Effects of Ginsenoside Metabolites on GABA<sub>A</sub> Receptor-Mediated Ion Currents

Byung-Hwan Lee<sup>1</sup>, Sun-Hye Choi<sup>1</sup>, Tae-Joon Shin<sup>1</sup>, Sung-Hee Hwang<sup>1</sup>, Jiyeon Kang<sup>1</sup>, Hyeon-Joong Kim<sup>1</sup>, Byung-Ju Kim<sup>2</sup>, and Seung-Yeol Nah<sup>*1</sup>

<sup>1</sup>Ginsentology Research Laboratory and Department of Physiology, College of Veterinary Medicine and Bio/Molecular Informatics Center, Konkuk University, Seoul 143-701, Korea
<sup>2</sup>Division of Longevity and Biofunctional Medicine, Pusan National University School of Korean Medicine, Yangsan 626-870, Korea

In a previous report, we demonstrated that ginsenoside Rc, one of major ginsenosides from <i>Panax ginseng</i>, enhances γ-aminobutyric acid (GABA) receptor<sub>A</sub> (GABA<sub>A</sub>)-mediated ion channel currents. However, little is known about the effects of ginsenoside metabolites on GABA<sub>A</sub> receptor channel activity. The present study investigated the effects of ginsenoside metabolites on human recombinant GABA<sub>A</sub> receptor (α<sub>1</sub>β<sub>1</sub>γ<sub>2s</sub>) channel activity expressed in <i>Xenopus</i> oocytes using a two-electrode voltage clamp technique. M4, a metabolite of protopanaxatriol ginsenosides, more potently inhibited the GABA-induced inward peak current (I<sub>GABA</sub>) than protopanaxadiol (PPD), a metabolite of PPD ginsenosides. The effect of M4 and PPD on I<sub>GABA</sub> was both concentration-dependent and reversible. The half-inhibitory concentration (IC<sub>50</sub>) values of M4 and PPD were 17.1±2.2 and 23.1±8.6 µM, respectively. The inhibition of I<sub>GABA</sub> by M4 and PPD was voltage-independent and non-competitive. This study implies that the regulation of GABA<sub>A</sub> receptor channel activity by ginsenoside metabolites differs from that of ginsenosides.

Keywords: Panax ginseng, Ginsenoside metabolites, Gamma-aminobutyric acid receptor, receptor, Xenopus oocytes

INTRODUCTION

Gamma-aminobutyric acid (GABA) receptor<sub>A</sub> (GABA<sub>A</sub>) receptors are members of the large ‘Cys-loop’ super-family of evolutionarily related and structurally similar ligand-gated ion channels that also includes the nicotinic acetylcholine, glycine, and 5-HT<sub>1</sub> receptors [1]. The GABA receptor is predominantly expressed in the central nervous system [2,3], and forms a chloride-selective transmembrane channel in the post-synaptic sites of nerve terminals. Thus, the GABA<sub>A</sub> receptor is responsible for fast inhibitory synaptic transmission [4,5].

Recent biochemical binding assays produced evidence that ginsenosides might regulate the GABA<sub>A</sub> receptor. For example, Kimura <i>et al.</i> [6] showed that ginsenosides differentially regulate [<sup>3</sup>H]-flunitrazepam or [<sup>3</sup>H]-muscimol binding to the GABA<sub>A</sub> receptor in a rat brain membrane fraction. Kim <i>et al.</i> [7] reported that prolonged infusion with ginsenoside Rc, but not ginsenoside Rg1, into rat brain elevates [<sup>3</sup>H]-muscimol binding to the GABA<sub>A</sub> receptor in a brain region-specific manner. Thus, ginsenosides may regulate the GABA<sub>A</sub> receptor by affecting ligand affinity for its receptor, but there is no direct evidence on the regulation of GABA<sub>A</sub> receptor channel activity by ginsenosides. On the other hand, Choi <i>et al.</i> [8] showed that ginsenoside Rc enhances GABA-mediated
ion currents in oocytes expressing the GABAA receptor. These results indicate the possibility that ginsenosides are closely related with GABAA receptor regulation. It is known that protopanaxadiol and protopanaxatriol ginsenosides are metabolized into protopanaxadiol (PPD) and M4, respectively, by gastric juice and intestinal microorganisms after ginseng intake [9]. However, little is known about whether PPD or M4 regulates GABAA receptor channel activity.

In the present study, we examined the effects of ginsenoside metabolites on GABAA receptor channel activity. We injected neuronal human GABAA (α1β1γ2) receptor cRNAs into Xenopus oocytes and examined the effect of ginsenoside metabolites on the GABA-elicited inward peak current in oocytes expressing α1β1γ2s GABAA receptors.

**Fig. 1.** Chemical structure of the ginsenoside metabolites M4 and protopanaxadiol (PPD) (A) and their effects in oocytes expressing γ-aminobutyric acid (GABA) receptors (B). M4 and PPD had no effect on GABA-induced inward peak current in oocytes expressing α1β1γ2s GABAA receptors.

**Materials and Methods**

**Materials**

The ginsenoside metabolites M4 and PPD were provided by the AMBO Institute (Seoul, Korea) (Fig. 1A). cDNAs for human GABAA receptor subunits were kindly provided by Dr. Whiting (Merck Sharp and Dohme Research Lab., Essex, UK). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Preparation of Xenopus oocytes and microinjection**

_Xenopus laevis_ frogs were purchased from _Xenopus_ I (Ann Arbor, MI, USA). Their care and handling were in accordance with the highest standards of institutional guidelines of Konkuk University. For isolation of oocytes, frogs were anesthetized with an aerated solution of 3-amino benzoic acid ethyl ester followed by removal of ovarian follicles. The oocytes were treated with collagenase and then agitated for 2 h in a Ca2+-free medium containing 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl2, 5 mM HEPES, 2.5 mM sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin. Stage V-VI oocytes were collected and stored in ND96 medium (in mM: 96 NaCl, 2 KCl, 1 MgCl2, 1.8 CaCl2, and 5 HEPES, pH 7.5) supplemented with 50 μg/mL gentamicin. The oocyte-containing solution was maintained at 18°C with continuous gentle shaking and renewed daily. Electrophysiological experiments were performed within 5 to 6 d of oocyte isolation, with ginsenoside metabolites added to the bath. For GABAA receptor activity experiments, each GABAA receptor subunit-encoding cRNA (40 nL) was injected into the animal or vegetal pole of each oocyte one day after isolation, using a 10 µL microdispenser (VWR Scientific, San Francisco, CA, USA) fitted with a tapered glass pipette tip 15 to 20 µm in diameter [8].

**Data recording**

A custom-made Plexiglas net chamber was used for two-electrode voltage-clamp recordings as previously reported [8]. The oocytes were impaled with two microelectrodes filled with 3M KCl (0.2-0.7 MΩ), and electrophysiological experiments were carried out at room temperature using an Oocyte Clamp (OC-725C; Warner Instruments, Hamsden, CT, USA). Stimulation and data acquisition were controlled with a pClamp 8 (Axon Instruments, Union City, CA, USA). For most electrophysiological experiments, oocytes were perfused initially with ND96 solution (in mM: 96 NaCl, 3 KCl, 2 CaCl2, and 5 HEPES; pH 7.4 with NaOH) and control current recordings were obtained. For most electrophysiological data, the oocytes were clamped at a holding potential of −80 mV. For current and voltage (I-V) relationship, voltage ramps were applied from −100 to +40 mV for 300 ms. In the different membrane-holding potential experiments, the oocytes were clamped at the indicated holding potentials. Linear leak and capacitance currents were corrected by means of the leak subtraction procedure.
Data analysis

To obtain the concentration-response curve for the effect of ginsenoside metabolites on the inward peak $I_{\text{GABA}}$ mediated by the GABA$_A$ receptor, the $I_{\text{GABA}}$ peak was plotted at different concentrations of GABA and Origin software (Origin, Northampton, MA, USA) was used to fit the plot to the Hill equation: $y/y_{\text{max}}=[A]^nH/(\text{IC}_{50}^\text{nH}+[A]^nH)$, where y is the peak current at a given concentration of ginsenoside metabolites, $y_{\text{max}}$ is the maximal peak current, half-inhibitory concentration ($\text{IC}_{50}$) is the concentration of ginsenoside metabolites producing a half-maximal effect, [A] is the concentration of ginsenoside metabolites, and nH is the Hill coefficient. All values are presented as means±SEM. The significance of differences between the mean control and treatment values was determined using Student’s t-test. A p-value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The addition of GABA (10 µM) to the bathing medium induced a large inward current ($I_{\text{GABA}}$) in oocytes injected with GABA$_A$ receptor subunits cRNAs, indicating that the GABA$_A$ receptor was functionally expressed (Fig. 1B). In oocytes expressing GABA$_A$, the treatment of ginsenoside metabolites such as M4 or PPD had no effect. Moreover, co- or pre-treatment with ginsenoside metabolites for 30 s with GABA induced an inhibition of $I_{\text{GABA}}$ in a reversible manner (Fig. 2A, B; n=15 from three different frogs). As shown in Fig. 2C, M4 more significantly inhibited $I_{\text{GABA}}$ than PPD.

In concentration-response experiments with M4 or PPD, co-treatment with M4 and PPD inhibited $I_{\text{GABA}}$ in a dose-dependent manner in oocytes expressing the GABA$_A$ receptor (Fig. 3A, B). The $\text{IC}_{50}$ of $I_{\text{GABA}}$ by M4 and PPD was 17.1±2.2 and 23.1±8.6 µM, respectively, in oocytes expressing the GABA$_A$ receptor (n=9-12 from three different frogs at each point) (Fig. 3B).

In current-voltage experiments, the membrane potential was held at –80 mV and a voltage ramp was applied from –100 to +40 mV for 300 ms. In the absence of GABA, the inward current at –100 mV was <0.3 µA and the outward current at +40 mV was 0.3-0.5 µA. The addition of GABA to the bathing medium resulted in an increase of the inward current at a potential more negative than –20 mV. In contrast, at a potential more positive than –20 mV, GABA caused a large increase in the outward current. Co-treatment of GABA with M4 or PPD inhibited both inward and outward currents as compared with those induced by GABA treatment alone. The reversal potential was near –20 mV in GABA alone and in GABA + M4 or PPD, which indicates that GABA induces the Cl$^-$ current [4]. Also, co-treatment of GABA with M4 or PPD did not affect the channel property of the GABA$_A$ receptor (Fig. 4A).

In addition, our results further revealed that the inhibitory effects of M4 and PPD on $I_{\text{GABA}}$ in oocytes expressing human GABA$_A$ receptors were independent of the membrane holding potential (Fig. 4B). At membrane holding potentials of –100, –80, –60, –40, and –20 mV, M4 inhibited $I_{\text{GABA}}$ by 53.8±2.5%, 52.4±4.0%, 52.8±1.5%,
54.1±1.7% and 53.9±2.9%, respectively (n=9-12, from three different frogs). PPD inhibited IGABA by 20.9±1.9%, 23.3±1.4%, 22.1±3.8%, 20.7±1.3% and 21.6±3.0%, same respective order (n=9-12, from three different frogs).

To begin studying the mechanism by which M4 or PPD inhibit IGABA in oocytes expressing human GABA receptors, we analyzed the effect of 100 μM M4 or PPD on the IGABA evoked by different GABA concentrations (Fig. 5A, B). Co-treatment of oocytes expressing human GABA receptors with 100 μM M4 or PPD plus different concentrations of GABA did not significantly shift the dose-response curve of GABA to the right. The EC50 values were 15.8±2.5, 18.3±2.4 and 15.4±2.6 μM for GABA alone, GABA+M4 and GABA+PPD, respectively, and the Hill coefficients were 1.6, 1.5 and 1.7, in the same respective order. Thus, M4 and PPD significantly inhibited the IGABA elicited by 10, 30, and 100 μM of GABA, independent of the GABA concentration (n=6-8 from three different frogs) (Fig. 5A, B).

Ginsenosides consist of aglycone and carbohydrate portions. Aglycone is the backbone of the ginsenoside, with a hydrophobic four-ring steroid-like structure that may be non-polar, whereas the carbohydrates on carbons 3, 6, and 20 of the backbone are polar (Fig. 1A). Therefore, ginsenosides are amphiphilic molecules. In vitro and in vivo studies have shown that ginsenosides administered orally are metabolized and finally become an aglycone such as M4 and PPD [9]. These ginsenoside

Fig. 3. Concentration-dependent effects of M4 and protopanaxadiol (PPD) on γ-aminobutyric acid (GABA)-induced inward peak current (IGABA) in oocytes expressing GABA receptors (GABA<sub>a</sub>) receptors. (A) The trace shows that PPD inhibited the currents elicited by GABA (GABA, 10 μM) in a dose-dependent manner. (B) The trace shows that M4 inhibited the currents elicited by GABA (GABA, 10 μM) in a dose-dependent manner. (C) Percent inhibition by M4 and PPD of IGABA was calculated from the average of the peak inward current elicited by GABA alone before M4 and PPD and the peak inward current elicited by GABA alone after co-treatment of M4 and PPD with GABA. The continuous line shows the curve fitted according to the equation.

Fig. 4. Current-voltage relationship and voltage-independent inhibition by M4 and protopanaxadiol (PPD). (A) Current-voltage relationships of γ-aminobutyric acid (GABA)-induced inward peak current (IGABA) inhibition by M4 and PPD in GABA receptor (GABA<sub>a</sub>) receptors. Representative current-voltage relationships were obtained using voltage ramps of –100 to +40 mV for 300 ms at a holding potential of –80 mV. Voltage steps were applied before and after application of 10 μM GABA in the absence or presence of 100 μM M4 or PPD. (B) Voltage-independent inhibition of IGABA in the GABA<sub>a</sub> receptors by M4 or PPD. The values were obtained from the receptors in the absence or presence of 100 μM M4 and PPD at the indicated membrane holding potentials.
metabolites might also induce apoptosis of cancer cells and play a role as anti-cancer agents [10,11], suggesting that ginsenosides are pro-drugs of these metabolites. However, relatively little is known regarding how ginsenoside metabolites regulate ion channel or receptor activity, especially on GABA\textsubscript{A} receptor.

In the present study, we investigated the effects of ginsenoside metabolites on human GABA\textsubscript{A} receptors heterologously expressed in Xenopus oocytes. We found that co- or pre-treatment with M4 rather than PPD induced a large inhibition of $I_{\text{GABA}}$ in reversible manners, treatment of M4 and PPD inhibited $I_{\text{GABA}}$ in oocytes expressing GABA\textsubscript{A} receptors in concentration-dependent manners, and inhibition of $I_{\text{GABA}}$ by treatment of M4 or PPD occurred in a voltage-independent and non-competitive manner in oocytes expressing GABA\textsubscript{A} receptors. These results indicate that the ginsenoside metabolites M4 and PPD might be novel GABA\textsubscript{A} receptor regulators.

GABA is one of major inhibitory neurotransmitters in the mammalian brain and it has long been known that many neuroactive drugs, such as the benzodiazepines, nonbenzodiazepines, barbiturates, ethanol, neuroactive steroids, anaesthetics, and picrotoxin interact with GABA\textsubscript{A} receptors by binding to modulatory sites of the receptor [12]. Thus, GABA\textsubscript{A} receptor regulators are clinically important for treatment of various mental dysfunctions. For example, benzodiazepines, which are known to potentiate $I_{\text{GABA}}$, have been used for their anxiolytic, sedative, and myorelaxant effects. However, many unwanted side effects such as amnesic-like effects, ataxia, ethanol and barbiturate potentiation, and tolerance and dependence, appear and result in a reduction in the therapeutic value of benzodiazepines [13-15]. On the other hand, we have shown that ginsenoside Rc enhances $I_{\text{GABA}}$ [8]; these results are well coupled to the report that some subsets of ginsenosides have anxiolytic-like effects in animal model [16]. Presently, M4 and PPD displayed inhibitory effects on $I_{\text{GABA}}$ rather than enhancing effects, indicating the possibility that ginsenoside metabolites are different from intact ginsenosides in the regulation of GABA\textsubscript{A} receptor-mediated ion channel regulations.

We have previously reported that ginsenoside metabolites such as compound K (CK), M4, and PPD differentially regulate ion channels and receptors. For example, CK, but not protopanaxatriol (PPT), potently inhibits the voltage-dependent $\alpha_{1}\text{G-type Ca}^{2+}$ channel [17]. Similarly, we also demonstrated that CK, but not M4 (PPT), inhibits a neuronal Na$^{+}$ (Nav1.2) channel [18], and that M4, but not CK, inhibits 5-HT\textsubscript{3A} receptor-mediated currents [19]. In addition, CK and PPT inhibit $\alpha_{3}\beta_{4}$ nicotinic acetylcholine receptor-mediated currents [20]. Recently, we also reported that, although PPD itself does not affect human HERG K$^{+}$ channel activity, it inhibits ginsenoside Rg$_{3}$-mediated decelerating effects of HERG K$^{+}$ channel currents [21]. In the present study, M4 more strongly inhibited GABA\textsubscript{A}-receptor-mediated ion cur-

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Fig. 5. Concentration-dependent effects of $\gamma$-aminobutyric acid (GABA) on M4 or protopanaxadiol (PPD)-mediated inhibition of GABA-induced inward peak current ($I_{\text{GABA}}$). (A) The representative traces were obtained from GABA receptor (GABA\textsubscript{A}) receptors expressed in oocytes. $I_{\text{GABA}}$ of the upper and lower panels were elicited from concentration of 10 µM and 100 µM GABA at a holding potential of –80 mV, respectively. (B) Concentration-response relationships for GABA in the GABA\textsubscript{A} receptors applied with GABA (1-100 µM) alone or with GABA plus co-treatment of 100 µM M4 or PPD. The $I_{\text{GABA}}$ of oocytes expressing the GABA\textsubscript{A} receptors was measured using the indicated concentration of GABA in the absence (□) or presence (○) of 100 µM M4 or presence (△) of 100 µM PPD. Oocytes were exposed to GABA alone or to GABA with M4 or PPD. Oocytes were voltage-clamped at a holding potential of –80 mV. Each point represents the mean±SEM (n=8-12/group).
rents than PPD. The previous and present findings indicate that ginsenoside metabolites as well as ginsenosides have regulatory effects on voltage-dependent ion channel and receptor activities, but they differentially affect the regulation of ion channels or receptors.

In summary, we have examined the effects of ginsenoside metabolites such as M4 and PPD on human GABA<sub>A</sub> receptor channel activities heterologously expressed in Xenopus oocytes. M4, rather than PPD, mainly inhibits I<sub>GABA</sub><sub>A</sub>. Since human GABA<sub>A</sub> receptors are mainly involved in the modulations of various physiological and pathophysiological activities in the central nervous system, the inhibitory effects of M4 on I<sub>GABA</sub><sub>A</sub> could provide a molecular basis for the pharmacological actions of ginsenoside metabolites in the nervous system.

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