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Effects of Ginsenoside Metabolites on $GABA_A$ Receptor-Mediated Ion Currents

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In a previous report, we demonstrated that ginsenoside Rc, one of major ginsenosides from *Panax ginseng*, enhances γ-aminobutyric acid (GABA) $receptor_A$ ($GABA_A$)-mediated ion channel currents. However, little is known about the effects of ginsenoside metabolites on $GABA_A$ receptor channel activity. The present study investigated the effects of ginsenoside metabolites on human recombinant $GABA_A$ receptor ($\alpha_1\beta_1\gamma_{2s}$) channel activity expressed in *Xenopus* oocytes using a two-electrode voltage clamp technique. M4, a metabolite of protopanaxatriol ginsenosides, more potently inhibited the GABA-induced inward peak current (I_{GABA}) than protopanaxadiol (PPD), a metabolite of PPD ginsenosides. The effect of M4 and PPD on I_{GABA} was both concentration-dependent and reversible. The half-inhibitory concentration (IC_{50}) values of M4 and PPD were 17.1±2.2 and 23.1±8.6 μM, respectively. The inhibition of I_{GABA} by M4 and PPD was voltage-independent and non-competitive. This study implies that the regulation of $GABA_A$ receptor channel activity by ginsenoside metabolites differs from that of ginsenosides.

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

INTRODUCTION

Gamma-aminobutyric acid (GABA) $receptor_A$ ($GABA_A$) 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_3 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 $GABA_A$ receptor is responsible for fast inhibitory synaptic transmission [4,5].

Recent biochemical binding assays produced evidence that ginsenosides might regulate the $GABA_A$ receptor. For example, Kimura *et al*. [6] showed that ginsenosides differentially regulate [^{3}H]-f1unitrazepam or [^{3}H]-muscimol binding to the $GABA_A$ receptor in a rat brain membrane fraction. Kim *et al*. [7] reported that prolonged infusion with ginsenoside Rc, but not ginsenoside Rg_1, into rat brain elevates [^{3}H]-muscimol binding to the $GABA_A$ receptor in a brain region-specific manner. Thus, ginsenosides may regulate the $GABA_A$ receptor by affecting ligand affinity for its receptor, but there is no direct evidence on the regulation of $GABA_A$ receptor channel activity by ginsenosides. On the other hand, Choi *et al*. [8] showed that ginsenoside Rc enhances GABA-mediated

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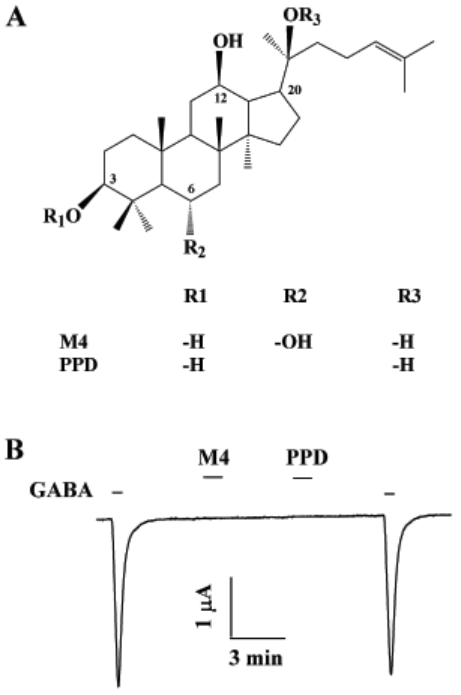

Fig. 1. Chemical structure of the ginsenoside metabolites M4 and protopanaxadiol (PPD) (A) and their effects in oocytes expressing γ-aminobutyric acid (GABA) receptor$_A$ ($GABA_A$) receptors. (B) M4 and PPD had no effect on GABA-induced inward peak current in oocytes expressing $\alpha_1\beta_1\gamma_{2s}$ $GABA_A$ receptors.

ion currents in oocytes expressing the $GABA_A$ receptor. These results indicate the possibility that ginsenosides are closely related with $GABA_A$ 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 $GABA_A$ receptor channel activity.

In the present study, we examined the effects of ginsenoside metabolites on $GABA_A$ receptor channel activity. We injected neuronal human $GABA_A$ ($\alpha_1\beta_1\gamma_{2s}$) receptor cRNAs into *Xenopus* oocytes and examined the effect of ginsenoside metabolites on the GABA-elicited inward peak currents (I_{GABA}). Treatment with ginsenoside metabolites inhibited I_{GABA} in a reversible, dose-dependent and non-competitive manner.

MATERIALS AND METHODS

Materials

The ginsenoside metabolites M4 and PPD were provided by the AMBO Institute (Seoul, Korea) (Fig. 1A). cDNAs for human $GABA_A$ 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 Ca^{2+}-free medium containing 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 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 $MgCl_2$, 1.8 $CaCl_2$, 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 $GABA_A$ receptor activity experiments, each $GABA_A$ 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 $CaCl_2$, 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.

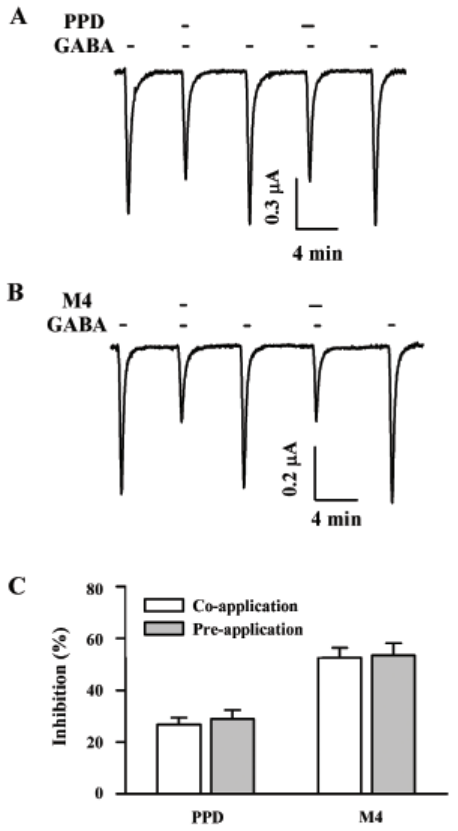

Fig. 2. Effect of M4 and protopanaxadiol (PPD) on γ-aminobutyric acid (GABA)-induced inward peak current (I_{GABA}) in oocytes expressing GABA $receptor_A$ ($GABA_A$) receptor. (A) GABA (10 μM) was first applied and then GABA was co- or pre-treated with PPD (100 μM). Co-treatment of PPD with GABA and pre-treatment of PPD before GABA application inhibited I_{GABA} in oocytes expressing $\alpha_1\beta_1\gamma_{2s}$ $GABA_A$ receptors. (B) GABA (10 μM) was first applied and then GABA was co- or pre-treated with M4 (100 μM). Thus, co-treatment of M4 with GABA and pre-treatment of M4 before GABA application inhibited I_{GABA} in oocytes expressing $\alpha_1\beta_1\gamma_{2s}$ $GABA_A$ receptors. The resting membrane potential of oocytes was about –35 mV and oocytes were voltage-clamped at a holding potential of –80 mV prior to drug application. Traces are representative of 8-12 separate oocytes from three different frogs. (C) Summary of percent inhibition by M4 and PPD of I_{GABA} was calculated from the average of the peak inward current elicited by GABA alone before M4 or PPD and the peak inward current elicited by GABA alone after co- and pre-treatment of M4 or PPD with GABA. Each point represents the mean±SEM (n=9-12 from three different frogs).

Data analysis

To obtain the concentration-response curve for the effect of ginsenoside metabolites on the inward peak I_{GABA} mediated by the $GABA_A$ receptor, the I_{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: $y/y_{max}=[A]^{nH}/([A]^{nH}+[IC_{50}]^{nH})$, where y is the peak current at a given concentration of ginsenoside metabolites, y_{max} is the maximal peak current, half-inhibitory concentration (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_{GABA}) in oocytes injected with $GABA_A$ receptor subunits cRNAs, indicating that the $GABA_A$ receptor was functionally expressed (Fig. 1B). In oocytes expressing $GABA_A$ receptor, 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_{GABA} in a reversible manner (Fig. 2A, B; n=15 from three different frogs). As shown in Fig. 2C, M4 more significantly inhibited I_{GABA} than PPD.

In concentration-response experiments with M4 or PPD, co-treatment with M4 and PPD inhibited I_{GABA} in a dose-dependent manner in oocytes expressing the $GABA_A$ receptor (Fig. 3A, B). The IC_{50} of I_{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_{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_{GABA} by 53.8±2.5%, 52.4±4.0%, 52.8±1.5%,

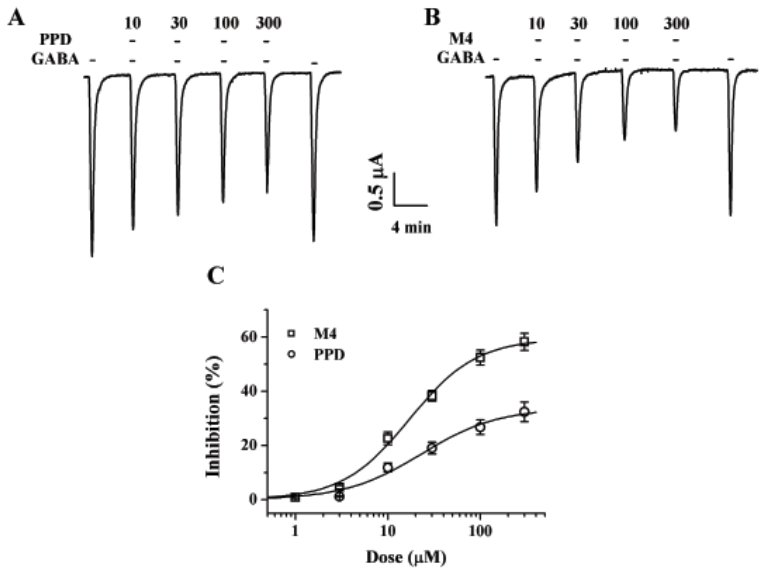

Fig. 3. Concentration-dependent effects of M4 and protopanaxadiol (PPD) on γ-aminobutyric acid (GABA)-induced inward peak current (I_{GABA}) in oocytes expressing GABA receptor$_A$ (GABA$_A$) receptors. (A) The trace shows that PPD inhibited the currents elicited by GABA (GABA, 10 μM) in a dose-dependent 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 I_{GABA} 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.

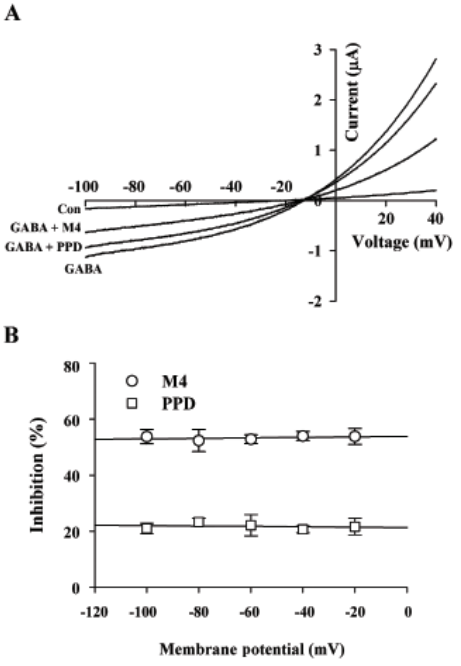

Fig. 4. Current-voltage relationship and voltage-independent inhibition by M4 and protopanaxadiol (PPD). (A) Current-voltage relationships of γ-aminobutyric acid (GABA)-induced inward peak current (I_{GABA}) inhibition by M4 and PPD in GABA receptor$_A$ (GABA$_A$) receptors. Representative current-voltage 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 I_{GABA} in the GABA$_A$ 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.

54.1±1.7% and 53.9±2.9%, respectively (n=9-12, from three different frogs). PPD inhibited I_{GABA} 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 I_{GABA} in oocytes expressing human GABA$_A$ receptors, we analyzed the effect of 100 μM M4 or PPD on the I_{GABA} evoked by different GABA concentrations (Fig. 5A, B). Co-treatment of oocytes expressing human GABA$_A$ 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 EC_{50} 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 I_{GABA} 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 agylcone such as M4 and PPD [9]. These ginsenoside

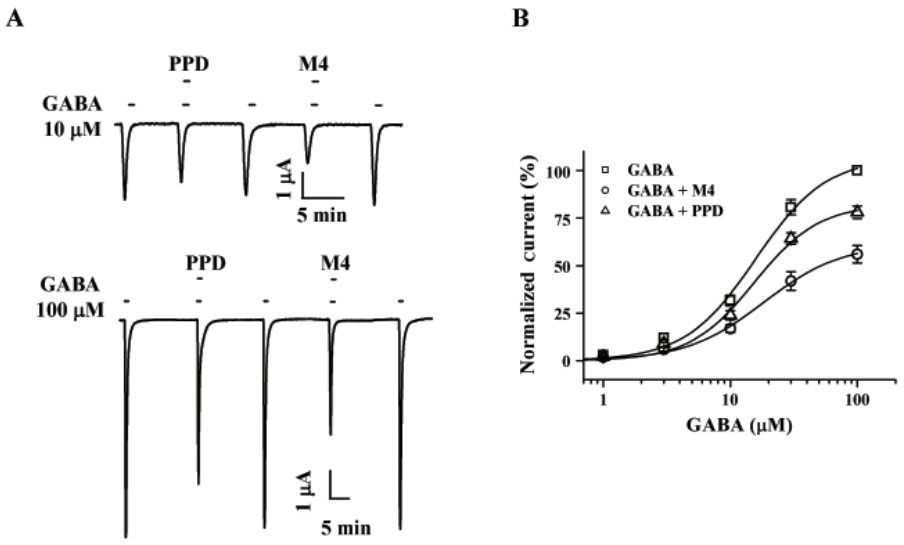

Fig. 5. Concentration-dependent effects of γ-aminobutyric acid (GABA) on M4 or protopanaxadiol (PPD)-mediated inhibition of GABA-induced inward peak current (I_{GABA}). (A) The representative traces were obtained from GABA $receptor_A$ ($GABA_A$) receptors expressed in oocytes. I_{GABA} of the upper 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 $GABA_A$ receptors applied with GABA (1-100 μM) alone or with GABA plus co-treatment of 100 μM M4 or PPD. The I_{GABA} of oocytes expressing the $GABA_A$ 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).

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 $GABA_A$ receptor.

In the present study, we investigated the effects of ginsenoside metabolites on human $GABA_A$ receptors heterologously expressed in *Xenopus* oocytes. We found that co- or pre-treatment with M4 rather than PPD induced a large inhibition of I_{GABA} in reversible manners, treatment of M4 and PPD inhibited I_{GABA} in oocytes expressing $GABA_A$ receptors in concentration-dependent manners, and inhibition of I_{GABA} by treatment of M4 or PPD occurred in a voltage-independent and non-competitive manner in oocytes expressing $GABA_A$ receptors. These results indicate that the ginsenoside metabolites M4 and PPD might be novel $GABA_A$ 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, anaethetics, and picrotoxin interact with $GABA_A$ receptors by binding to modulatory sites of the receptor [12]. Thus, $GABA_A$ receptor regulators are clinically important for treatment of various mental dysfunctions. For example, benzodiazepines, which are known to potentiate I_{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_{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_{GABA} rather than enhancing effects, indicating the possibility that ginsenoside metabolites are different from intact ginsenosides in the regulation of $GABA_A$ 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 α1G-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_{3A} receptor-mediated currents [19]. In addition, CK and PPT inhibit α3β4 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 $GABA_A$-receptor-mediated ion cur-

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 $GABA_A$ receptor channel activities heterologously expressed in *Xenopus* oocytes. M4, rather than PPD, mainly inhibits I_{GABA}. Since human $GABA_A$ 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_{GABA} could provide a molecular basis for the pharmacological actions of ginsenoside metaobolites in the nervous system.

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