTX were used to eliminate outward K+ currents. TTX was used to eliminate inward Na+ currents.

We recorded the voltage-gated calcium channel Ba2+ 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±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:

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

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 $I_{\text{Ba}}$ peak current was calculated as follows: [(maximum current value before administration – maximum current value after administration)/maximum current value before administration]×100%. The control group was treated with the extracellular solution, and the experimental groups were treated with 1, 2, 5, 10, and 100 μmol/L Rb1. Both the control group and the 1 μmol/L Rb1 group showed no inhibitory effects on the $I_{\text{Ba}}$ (Figure 2A, 2B). However, the other experimental groups with 2, 5, 10, and 100 μmol/L Rb1 demonstrated inhibitory effects on the $I_{\text{Ba}}$. The inhibition rates were 2%±0.87% ($n$=6), 5%±1.78% ($n$=4), 20%±3.96% ($n$=9), and 40%±6.71% ($n$=5), respectively. The $I_{\text{Ba}}$ 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 $I_{\text{Ba}}$ were partially reversible after wash-out with the bathing solution (Figure 2C, 2D).

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

The $I_{\text{Ba}}$ 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 μmol/L ginsenoside Rb1 inhibited the $I_{\text{Ba}}$ by $21.53%±2.81%$ ($n$=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 μmol/L Rb1 inhibited the $I_{\text{Ba}}$ by $20.19%±2.98%$ ($n$=5) before ω-conotoxin-GVIA treatment and inhibited the $I_{\text{Ba}}$ by $20.51%±3.15%$ ($n$=5) in the presence of ω-conotoxin-GVIA ($P>0.05$ compared to Rb1 treatment alone). As shown in Figure 3C, 10 μmol/L Rb1 inhibited the $I_{\text{Ba}}$ by $19.80%±3.21%$ ($n$=5) before ω-agatoxin IVA treatment and inhibited the $I_{\text{Ba}}$ by $20.34%±2.58%$ ($n$=5) in the presence of ω-agatoxin IVA.

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

| Groups      | $I_{\text{Ba}}$ inhibition (%) |
|-------------|-------------------------------|
| Control     | 0±0.74                        |
| 1 μmol/L Rb1| 0±1.12                        |
| 2 μmol/L Rb1| 2±0.87*                       |
| 5 μmol/L Rb1| 5±1.78*                       |
| 10 μmol/L Rb1| 20±3.96*                      |
| 100 μmol/L Rb1| 40±6.71*                    |

$*P<0.01$ compared with the previous group.
of ω-agatoxin IVA ($P>0.05$ compared to Rb1 treatment alone). Thus, neither ω-conotoxin-GVIA nor ω-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 μmol/L ginsenoside Rb1 inhibited the $I_{Ba}$ at the maximum amplitude (control: $I_{Ba}=752.56\pm48.42$ pA, Rb1: $I_{Ba}=600\pm40.70$ 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 μmol/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%±3.96% ($n=9$), while that with the bath application of forskolin (10 µmol/L) and Rb1 was 22.5%±2.95% ($n=11$). Forskolin did not offset the inhibitory effect of Rb1. In the presence of H-89 (10 µmol/L), the percent inhibitory action by Rb1 was reduced to 20.85%±3.78% ($n=12$), 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).
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 Gα\_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\[^{27}\]. Chen also showed that it could decrease the Aβ-induced elevation of intracellular calcium and stabilize microtubule integrity\[^{18}\]. It is widely known that postsynaptic [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 [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 Aβ-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-

![Figure 4](image1.png)

**Figure 4.** The I-V relationships of the \(I_{\text{Ba}}\) showed the inhibitory effects of 10 µmol/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](image2.png)

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

![Figure 6](image3.png)

**Figure 6.** (A) The percentage of inhibitory action by 10 µmol/L Rb1 and Rb1 co-administered with forskolin. (B) The percentage of inhibitory action by 10 µmol/L Rb1 in the presence and absence of H-89.
state inactivation curves of the $I_{na}$ 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\cite{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, Limin 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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