

## Inhibitory Effects of Ginsenoside Metabolites, Compound K and Protopanaxatriol, on GABA<sub>C</sub> Receptor-Mediated Ion Currents

Byung-Hwan Lee<sup>1</sup>, Sung-Hee Hwang<sup>1</sup>, Sun-Hye Choi<sup>1</sup>, Hyeon-Joong Kim<sup>1</sup>, Joon-Hee Lee<sup>2</sup>, Sang-Mok Lee<sup>1</sup>, Yun Gyong Ahn<sup>3</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, <sup>2</sup>Department of Physical Therapy, Sehan University, Yeongam 526-702, <sup>3</sup>Seoul Center, Korea Basic Science Institute, Seoul 136-701, Korea

Ginsenosides, one of the active ingredients of *Panax ginseng*, show various pharmacological and physiological effects, and they are converted into compound K (CK) or protopanaxatriol (M4) by intestinal microorganisms. CK is a metabolite derived from protopanaxadiol (PD) ginsenosides, whereas M4 is a metabolite derived from protopanaxatriol (PT) ginsenosides. The  $\gamma$ -aminobutyric acid receptor<sub>C</sub> (GABA<sub>C</sub>) is primarily expressed in retinal bipolar cells and several regions of the brain. However, little is known of the effects of ginsenoside metabolites on GABA<sub>C</sub> receptor channel activity. In the present study, we examined the effects of CK and M4 on the activity of human recombinant GABA<sub>C</sub> receptor ( $\rho$  1) channels expressed in *Xenopus* oocytes by using a 2-electrode voltage clamp technique. In oocytes expressing GABA<sub>C</sub> receptor cRNA, we found that CK or M4 alone had no effect in oocytes. However, co-application of either CK or M4 with GABA inhibited the GABA-induced inward peak current ( $I_{GABA}$ ). Interestingly, pre-application of M4 inhibited  $I_{GABA}$  more potently than CK in a dose-dependent and reversible manner. The half-inhibitory concentration (IC<sub>50</sub>) values of CK and M4 were 52.1±2.3 and 45.7±3.9  $\mu$ M, respectively. Inhibition of  $I_{GABA}$  by CK and M4 was voltage-independent and non-competitive. This study implies that ginsenoside metabolites may regulate GABA<sub>C</sub> receptor channel activity in the brain, including in the eyes.

**Key Words:** GABA<sub>C</sub> receptor, Ginsenoside metabolites, *Panax ginseng*, *Xenopus* oocytes

### INTRODUCTION

Ginseng, the root of *Panax ginseng* C.A. Meyer, has been used as a general tonic to promote longevity and to enhance bodily functions to protect against stress, fatigue, and diseases such as cancer and diabetes mellitus [1]. Recent studies have shown that among the various ginseng components, ginsenosides (also called ginseng saponins) exhibit neuroprotective effects against various excitatory stimulants [2]. Approximately 30 different forms have been isolated and identified from the root of *Panax ginseng*. They are classified into protopanaxadiol (PD) or protopanaxatriol (PT) ginsenosides according to the position of the carbohydrate component at carbon-3 and -6 [3].

Hasegawa et al. showed that ginsenosides administered via the oral route may pass into the large intestine without decomposition by either gastric juices or digestive enzymes [4]. PD ginsenosides are converted into compound K (CK) by intestinal microorganisms, with glucose at the C-20 position, whereas PT ginsenosides are converted to protopanaxatriol (M4), with only a ginsenoside backbone without a carbohydrate component (Fig. 1). These metabolites are absorbed into the blood of humans and rats [5,6]. Recently, reports have shown that these ginsenoside metabolites might also cause apoptosis of cancer cells and function as anti-cancer agents [7,8], suggesting that ginsenosides may have a role as prodrugs.

GABA receptors, which are activated by GABA, an inhibitory neurotransmitter, exhibit inhibitory actions at the neuronal synaptic membrane and they are classified into 3 types of receptors: GABA<sub>A</sub>, GABA<sub>B</sub>, and GABA<sub>C</sub>. GABA<sub>A</sub> and GABA<sub>C</sub> receptors function as anion-selective channels that are permeable to chloride (Cl<sup>-</sup>) ions, whereas GABA<sub>B</sub> receptors are G-protein coupled receptors [9,10]. GABA<sub>C</sub> receptors are members of the large "Cys-loop" super family and form homopentameric Cl<sup>-</sup> channels by the rho ( $\rho$ ) subunit [11]. The GABA<sub>C</sub> receptor is predominantly expressed in retinal bipolar cells [12,13] and less abundantly in the

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Corresponding to: Seung-Yeol Nah, Ginsentology Research Laboratory and Department of Physiology, College of Veterinary Medicine and Bio/Molecular Informatics Center, Konkuk University, 120 Neungdong-ro, Gwangjin-gu, Seoul 143-701, Korea. (Tel) 82-2-450-4154, (Fax) 82-2-450-3037, (E-mail) synah@konkuk.ac.kr



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**ABBREVIATIONS:** CK, compound K; GABA<sub>C</sub>,  $\gamma$ -aminobutyric acid receptor<sub>C</sub>; PD, protopanaxadiol; PT, protopanaxatriol.

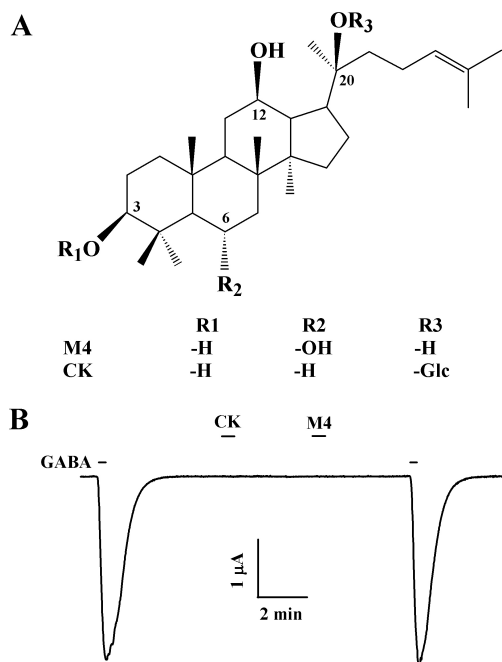
cerebellum [14] and hippocampus [15]. They have important roles in vision, sleep, cognition, and memory [16]. In previous studies, we demonstrated that the ginsenoside Rc (20-[(6-O- $\alpha$ -L-Arabinofuranosyl- $\beta$ -D-glucopyranosyl)oxy]-12 $\beta$ -hydroxydammar-24-en-3 $\beta$ -yl 2-O- $\beta$ -D-glucopyranosyl- $\beta$ -D-glucopyranoside) enhanced GABA-elicited inward peak currents ( $I_{GABA}$ ) and that ginsenoside metabolites inhibited  $I_{GABA}$  in oocytes expressing the GABA<sub>A</sub> ( $\alpha_1\beta_1\gamma_{2s}$ ) receptor [17,18]. However, little is known regarding the effects of CK or M4 on GABA<sub>C</sub> receptor channel activity.

In the present study, we examined the effects of CK or M4 on GABA<sub>C</sub> receptor channel activity. We injected neuronal human GABA<sub>C</sub> ( $\rho 1$ ) receptor cRNAs into *Xenopus* oocytes and examined the effect of CK or M4 on  $I_{GABA}$ . We found that treatment of CK or M4 inhibited  $I_{GABA}$  in a reversible, dose-dependent, and non-competitive manner. We further discussed the possible role of ginsenoside metabolites in the regulation of human GABA<sub>C</sub> ( $\rho 1$ ) receptor.

## METHODS

### Materials

Ginsenoside metabolites were provided by the AMBO Institute (Seoul, Korea) (Fig. 1A). cDNAs for the human GABA<sub>C</sub> receptor  $\rho 1$  subunit were purchased from Thermo Fisher Scientific Inc. (Wyman Street Waltham, MA, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).



**Fig. 1.** Chemical structure of the ginsenoside metabolites CK and M4 (A) and their effect on oocytes expressing GABA<sub>C</sub> receptors (B). CK and M4 had no effect on  $I_{GABA}$  in oocytes expressing GABA<sub>C</sub> receptors.

### 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 the institutional guidelines of Konkuk University. For the 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 Ca<sup>2+</sup>-free medium containing 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 5 mM HEPES, 2.5 mM sodium pyruvate, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. Stage V~VI oocytes were collected and stored in ND96 medium (in mM: 96 NaCl, 2 KCl, 1 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, and 5 HEPES, pH 7.5) supplemented with 50  $\mu$ g/ml gentamicin. The oocyte-containing solution was maintained at 18°C with continuous gentle shaking and renewed daily. Electrophysiological experiments were performed within 5~6 days of oocyte isolation, with ginsenoside metabolites added to the bath. For GABA<sub>C</sub> receptor activity experiments, GABA<sub>C</sub> receptor-encoding cRNAs (1~10 ng/ oocyte) were injected into the animal or vegetal pole of each oocyte one day after isolation, using a 10  $\mu$ l microdispenser (VWR Scientific, San Francisco, CA, USA) fitted with a tapered glass pipette tip (15~20  $\mu$  m in diameter) [17].

### cRNA preparation of the GABA<sub>C</sub> receptor $\rho 1$ subunit

cDNA constructs were linearized at the 3' ends by digestion with *NotI*, and run-off transcripts were prepared using the methylated cap analogue m<sup>7</sup>G(5')ppp(5')G. cRNAs were prepared using the mMessage mMachine transcription kit (Ambion, Austin, TX, USA) with T3 RNA polymerase. The absence of degraded RNA was confirmed by denaturing agarose gel electrophoresis followed by ethidium bromide staining. The final cRNA products were resuspended at a concentration of 1  $\mu$ g/ $\mu$ l in RNase-free water and stored at -80°C [17].

### Data recording

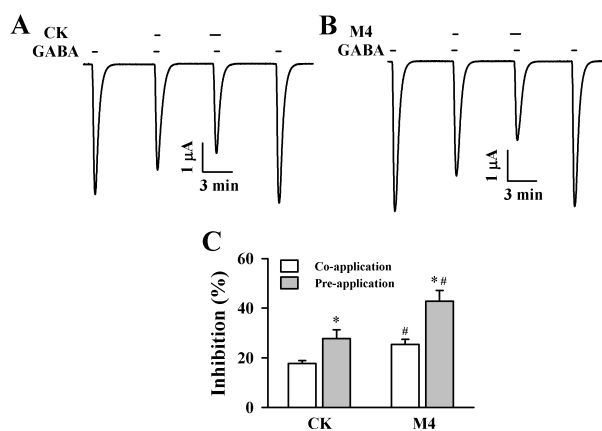
A custom-made Plexiglas net chamber was used for 2-electrode voltage-clamp recordings as previously reported [18]. The oocytes were impaled with 2 microelectrodes filled with 3 M KCl (0.2~0.7 M $\Omega$ ), and electrophysiological experiments were carried out at room temperature using an Oocyte Clamp (OC-725C, Warner Instruments, Hamden, 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<sub>2</sub>, 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 various membrane-holding potential experiments, oocytes were clamped at the indicated holding potentials. Linear leak and capacitance currents were corrected by the leak subtraction procedure.

### Data analysis

To obtain the concentration-response curve for the effect of ginsenoside metabolites on inward peak  $I_{GABA}$  mediated by the GABA<sub>C</sub> 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,  $IC_{50}$  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  $\pm$  S.E.M. The significance of differences between the mean control and treatment values was determined using Student's  $t$ -test, linear regression analysis, and one-way ANOVA.  $p < 0.05$  was considered statistically significant.

## RESULTS

The addition of GABA ( $2 \mu M$ ) to the bathing medium induced a large inward current ( $I_{GABA}$ ) in oocytes injected with GABA<sub>C</sub> receptor subunits cRNAs, indicating that the GABA<sub>C</sub> receptor was functionally expressed in this system (Fig. 1B). In oocytes expressing the GABA<sub>C</sub> receptor, treatment with ginsenoside metabolites such as CK or M4 had

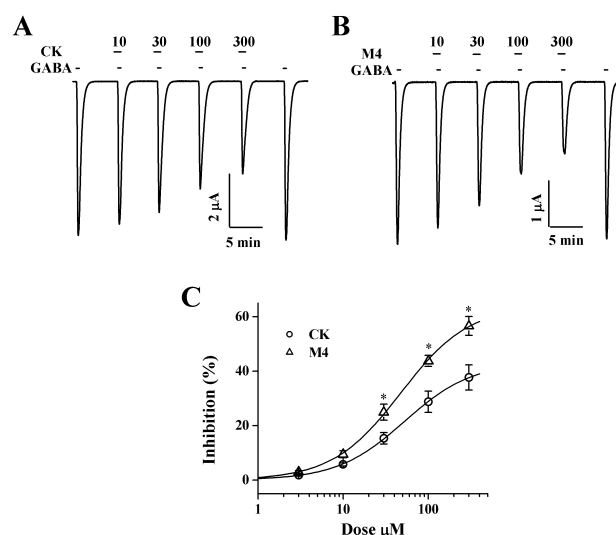


**Fig. 2.** Effect of CK and M4 on  $I_{GABA}$  in oocytes expressing the  $\rho 1$  GABA<sub>C</sub> receptor. (A) GABA ( $2 \mu M$ ) was first applied and then was co- or pre-treated with CK ( $100 \mu M$ ). Co-treatment of CK with GABA and pre-treatment of CK before GABA application inhibited  $I_{GABA}$  in oocytes expressing  $\rho 1$  GABA<sub>C</sub> receptors. (B) GABA ( $2 \mu M$ ) was first applied and then co- or pre-treated with M4 ( $100 \mu M$ ). Co-treatment of M4 with GABA and pre-treatment of M4 before GABA application inhibited  $I_{GABA}$  in oocytes expressing  $\rho 1$  GABA<sub>C</sub> receptors. The resting membrane potential of oocytes was approximately  $-35$  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 3 different frogs. (C) Summary of percent inhibition by CK and M4 of  $I_{GABA}$  calculated from the average of the peak inward current elicited by GABA alone before CK and M4 and the peak inward current elicited by GABA alone after co- and pre-treatment of CK and M4 with GABA. Each point represents the mean  $\pm$  S.E.M. ( $n=9\sim 12$  from 3 different frogs). \* $p < 0.05$  as compared to co-treatment of CK and M4, and # $p < 0.05$  as compared to CK.

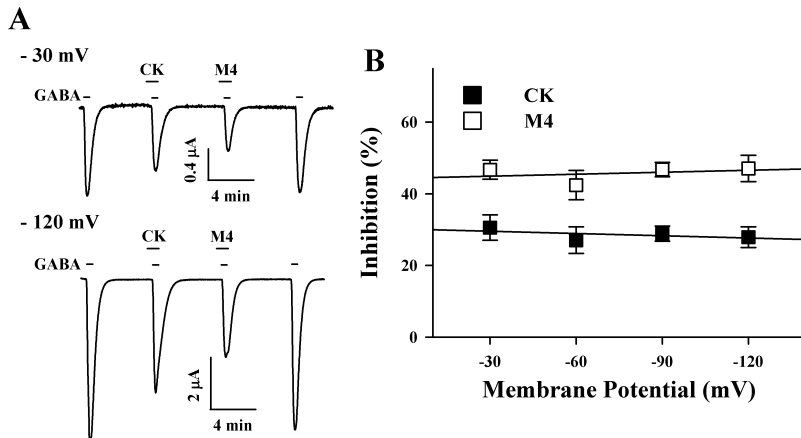
no effect at a holding potential of  $-80$  mV. Co-treatment of CK and M4 ( $100 \mu M$  each) for 30 s with GABA inhibited  $I_{GABA}$  by  $17.7 \pm 1.2\%$  and  $25.4 \pm 2.2\%$ , respectively. Interestingly, pre-treatment of CK or M4 ( $100 \mu M$ ) induced a much larger inhibition of  $I_{GABA}$  (Fig. 2A and 2B,  $n=9\sim 12$  from 3 different frogs). Thus, CK or M4 inhibited  $I_{GABA}$  by  $27.8 \pm 3.5\%$  or  $42.8 \pm 4.4\%$  for GABA<sub>C</sub> receptors. As shown in Fig. 2C, M4 inhibited  $I_{GABA}$  more strongly than CK.

Since pre-treatment of CK and M4 induced further inhibition on  $I_{GABA}$  as compared to co-treatment, in the following experiments, we examined the effects of CK and M4 on  $I_{GABA}$  after pre-treatment. In concentration-response experiments, pre-treatment of CK and M4 for 30 s inhibited  $I_{GABA}$  in a concentration-dependent manner (Fig. 3A and 3B). Pre-treatment of CK inhibited  $I_{GABA}$  by  $1.9 \pm 0.5\%$ ,  $5.8 \pm 0.6\%$ ,  $15.4 \pm 2.1\%$ ,  $28.8 \pm 3.9\%$ , and  $37.7 \pm 4.6\%$ , and pre-treatment of M4 inhibited  $I_{GABA}$  by  $3.0 \pm 0.5\%$ ,  $9.5 \pm 1.2\%$ ,  $24.9 \pm 2.9\%$ ,  $43.8 \pm 2.1\%$ , and  $56.6 \pm 3.5\%$  at 3, 10, 30, 100, and 300  $\mu M$ , respectively. The  $IC_{50}$  of  $I_{GABA}$  by CK and M4 was 52.1 and 45.7  $\mu M$  in oocytes expressing the GABA<sub>C</sub> receptor ( $n=9\sim 12$  from 3 different frogs for each point) (Fig. 3C).

Next, we determined that the inhibitory effects of CK and M4 on  $I_{GABA}$  were independent of the membrane holding potential (Fig. 4). At membrane holding potentials of  $-120$ ,  $-90$ ,  $-60$ , and  $-30$  mV, CK inhibited  $I_{GABA}$  by  $27.9 \pm 2.9\%$ ,  $28.9 \pm 2.1\%$ ,  $27.1 \pm 3.7\%$ , and  $30.6 \pm 3.5\%$ , respectively ( $n=9\sim 12$ , from 3 different frogs). M4 also inhibited  $I_{GABA}$  by  $47.1 \pm 3.7\%$ ,  $46.8 \pm 2.0\%$ ,  $42.4 \pm 4.1\%$ , and  $46.7 \pm 2.6\%$ , respectively ( $n=9\sim 12$ , from 3 different frogs), indicating that CK or M4 action on  $I_{GABA}$  is not dependent on membrane holding potentials.



**Fig. 3.** Concentration-dependent effects of CK and M4 on  $I_{GABA}$  in oocytes expressing  $\rho 1$  GABA<sub>C</sub> receptors. (A, B) The trace shows that CK and M4 inhibited the currents elicited by GABA ( $2 \mu M$ ) in a dose-dependent manner. (C) Percent inhibition by CK and M4 of  $I_{GABA}$  was calculated from the average of the peak inward current elicited by GABA alone before CK and M4 and the peak inward current elicited by GABA alone after pre-treatment of CK and M4 before GABA. The continuous line shows the curve fitted according to the equation. Each point represents the mean  $\pm$  S.E.M. ( $n=9\sim 12$  from 3 different frogs). \* $p < 0.005$  as compared to CK.

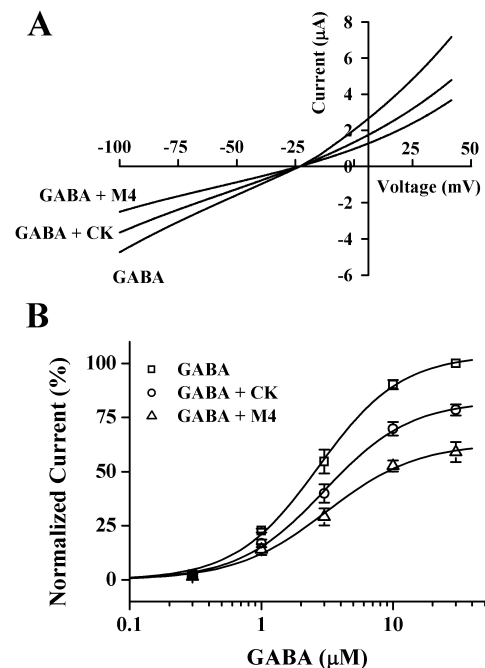


**Fig. 4.** Voltage-independent inhibition by CK and M4. (A) GABA ( $2 \mu\text{M}$ ) was first applied and pre-treatment of CK ( $100 \mu\text{M}$ ) or M4 ( $100 \mu\text{M}$ ) before GABA application inhibited  $I_{\text{GABA}}$  in oocytes expressing  $\rho 1$  GABA<sub>C</sub> receptors. The inhibitory degrees were very similar at  $-30 \text{ mV}$  and  $-120 \text{ mV}$  of membrane potential. (B) Voltage-independent inhibition of  $I_{\text{GABA}}$  in GABA<sub>C</sub> receptors by CK and M4. Values were obtained from the receptors in the absence or presence of  $100 \mu\text{M}$  CK and M4 at the indicated membrane holding potentials. The observed effects of CK and M4 were correlated with membrane potential. No significant effects were noted for CK ( $r^2=0.28$ ,  $p>0.31$ ) and M4 ( $r^2=0.10$ ,  $p>0.44$ ).

In experiments exploring current-voltage, the membrane potential was held at  $-80 \text{ mV}$ , and a voltage ramp was applied from  $-100$  to  $+40 \text{ mV}$  for  $300 \text{ ms}$ . Leakage correction was executed by subtraction of the  $I\sim V$  curve obtained by the same voltage protocol before the application of GABA. In the absence of GABA, the inward current at  $-100 \text{ mV}$  was  $<0.3 \mu\text{A}$ , and the outward current at  $+40 \text{ mV}$  was  $0.3\sim 0.5 \mu\text{A}$ . The addition of GABA to the bathing medium resulted in an increase in the inward current at a potential more negative than  $-20 \text{ mV}$ . In contrast, at a potential more positive than  $-20 \text{ mV}$ , GABA caused a large increase in the outward current. Pre-treatment of GABA with CK or M4 inhibited both inward and outward currents as compared with those induced by GABA treatment alone. The reversal potential was near  $-20 \text{ mV}$  in GABA alone and in GABA plus CK or M4, which indicates that GABA induces the  $\text{Cl}^-$  current [19]. Additionally, pre-treatment of CK or M4 with GABA did not affect the channel properties of the GABA<sub>C</sub> receptor (Fig. 5A). To further study the mechanism by which CK or M4 inhibits  $I_{\text{GABA}}$ , we analyzed the effect of  $100 \mu\text{M}$  CK or M4 on  $I_{\text{GABA}}$  evoked by different GABA concentrations (Fig. 5B). Pre-treatment of CK and M4 with different concentrations of GABA did not significantly shift the dose-response curve of GABA to the right ( $p>0.05$  compared to GABA alone). The  $\text{ED}_{50}$  values were  $2.70\pm 0.21$ ,  $3.02\pm 0.29$  and  $3.12\pm 0.42 \mu\text{M}$  for GABA alone, GABA+CK, and GABA+M4, respectively, and the Hill coefficients were 1.37, 1.34, and 1.28, respectively. Thus, CK and M4 significantly inhibited  $I_{\text{GABA}}$  elicited by 3, 10, and  $30 \mu\text{M}$  of GABA, independent of the GABA concentration in oocytes expressing the GABA<sub>C</sub> receptors, indicating that CK and M4 regulate GABA<sub>C</sub> receptor channel activity in a non-competitive manner ( $n=8\sim 12$  from 3 different frogs; Fig. 5B).

## DISCUSSION

Ginsenosides consist of aglycone and carbohydrate sections. The aglycone is the backbone of the ginsenoside, with a hydrophobic four-ring steroid-like structure that may be non-polar, whereas the carbohydrates on carbon-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 via the oral route were metabolized, and they ultimately become an



**Fig. 5.** Current-voltage relationships and concentration-dependent effects of GABA on CK- and M4-mediated inhibition of  $I_{\text{GABA}}$ . (A) Current-voltage relationships of  $I_{\text{GABA}}$  inhibition by CK and M4 in GABA<sub>C</sub> receptors. Representative current-voltage relationships were obtained using voltage ramps of  $-100$  to  $+40 \text{ mV}$  for  $300 \text{ ms}$  at a holding potential of  $-80 \text{ mV}$ . Voltage steps were applied before and after application of  $2 \mu\text{M}$  GABA in the absence or presence of  $100 \mu\text{M}$  CK and M4. (B) Concentration-response relationships for GABA in GABA<sub>C</sub> receptors with GABA applied ( $0.3\sim 30 \mu\text{M}$ ) alone or with GABA plus pre-treatment of  $100 \mu\text{M}$  CK and M4 before GABA application.  $I_{\text{GABA}}$  of oocytes expressing the GABA<sub>C</sub> receptors was measured using the indicated concentration of GABA in the absence ( $\square$ ) or presence ( $\circ$ ) of  $100 \mu\text{M}$  CK or presence ( $\triangle$ ) of  $100 \mu\text{M}$  M4. Oocytes were voltage-clamped at a holding potential of  $-80 \text{ mV}$ . Each point represents the mean $\pm$ S.E.M. ( $n=8\sim 12/\text{group}$ ).

aglycone such as CK and M4 [4]. Previous studies also showed that ginsenosides and ginsenoside metabolites regulated GABA<sub>A</sub> receptor activity [17,18]. However, relatively little is known regarding how ginsenoside metabo-

lites regulate GABA<sub>C</sub> receptor ion channel activity.

In the present study, we investigated the effects of ginsenoside metabolites, CK and M4, on human GABA<sub>C</sub> receptors heterologously expressed in *Xenopus* oocytes. We found that (1) co- or pre-treatment with M4 rather than CK induced a large inhibition of  $I_{GABA}$  in a reversible manner, (2) pre-treatment of CK or M4 inhibited  $I_{GABA}$  in oocytes expressing GABA<sub>C</sub> receptors dependent on concentration, and (3) inhibition of  $I_{GABA}$  by pre-treatment of CK or M4 occurred in a membrane holding potential-independent and non-competitive manner in oocytes expressing GABA<sub>C</sub> receptors. These results indicate that the ginsenoside metabolites, CK and M4, may be novel GABA<sub>C</sub> receptor regulators.

GABA is one of the major inhibitory neurotransmitters in the mammalian brain, and it has long been known that many neuroactive drugs such as benzodiazepines, nonbenzodiazepines, barbiturates, ethanol, neuroactive steroids, anaesthetics, and picrotoxin, interact with GABA<sub>A</sub> receptors by binding to modulatory sites of the receptor [12]. However, GABA<sub>C</sub> receptors are not sensitive to the GABA<sub>A</sub> receptor antagonist bicuculline and are not modulated by GABA<sub>A</sub> receptor modulators such as benzodiazepines and barbiturates [11,20,21]. GABA<sub>A</sub> receptors are generally composed of heteropentameric ion channels, whereas GABA<sub>C</sub> receptors can form homopentameric ion channels by  $\rho$  subunits [20]. The GABA<sub>C</sub> receptor is predominantly expressed in retinal bipolar cells [12,13].

In previous studies, we have shown that the ginsenoside Rc enhanced  $I_{GABA}$  [17], whereas M4 and PPD showed inhibitory effects on  $I_{GABA}$  in oocytes expressing GABA<sub>A</sub> receptors [18]. Similarly, we have also demonstrated that CK, but not M4, inhibited a neuronal Na<sup>+</sup> (Nav1.2) channel [22]. We reported that M4, but not CK, displayed an inhibitory effect on 5-HT<sub>3A</sub> receptor-mediated currents [23]. Recently, we also reported that although PPD itself had no effects on human HERG K<sup>+</sup> channel activity, it inhibited ginsenoside Rg<sub>3</sub>-mediated decelerating effects on HERG K<sup>+</sup> channel currents [24]. In the present study, we found that M4 inhibited GABA<sub>C</sub> receptor-mediated ion currents more strongly than CK did. The previous and present findings indicate that ginsenoside metabolites as well as ginsenosides themselves have regulatory effects on ligand-gated ion channels and voltage-gated ion channel activity but they show differential effects on the regulation of ligand-gated ion channels and voltage-gated ion channel activity.

Interestingly, the inhibitory effect of CK or M4 was stronger with pre-application of CK or M4 than with co-application with GABA (Fig. 2), indicating that it is possible that the CK- or M4-mediated regulation of GABA<sub>C</sub> receptor may be indirectly achieved through phosphorylation of the GABA<sub>C</sub> receptor, as Feigenspan and Bormann reported that the GABA<sub>C</sub> receptor is regulated by GTP binding protein-coupled receptors [25]. However, ginsenoside metabolite action on GABA<sub>C</sub> receptor channel activity was not sensitive to staurosporine, a protein kinase C inhibitor (data not shown), indicating that CK- or M4-mediated regulation of GABA<sub>C</sub> receptor involves mechanism(s) besides activation of GTP binding protein-coupled receptors (data not shown).

It is unclear from the present results exactly how CK and M4 act to inhibit  $I_{GABA}$ . Though CK and M4 do not have charges, one possible mechanism is that CK and M4 may act as open channel blockers of the GABA<sub>C</sub> receptor. However, the inhibitory effect of CK and M4 on  $I_{GABA}$  was

not voltage-dependent, as shown by the linear regression analysis (Fig. 4). These results suggest that CK and M4 may not be open channel blockers because common open channel blockers are strongly voltage-dependent [26]. Another possibility is that CK and M4 may act as competitive inhibitors by inhibiting GABA binding to its binding site(s) in GABA<sub>C</sub> receptors. In competition experiments, we observed that the inhibitory effects of CK and M4 on  $I_{GABA}$  were not affected by increasing concentrations of GABA without changing the Hill coefficient (Fig. 5). Thus, these results indicate that CK or M4 interaction site(s) with the GABA<sub>C</sub> receptor might not be related to GABA-binding sites, which is similar to results obtained from other ligand-gated ion channels [27-29]. However, though the chemical structure of CK with a glucose at carbon-20 is different from that of M4 (Fig. 1), their regulatory patterns on GABA<sub>C</sub> receptor channel activity are very similar with slightly different IC<sub>50</sub> values (Fig. 3). These results indicate that the aglycone rather than the carbohydrate portion of CK might play a major role in the regulation of GABA<sub>C</sub> receptors.

Currently, there are only limited reports on the effects of ginseng on vision. For example, the administration of red ginseng extract to bullfrog improves visual processes by increasing both dark-adapted and light-adapted peak amplitude, as recorded by the electroretinogram [30]. An herbal mixture, including ginseng extract, increased the survival and regeneration of axotomized retinal ganglion cells [31]. Ginseng extract also exhibited ameliorating effects on diabetic-induced retinopathy [32]. Thus, it is likely that ginseng extract may have beneficial effects on visual systems in both normal and pathological conditions. In the present study, we found that ginsenoside metabolites affect GABA<sub>C</sub> receptor channel activity. It is known that inhibition of GABA<sub>C</sub> receptor channel activity is closely related to the regulation of vision in retinal bipolar cells [13]. However, we currently do not know whether CK- and M4-induced regulation of GABA<sub>C</sub> receptor channel activity could be responsible for the beneficial effects exhibited by ginseng extract. Future studies will be needed to elucidate the role of ginsenoside metabolites in the regulation of GABA<sub>C</sub> receptor channel activity.

In summary, we have examined the effects of ginsenoside metabolites such as CK and M4 heterologously expressed in *Xenopus* oocytes on human GABA<sub>C</sub> receptor channel activity. We found that M4, and not CK, mainly inhibits  $I_{GABA}$ . Since human GABA<sub>C</sub> receptors are primarily involved in the modulation of various physiological and pathophysiological activities in the central nervous system, the regulatory effects of M4 on  $I_{GABA}$  could provide a molecular basis for the pharmacological actions of ginsenoside metabolites in the nervous system, including the visual system.

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