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Ginsenosides Inhibit N-, P-, and Q-types but not L-type of Ca^{2+} Channel in Bovine Chromaffin cells

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Abstract : In previous reports we have shown that ginsenosides inhibit high threshold voltage-dependent Ca^{2+} channels in neuronal cells. However, these studies did not show whether ginsenosides-induced inhibition of Ca^{2+} currents discriminates among the various Ca^{2+} channel subtypes, although it is known that there are at least five different Ca^{2+} channel subtypes in neuronal cells. In this study we investigated the effect of ginsenosides on high threshold voltage-dependent Ca^{2+} channel subtypes using their selective Ca^{2+} channel blockers nimodipine (L-type), ω-conotoxin GVIA (N-type), or ω-agatoxin IVA (P-type) in bovine chromaffin cells. We could observe that ginsenosides inhibited high threshold voltage-dependent Ca^{2+} currents in a dose-dependent manner. The IC_{50} was about 120 μg/m*l*. Nimodipine had no effect on ginsenosides response. However, the effect of ginsenosides on Ca^{2+} currents was reduced by ω-conotoxin GVIA, ω-agatoxin IVA, and mixture of nimodipine, ω-contoxin GVIA, and ω-agatoxin IVA. These data suggest that ginsenosides are negatively coupled to three types of calcium channels in bovine chromaffin cell, including an ω-conotoxin GVIA-sensitive (N-type) channel, an ω-agatoxin IVA-sensitive (P-type) channel and nimodipine/ω-conotoxin GVIA/ω-agatoxin IVA-resistant (presumptive Q-type) channel.

Key words : Ginsenosides, Ca^{2+} channels subtypes, chromaffin cells, anti-stress.

INTRODUCTION

Ginseng, the root of *Panax ginseng* C.A. Meyer, is a well-known folk medicine that has been shown to produce a variety of medicinal effects, both within and out of nervous systems. A recent study showed that ginsenosides or ginseng saponins are the main molecules responsible for the actions of ginseng. These ginsenosides are well characterized and have a four-ring, steroid-like structure with sugar moieties attached and about thirty ginsenosides have been isolated and identified from the root of *Panax* ginseng.[1]

Ca^{2+} is an important regulator for many neuronal functions, including exocytosis and excitability. Voltage-dependent Ca^{2+} channels play a key role in control of free cytosolic Ca^{2+}.[2] Now, it is known that there are at least more than five different subtypes of voltage-dependent Ca^{2+} in nervous system such as L-, N-, P-, Q-, and T-type of Ca^{2+} channels. N-type Ca^{2+} channels are pharmacologically identified and characterized by irreversible blocker ω-conotoxin GVIA.[3] P-type channels are blocked potently by ω-agatoxin IVA but with a somewhat lower affinity and exhibit little or no inactivation during prolonged depolarization,[4] whereas Q-type channels show inactivation even after 100 ms.[5]

Recent reports demonstrated that ginseng root extract has shown to inhibit voltage-dependent Ca^{2+} channels in sensory neurons and ginsenoside Rf, one of ginsenosides, was more potent than several other ginsenosides in inhibiting Ca^{2+} channels via pertussis toxin (PTX)-sensitive G proteins.[6-8] It is well known that adrenal chromaffin cells secrete catecholamines in response to acetylcholine (ACh) released from the terminal of splanchnic nerve. Binding of ACh to the nicotinic receptors leads to depolarization of the cell by influx of Na^+ through ACh-gated cation channels, causes the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels, and results in catecholamine secretion by exocytosis. Ginsenosides and several individual ginsenosides such as Rb_1, Rc, Re, Rf, and Rg_1 were also found to inhibit voltage-dependent Ca^{2+} channels in rat adrenal

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chromaffin cells.[9] Thus, although we have shown in previous studies that ginsenosides inhibit voltage-dependent Ca^{2+} channels in sensory neurons and chromaffin cells, we did not demonstrated that which subtype(s) of Ca^{2+} channels are mainly affected by ginsenosides. Therefore, this study was performed to test the effect of ginsenosides on voltage-dependent Ca^{2+} channels subtypes using bovine chromaffin cells, which contain four different Ca^{2+} channel subtype such as L-, N-, P-, and Q-type.[10]

MATERIALS AND METHODS

1. Materials

Ginsenosides were kindly obtained from Korea Ginseng and Tobacco Research Institute. Ginsenosides contained at least 11 glycosides such as Rb_1 (18.26%), Rb_2 (9.07%), Rc (9.65%), Rd (8.24%), Re (9.28%), Rf (3.48%), Rg_1 (6.42%), Rg_2 (3.62%), Rg_3 (4.7%), Ro (3.82%), Ra (2.91 %), and other minor ginsenosides (20.55%) and were dissolved in extracellular solution before use. Nimodipine (RBI, USA) was dissolved into 100 mM stock solution in DMSO and was diluted for final concentration and ω-conotoxin GVIA (RBI, USA) and ω-agatoxin IVA (Peptide Institute, Japan) were dissolved in 0.1% BSA.

2. Methods

Adrenal chromaffin cells were isolated from bovine adrenal medulla by collagenase digestion as previously described with slight modification.[11] Briefly, medulla part of adrenal glands was minced into small pieces and was incubated in Ca^{2+}+Mg^{2+}-free Lockes solution (157.4 mM NaCl, 5.6 mM KCl, 5 mM Hepes, 5.6 mM glucose, 200 U/ m*l* penicillin, and 40 mg/ml gentamicin, pH 7.4) containing 0.5% collagenase (Type II; Worthington, USA) for 50 min at 37 with gentle stirring. After removing tissue clumps using nylon mesh, cells were washed with Ca^{2+}+Mg^{2+}-free Lockes solution by three times and cells were further washed with Ca^{2+}+Mg^{2+}-containing Lockes solution (157.4 mM NaCl, 5.6 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, 5.6 mM glucose, 200 U/m*l* penicillin, and 40 mg/m*l* gentamicin, pH 7.4) by three times. Cells were resuspended with DMEM/F12 media (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) and were seeded into poly-L-lysine coated dish. Cells were placed in a 37 incubator and gassed with 5% CO_2.

3. Recordings and experimental solutions

All recordings used standard whole-cell patch clamp at room temperature.[12] Whole-cell currents were recorded with Axopatch 1D amplifier and Digidata 1200 interface. Data acquisition and analysis was performed using pClamp 6.0. Test pulses of 200 ms duration were applied every 15s. Records were filtered at 2 kHz with an 8-pole Bessel filter. Leak, capacity, and other contaminating currents were eliminated by subtracting recordings in 1 mM Cd^{2+}, a selective Ca^{2+} channel blocker. The resistance of the electrode was 2-4 MΩ. The application of solutions was performed to according to the method of Taddese *et al.* (1995).[13] The extracellular solution contained 5 mM $CaCl_2$, 150 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes (titrated to pH 7.4 with TEA-OH), and 0.1% glucose. Compared with Na^+ containing solution diminishes the rate of Ca^{2+} channel rundown during prolonged recordings. The recording electrode contained 100 mM CsCl, 1 mM Na_2ATP, 0.3 mM Na_3GTP, 10 mM EGTA, 2.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 80 μM leupeptin, 8 mM creatine phosphate (Na salt) and 40 mM HEPES (pH 7.0). Unless otherwise indicated, Ca^{2+} current amplitude was measured by averaging the data between 180 ms and 200 ms after the onset of the pulse. This procedure avoids contamination due to voltage-dependent Na^+ current. Data were presented as means ± S.E.M. Statistical significance was measured as a paired Students *t*-test.

RESULTS AND DISCUSSION

First, we investigated the dose-dependent effect of ginsenosides on voltage-dependent Ca^{2+} channels in bovine chromaffin cells. As shown in Fig. 1, ginsenosides had no effect on Ca^{2+} currents in the range of 10 to 25 μg/m*l* and at dose of 50 μg/m*l* ginsenosides inhibit Ca^{2+} current slightly. However, at dose over 100 μg/m*l* ginsenosides inhibit Ca^{2+} current by 17~25% and the IC_{50} was about 120 μg/m*l*.

We investigated the effect of ginsenosides on various Ca^{2+} channel subtype antagonists such as ω-conotoxin GVIA (N-type Ca^{2+} channel blocker, 1 μM), nimodipine (L-type Ca^{2+} channel blocker, 10 μM), ω-agatoxin IVA (P-type Ca^{2+} channel blocker, 100 nM), and mixture of nimodipine, ω-conotoxin GVIA (1 μM), and ω-agatoxin IVA (100 nM) for blocking presumptive Q-type Ca^{2+} channels. As shown in Fig. 2, ginsenosides (100 μg/m*l*) inhibited Ca^{2+} current by 24.1 ± 3.0% (n=8) before ω-conotoxin treatment. ω-Conotoxin GVIA inhibited Ca^{2+} current by 36.0 ± 8.0% (n=8) irreversibly and ginsenosides in the presence of ω-conotoxin GVIA inhibited 6.0

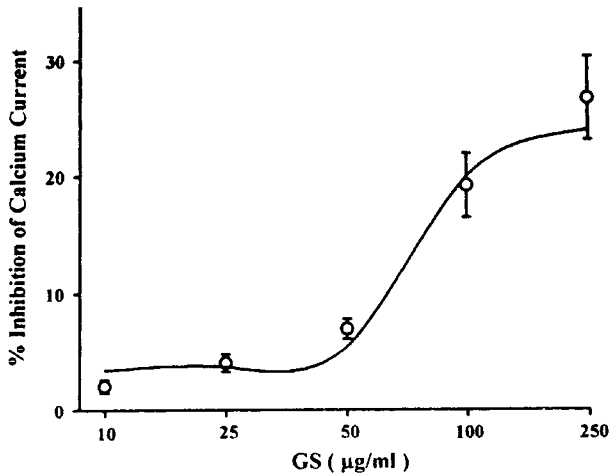

Fig. 1. Dose dependence of Ca^{2+} channel inhibition by ginsenosides (GS). Average percentage inhibition (±SEM; six to eight cells used for each data point) of ICa vs. GS concentration. The curve is the best least-squares fit of the Michalis-Menten equation: $y/y_{max}=[GS]/([GS]+K_{1/2})$, where y_{max} is the maximum inhibition (33±4%, SD), $K_{1/2}$ is the con- centration for half-maximal inhibition (120±17 μg/m*l*, SD), and [GS] is the concentration of GS.

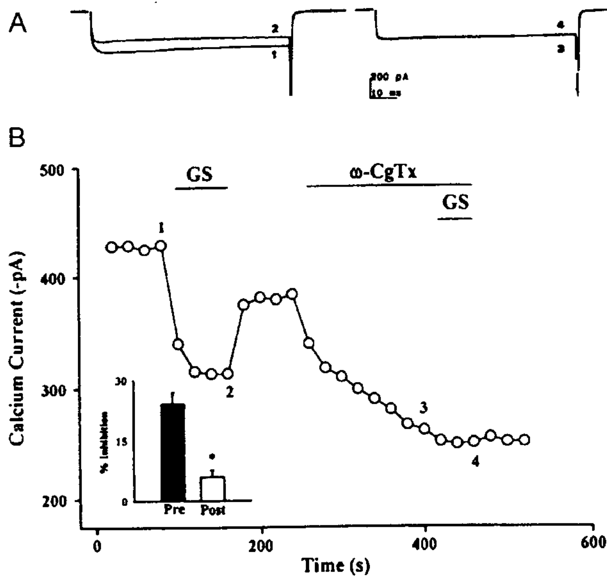

Fig. 2. GS inhibits high threshold N-type Ca^{2+} channels. (A) Pairs of inward currents evoked by pulses from -70 mV to +10 mV at the times indicated in (B). Leak and capacity currents were eliminated by subtracting records obtained in 1 mM Cd^{2+}, a Ca^{2+} channel blocker. (B) Time course of effects of 100 μg/m*l* ginsenosides (GS) on Ca^{2+} current amplitude before and after application of ω-conotoxin-GVIA (1 μM), a selective, irreversible antagonist of N-type Ca^{2+} channels. *Inset*: The bar graphs of average percentage inhibition (mean±SEM, n=for GS) of Ca^{2+} current by GS in cells either untreated or after treated with ω-conotoxin GVIA. *P<0.005 compared to ginsenosides alone treatment.

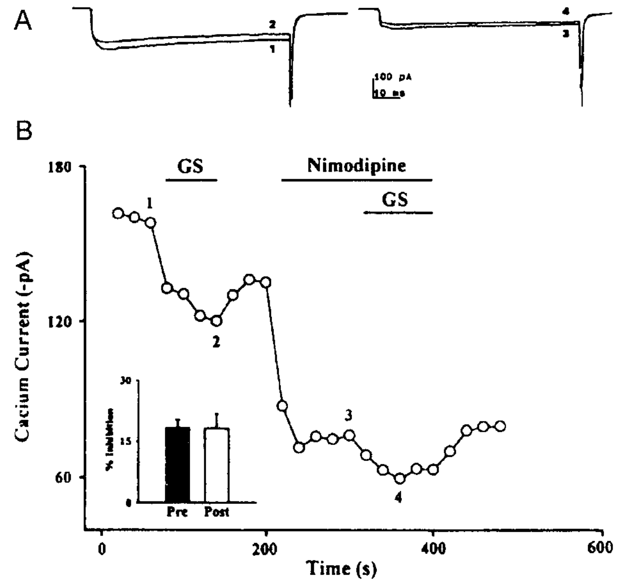

Fig. 3. GS spares high threshold L-type Ca^{2+} channels. (A) Pairs of inward currents evoked by pulses from -70 mV to +10 mV at the times indicated in (B). Leak and capacity currents were eliminated by subtracting records obtained in 1 mM Cd^{2+}, a Ca^{2+} channel blocker. (B) Time course of effects of 100 μg/m*l* ginsenosides (GS) on Ca^{2+} current amplitude before and after application of nimodipine (10 μM), a selective antagonist of L-type Ca^{2+} channels. *Inset*: The bar graphs of average percentage inhibition (mean±SEM, n= for GS) of Ca^{2+} current by GS in cells either untreated or after treated with nimodipine. P<0.956 compared to ginsenosides alone treatment.

1.7% (n=8) (p<0.005 compared to ginsenoside treatment alone). As shown in Fig. 3, ginsenosides (100 μg/m*l*) inhibited Ca^{2+} current by 18.3±2.0% (n=8) before nimodipine treatment. Nimodipine inhibited Ca^{2+} current reversibly by 26.8±4.0% (n=8) and ginsenosides in the presence of nimodipine inhibited 18.1±3.6% (n=8) (p<0.956 compared to ginsenosides treatment alone). As shown in Fig. 4, ginsenosides (100 μg/m*l*) inhibited Ca^{2+} current by 17.3±3.3% (n=7) before ω-agatoxin IVA treatment. ω-agatoxin IVA inhibited Ca^{2+} current in a slightly reversible manner by 17.4±1.7% (n=7) and ginsenosides in the presence of ω-agatoxin IVA inhibited 7.6±1.4% (n=7) (p<0.05 compared to ginsenosides treatment alone). As shown in Fig. 5, ginsenosides (100 μg/m*l*) inhibited Ca^{2+} current by 16.7±2.7% (n=8) before ω-conotoxin GVIA, ω-agatoxin IVA, and nimodipine cotreatment. ω-Conotoxin GVIA, ω-agatoxin IVA, and nimodipine cotreatment inhibited Ca^{2+} current irreversibly by 81.5±5.2% (n=8) and ginsenosides in the presence of both toxins and nimo-

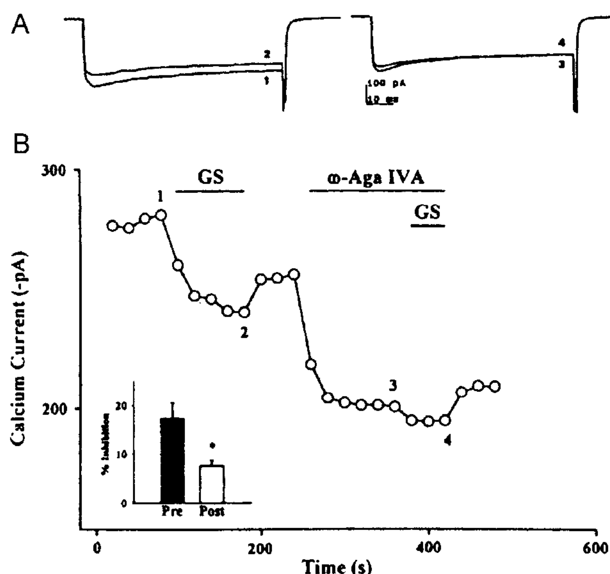

Fig. 4. GS inhibits high threshold P-type Ca^{2+} channels. (A) Pairs of inward currents evoked by pulses from -70 mV to +10 mV at the times indicated in (B). Leak and capacity currents were eliminated by subtracting records obtained in 1 mM Cd^{2+}, a Ca^{2+} channel blocker. (B) Time course of effects of 100 μg/m*l* ginsenosides (GS) on Ca^{2+} current amplitude before and after application of ω-agatoxin IVA (100 nM), a selective antagonist of P-type Ca^{2+} channels. *Inset*: The bar graphs of average percentage inhibition (mean±SEM, n = for GS) of Ca^{2+} current by GS in cells either untreated or after treated with ω-agatoxin IVA. *P<0.05 compared to ginsenosides alone treatment.

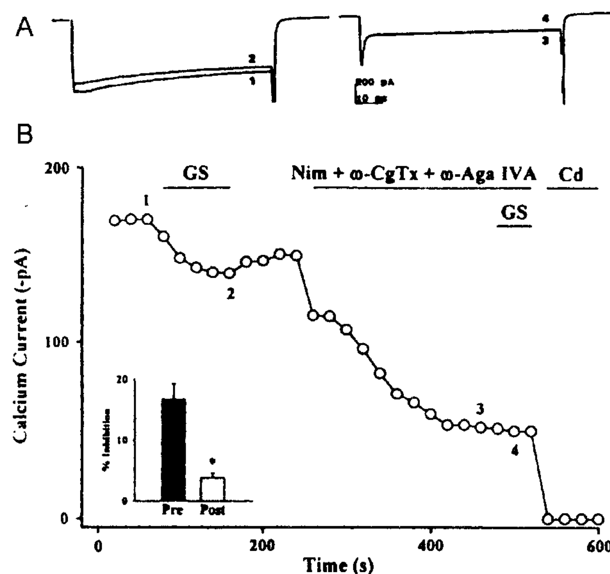

Fig. 5. GS inhibits high threshold presumptive Q-type Ca^{2+} channels. (A) Pairs of inward currents evoked by pulses from -70 mV to +10 mV at the times indicated in (B). Leak and capacity currents were eliminated by subtracting records obtained in 1 mM Cd^{2+}, a Ca^{2+} channel blocker. (B) Time course of effects of 100 μg/m*l* ginsenosides (GS) on Ca^{2+} current amplitude before and after application of nimodipine (10 μM), ω-conotoxin GVIA (1 μM), and ω-agatoxin IVA (100 nM). *Inset*: The bar graphs of average percentage inhibition (mean±SEM, n=for GS) of Ca^{2+} current by GS in cells either untreated or after treated with three Ca^{2+} channel antagonists. *P<0.0004 compared to ginsenosides alone treatment.

dipine inhibited 3.84±0.8% (n=8) (p<0.0004 compared to ginsenosides treatment alone) and treatment of Cd^{2+} blocked almost of residual Ca^{2+} currents.

We showed that ginsenosides inhibited high threshold Ca^{2+} currents in bovine chromaffin cells in present study and rat chromaffin cells in previous study.[6] Interestingly, in both studies the effect of ginsenosides on Ca^{2+} currents was partially reversible even after washing out ginsenosides. In contrast, previous study using rat sensory neurons the effect of ginsenosides on Ca^{2+} current was reversible after washing out ginsenosides.[6-8] Thus, the reversibility of ginsenosides on Ca^{2+} current inhibition might depend on neuronal cell type.

In previous study we have shown a possibility that ginsenosides might regulate more than one type of Ca^{2+} channels, since ginsenoside Rf inhibits N-type of Ca^{2+} channels as well as other types of Ca^{2+} channels in sensory neurons.[8] In present study we did further investigate to know which type of Ca^{2+} channels are regulated by ginsenosides in bovine chromaffin cells, which are well-known to have four subtypes of Ca^{2+} channel such as L-, N-, P-, and Q-type, using various Ca^{2+} channel antagonists. As shown in Figs. 2~5, ginsenosides inhibited mainly N-, P-, and Q-types but not L-types of Ca^{2+} channels. Interestingly, it is known that calcium influx through the N-, P-, and Q-subtypes among various voltage-dependent Ca^{2+} channels triggers neurotransmission at central and peripheral synapses.[14,15] However, it was not clearly reported that which subtypes of Ca^{2+} channel are involved in exocytosis of bovine chromaffin cells. Recent reports showed that non-L-type Ca^{2+} channels (N- and P/Q-types) are about 80% of the total Ca^{2+} current and L-type channels contribute about 20% in bovine chromaffin cells.[10] Opioids and ATP inhibit N- and P/Q-types of Ca^{2+} channel in these cells and it seems that these non-L-type Ca^{2+} channels are involved in catecholamines secretion.[10] However, Artalejo *et al.* (1991) also showed that the 14pS

Ca^{2+} channels (which are probably P-type or Q-type of Ca^{2+} channels) are involved in regular secretion of catecholamine in the absence of stress and 27pS Ca^{2+} channel (which are L-type of Ca^{2+} channels) are activated under the situation of strong depolarization and might be involved in massive secretion of catecholamine under stress.[16] Thus, it looks that all L-type and non-L-type Ca^{2+} channels might be involved in catecholamine secretion. On the other hand, ginseng has been used as an anti-stress agent[1] and ginsenosides as active ingredients of ginseng might be responsible for its anti-stress efficacy by selective regulation of voltage-dependent Ca^{2+} channel subtypes, which are mainly involved in catecholamine secretion in chromaffin cells.

4. Acknowledgements

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요 약

앞의 연구에서 우리는 진세노사이드가 신경세포에 존재하는 high-threshold voltage-dependent Ca^{2+} channel을 억제한다는 것을 발표하였다. 그러나, 이러한 연구는 진세노사이드가 여러 칼슘 채널 subtypes중 어느 특정 칼슘 채널만을 선택적으로 조절한다는 것을 보여주지는 않았다. 따라서 이 연구에서 우리는 여러 칼슘 채널 subtypes에 선택적으로 작용하는 약물 혹은 toxins을 이용하여 진세노사이드가 어느 종류의 칼슘 채널 subtypes를 억제하는가를 bovine chromaffin cell을 이용하여 연구하였다. 사용한 물질은 nimodipine(L-type 칼슘 채널 길항제), ω-conotoxin GVIA(N-type Ca^{2+} channel 길항제), ω-agatoxin IVA(P-type 칼슘 채널 길항제) 이었다. 연구 결과 진세노사이드는 bovine chromaffin 세포에 존재하는 high-threshold 칼슘 current을 투여 농도별로 억제하였다. IC_{50}은 약 120 μg/ml인 것으로 나타났다. nimodipine은 진세노사이드에 의한 칼슘 currents 억제 작용에 영향을 미치지 않은 것으로 나타났다. 그러나, ω-conotoxin GVIA, ω-agatoxin IVA 및 nimodipine+ω-conotoxin GVIA+ω-agatoxin IVA을 처리한 세포에서는 진세노사이드에 의한 칼슘 currents 억제 작용이 현저하게 줄어 들었다. 이러한 연구 결과들은 진세노사이드가 L-type 칼슘 채널은 억제하지 않고, 주로 N-, P-, 및 Q-type 칼슘 채널을 억제한다는 것을 보여주고 있다.

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