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Effects of ginsenoside on G protein-coupled inwardly rectifying K^+ channel activity expressed in *Xenopus* oocytes

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Abstract

Recently, we provided evidence that ginsenoside, the active component of *Panax* ginseng, uses the pertussis toxin-insensitive $G\alpha_{q/11}$–phospholipase C-β3 signal transduction pathway to increase Ca^{2+}-activated Cl^- currents in the *Xenopus* oocyte. Other investigators have shown that stimulation of receptors linked to the $G\alpha_q$–phospholipase C pathway inhibits the activity of G protein-coupled inwardly rectifying K^+ (GIRK) channels. In the present study, we sought to determine whether ginsenoside influenced the activity of GIRK 1 and GIRK 4 (GIRK 1/4) channels expressed in the *Xenopus* oocyte, and if so, the underlying signal transduction mechanism. In oocytes injected with GIRK 1/4 channel cRNA, bath-applied ginsenoside inhibited the high K^+ solution-elicited GIRK current (EC_{50}: 4.9 ± 4.3 μg/ml). Pretreatment of the oocyte with pertussis toxin reduced the high K^+ solution-elicited GIRK current by 49%, but it did not alter the inhibitory effect of ginsenoside on the GIRK current. Prior intraoocyte injection of cRNA(s) coding $G\alpha_q$, $G\alpha_{11}$ or $G\alpha_q/G\alpha_{11}$, but not $G\alpha_{i2}$ or $G\alpha_{oA}$, attenuated the inhibitory ginsenoside effect. Injection of cRNAs coding $G\beta_1\gamma_2$ also attenuated the ginsenoside effect. Preincubation of GIRK channel-expressing oocytes with phospholipase C inhibitor, {1-[6-((17b-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1*H*-pyrrole-2,5-dione} (U73122), or protein kinase C inhibitor, staurosporine or chelerythrine, blocked the inhibitory ginsenoside effect on the GIRK current. Intraoocyte injection of bis (*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetracetic acid (BAPTA), a free Ca^{2+} chelator, had no significant effect on the action of ginsenoside. Taken together, these results suggest that ginsenoside inhibits the activity of the GIRK 1/4 channel expressed in the *Xenopus* oocyte through a pertussis toxin-insensitive and $G\alpha_{q/11}$-, phospholipase C- and protein kinase C-mediated signal transduction pathway.

Keywords: Ginseng; Ginsenoside; Protein kinase C; K^+ channel; G protein-coupled inwardly rectifying; *Xenopus* oocyte

1. Introduction

Ginseng (also called *Panax* ginseng C.A. Meyer after a Russian botanical taxonomist who named it) is a well-known folk medicine and has been used as a tonic (Brekhman and Dardymov, 1969). The main molecular component responsible for the actions of ginseng is ginsenoside, which is also known as ginseng saponin. Ginsenoside is one of the derivatives of the triterpenoid dammarane, which is consists of 30 carbon atoms (Nah, 1997). Ginsenoside has a four-ring, steroid-like structure with sugar moieties attached, and about 30 different forms have been isolated and identified from the root of *Panax* ginseng.

Ginsenoside has been shown to exert various effects on diverse cells and tissues (Nah, 1997). Ginsenoside increases the intracellular Ca^{2+} concentration in macrophages, NIH3T3 and endothelial cells (Shin et al., 1996; Hong et al., 1998; Li et al., 2000). Also, ginsenoside inhibits high-threshold voltage-gated Ca^{2+} channels in chromaffin cells (Kim et al., 1998) and sensory neurons (Nah and McCleskey, 1994; Nah et al., 1995) and activates Ca^{2+}-activated K^+ channels in vascular smooth muscle cells (Li et al., 2001). Further, ginsenoside inhibits acetylcholine-stimulated catecholamine release from chromaffin cells (Tachikawa et al., 1995; Kudo et al., 1998), and when given chronically to mice,

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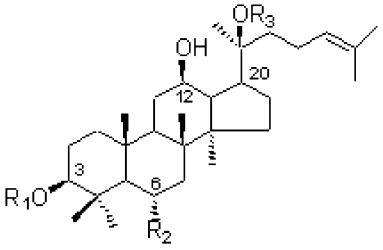
it increases phosphate incorporation into inositol phospholipids in the liver (Rim et al., 1997). Recent studies have suggested that G proteins mediate some of the effects of ginsenoside. Nah and McCleskey (1994) and Nah et al. (1995) showed that the inhibitory effect of ginsenoside on voltage-dependent Ca^{2+} currents in sensory neurons was mediated through the activation of pertussis toxin-sensitive G protein. Further, Choi et al. (2001a) have provided evidence that ginsenosides enhance Ca^{2+}-activated Cl^- currents by releasing intracellular Ca^{2+} via a pertussis toxin-insensitive $G\alpha_{q/11}$ coupled to phospholipase C-β3 in the *Xenopus* oocyte.

G protein-coupled inwardly rectifying K^+ (GIRK) channels play an important role in regulating cell excitability in both the heart and the nervous system (Dascal, 1987; Karschin, 1999). In the nervous system, GIRK channels are linked to a variety of pertussis toxin-sensitive, G protein-coupled receptors, including cannabinoid, $GABA_B$, muscarinic, opioid, $5\text{-}HT_{1A}$ and somatostatin receptors (Andrade et al., 1986; Dascal et al., 1993; Penington et al., 1993; Chen and Yu, 1994; McAllister et al., 1999). Stimulation of these receptors catalyzes the turnover of heterotrimeric $G\alpha_i/\alpha_o\beta\gamma$ proteins, liberating $G\beta\gamma$ subunits. This complex directly binds to the cytoplasmic domains of both GIRK 1 and GIRK 4 channels with subsequent channel activation (Reuveny et al., 1994). GIRK channels are also linked to pertussis toxin-insensitive G protein-coupled receptors such as bombesin, endothelin, $5\text{-}HT_{2C}$, metabotropic glutamate1a and m1 muscarinic receptors. Agonist stimulation of these receptors activates the $G\alpha_q$–phospholipase C signal transduction pathway, leading to the inhibition of GIRK channel activity (Stevens et al., 1999; Rogalski and Chavkin, 2001; DiMagno et al., 1996; Sharon et al., 1997; Hill and Peralta, 2001). In the present study, we examined whether ginsenoside influenced the activity of GIRK channels expressed in the *Xenopus* oocyte. Finding this to be the case, we further investigated the underlying signal transduction mechanism. Here, we present results suggesting that ginsenoside inhibits GIRK channel activity in a pertussis toxin-insensitive fashion through the $G\alpha_{q/11}$–phospholipase C–protein kinase C pathway.

2. Materials and methods

2.1. Materials

cDNAs coding Gβ1, Gγ2, $G\alpha_{oA}$, $G\alpha_{11}$ and $G\alpha_q$, were purchased from Guthrie Research Institute (Sayre, PA). GIRK1 and GIRK4 (GIRK1/4) channel cDNAs were kindly provided by Dr. N. Dascal (Tel Aviv University, Israel). Mouse brain IRK1 and IRK2 channel cDNAs were kindly provided by Dr. Y. Kurachi (Osaka University, Japan). BAPTA, niflumic acid, 4-phorbol 12-myristate 13-acetate (PMA) and 4-phorbol 12,13-didecanoate (PDD), staurosporine, and chelerythrine were obtained from Sigma (St. Louis, MO). {1-[6-((17b-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1*H*-pyrrole-2,5-dione} (U73122) (active PLC inhibitor) and {1-[6-((17b-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione} (U73343) (inactive PLC inhibitor) were obtained from Calbiochem (La Jolla, CA), and pertussis toxin was from List Biological Laboratories (Campbell, CA). Ginsenoside was kindly provided by Korea Ginseng and Tobacco Research Institute (Taejon, Korea). Fig. 1 shows the chemical structure of representative ginsenosides. Ginsenoside stock solution



Ginsenosides	R_1	R_2	R_3	PD or PT
Rb_1	-Glc^{2}-Glc	-H	-Glc^{6}-Glc	PD
Rb_2	-Glc^{2}-Glc	-H	-Glu^{6}-Ara(pyr)	PD
Rc	-Glc^{2}-Glc	-H	-Glc^{6}-Ara(fur)	PD
Rd	-Glc^{2}-Glc	-H	-Glc	PD
Re	-H	-O-Glc^{2}-Rha	-Glc	PT
Rf	-H	-O-Glc^{2}-Glc	-H	PT
Rg_1	-H	-O-Glc	-Glc	PT
Rg_2	-H	-O-Glc^{2}-Rha	-H	PT

Fig. 1. Structure of the eight representative ginsenosides. They differ at three side chains attached to the common steroid ring. Abbreviations for carbohydrates are as follows: Glc, glucopyranoside; Ara(pyr), arabinopyranoside; Rha, rhamnopyranoside. Superscripts indicate the carbon in the glucose ring that links the two carbohydrates.

(10 mg/ml) was prepared in the oocyte-bathing medium ND96 (see below). The stock solutions (10 mM) of chelerythrine, PDD, PMA, staurosporine, U73122 and U73343 were prepared in DMSO. These stock solutions were further diluted before use with ND96. Final DMSO concentration was $<0.05\%$.

2.2. In vitro synthesis of cRNA

Recombinant plasmids containing cDNA inserts for various G protein subunits, GIRK 1/4 and IRK 1/2 were linearized by digestion with appropriate restriction enzymes. The cRNAs from linearized templates were obtained with an in vitro transcription kit (mMessage mMachine; Ambion, Austin, TX, USA) using a SP6, T3 or T7 RNA polymerase. The RNA was dissolved in RNase-free water at 1 μg/μl, divided into aliquots and stored at -70 °C until used.

2.3. Oocyte preparation

Xenopus laevis frogs were obtained from Xenopus I (Ann Arbor, MI, USA). Before being operated on for oocyte extraction, they were kept in a temperature-controlled aquarium (18 ± 1 °C) with a 12:12 h light/dark cycle, and food was given every 2 days. Oocytes were extracted under deep anesthesia, which was induced by immersing frogs in an aerated solution of 0.15% 3-amino benzoic acid ethyl ester. Following oocyte extraction, frogs were killed by overdosing with the anesthetic. The extracted oocytes were separated by treatment with collagenase and agitation 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 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$ and 5 mM HEPES, pH 7.5) supplemented with 0.5 mM theophylline and 50 μg/ml gentamicin. This oocyte-containing solution was maintained at 18 °C with continuous gentle shaking and changed everyday.

2.4. Oocyte treatment before electrophysiological recordings

In the first series of experiments, the oocytes were injected with cRNA(s) prepared as above or vehicle (i.e. distilled water), and electrophysiological recordings were made 2 days after. The intraoocyte injections were made with glass micropipettes pulled from capillary tubing whose tips were broken to an outer diameter of about 20 μm.

2.5. Oocyte recording

A custom-made Plexiglas net chamber was used for two-electrode voltage-clamp recordings. The chamber was constructed by milling two concentric wells into the chamber bottom (diameter/height: upper well, 8/3 mm; lower well: 6/5 mm) and gluing plastic meshes (~ 0.4 mm grid diameter) onto the bottom of the upper well. The perfusion inlet (~ 1 mm diameter) was formed through the wall of the lower well, and a suction tube was placed on the edge of the upper well. An oocyte was placed on the net that separates the upper and lower wells. The grids of the net served as dimples that kept the oocyte in place during electrophysiological recordings. Oocytes were impaled with two microelectrodes filled with 3 M KCl (0.2–0.7 MΩ) and voltage-clamped at -80 mV. After stabilization of oocytes with ND96, oocytes were then bathed with a high K^+ solution (composition in mM: KCl 96, NaCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5, pH 7.5). In this solution, the K^+ equilibrium potential (E_K) was close to 0 mV, enabling K^+ inward currents to flow through inwardly rectifying K^+ channels at negative holding potentials. IRK currents and some GIRK currents were recorded in solution (composition in mM: KCl 90, $MgCl_2$ 3, HEPES 5, pH 7.4) as described previously (Dascal et al., 1993). The electrophysiological experiments were done at room temperature with an Oocyte Clamp (OC-725C, Warner Instrument, CT).

2.6. Data analysis

All numerical values are presented as means ± S.E.M. The differences between control and experimental data were analyzed using unpaired *t*-test. $P<0.05$ was considered significant. In the indicated cases, data were analyzed by analysis of variance (ANOVA) and Dunnett's procedure.

3. Results

3.1. Effect of ginsenoside on the activity of GIRK 1/4 channels expressed in the Xenopus oocytes

In oocytes injected with vehicle (i.e. distilled water), superfusion of a high K^+ solution (96 mM KCl) elicited only a slight inward current (Fig. 2A). The high K^+ solution-elicited inward current was much larger in oocytes injected with both GIRK 1 and 4 channel cRNAs than in those injected with distilled water (Fig. 2B and C). These results are very consistent with previous reports that heterologous expression of GIRK 1/4 channels was needed to give rise to a functionally active GIRK channel (Kofuji et al., 1995; Krapivinsky et al., 1995; Chan et al., 1996). Ginsenoside (50 μg/ml) application during superfusion of the high K^+ solution induced a large transient inward current (which was sensitive to niflumic acid, a Cl^- channel blocker; data not shown) (Fig. 2C). In addition, ginsenoside application resulted in a significant decrease in the high K^+ solution-elicited inward current ($58\pm4.3\%$ inhibition, respectively; $n=18$ each, $P<0.01$, Fig. 2C inset). The high K^+ solution-elicited inward current disappeared almost completely when Ba^{2+} (300 μM) was applied (Fig. 2C). Ginsenoside had no effect on the

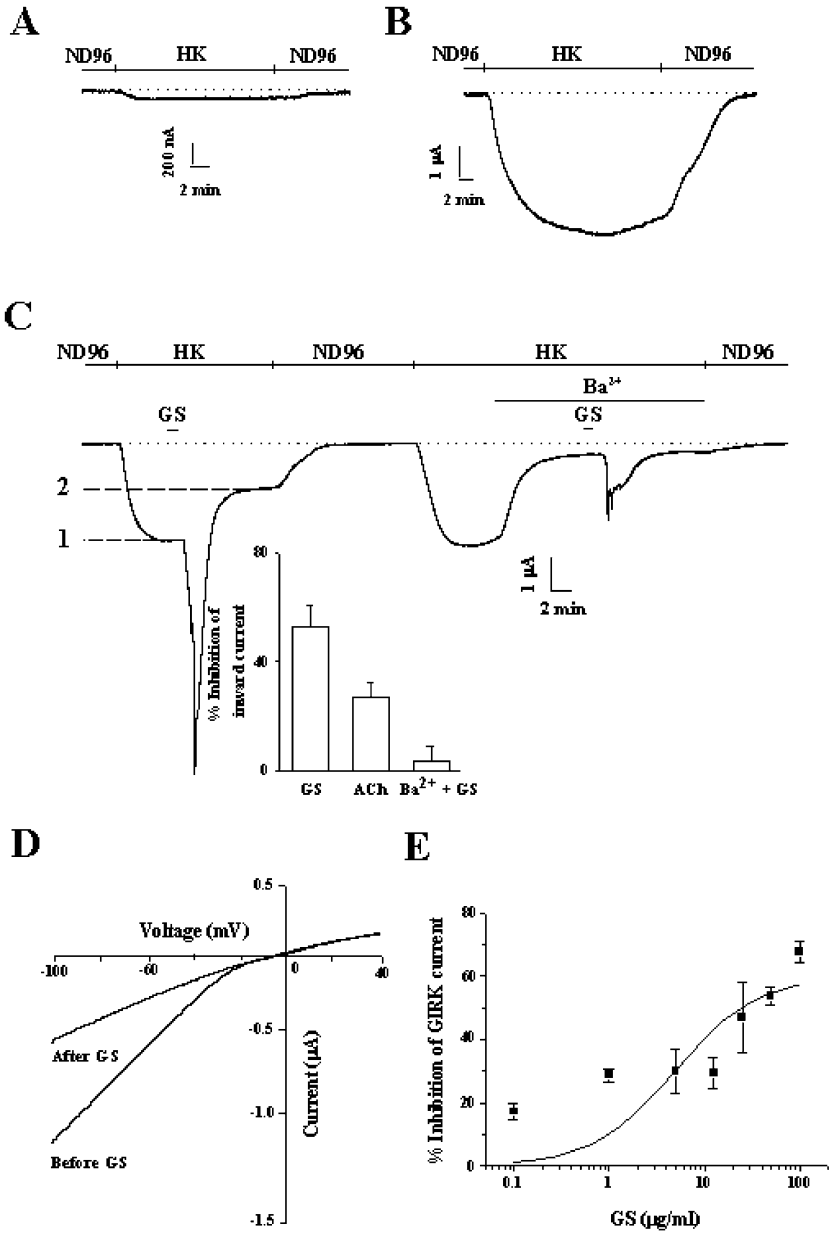


Fig. 2. Effect of Ginsenoside (GS) on GIRK1/4 current. (A–C) Representative current traces from individual oocytes (voltage-clamped at −80 mV for A–C) that were injected with vehicle (i.e. distilled water) (A), 4 ng of GIRK 1/4 channel cRNA(s) (B and C). Application of high K^+ (HK) solution evoked slight (A) and large inward currents (B and C). Inset: graphic representation of the mean inhibition of HK solution-induced inward current by GS, acetylcholine (ACh) and GS in the presence of 300 μM Ba^{2+} (10 μM ACh for 60 s; 5 ng/oocyte injection of m1 muscarinic acetylcholine receptor cRNA in ACh experiments). Application of GS (50 μg/ml for 60 s in this and following experiments, unless stated otherwise) induced a transient inward current and caused a decline in the HK solution-elicited inward current (C). The dotted lines (A–C) indicate the 0 current levels. (D) Current–voltage relationships in oocytes injected with GIRK 1/4 channel cRNAs before and after GS treatment. These data were obtained using a voltage ramp protocol (−100 to +40 mV). (E) Dose dependence of the inhibitory GS effect on the high K^+ solution-elicited inward current. Oocytes were voltage-clamped at −80 mV. Each data point in the graph is the mean (±S.E.M) of 9–12 independent experiments performed using different batches of oocytes.

residual inward current that was insensitive to Ba^{2+} (Fig. 2C, inset), indicating that ginsenoside inhibits Ba^{2+}-sensitive inward currents. As a positive control, we also tested the effect of acetylcholine in oocytes injected with cRNAs coding GIRK 1/4 channel and m1 muscarinic acetylcholine receptor, which is known to utilize the $G\alpha_q$–phospholipase C pathway (Hill and Peralta, 2001). Acetylcholine (10 μM) induced a large transient inward current, as do ginsenoside and other receptor agonists (Stevens et al., 1999; Rogalski and Chavkin, 2001; DiMagno et al., 1996; Sharon et al., 1997) and inhibited the high K^+ solution-elicited inward current (27.3 ± 5.3% inhibition; $n=12$, $P<0.01$, Fig. 2C inset). Fig. 2D shows the representative current–voltage relationships of the high K^+ solution-elicited inward cur-

rents before and after the application of ginsenoside ($n=9$, from three different batch of oocytes). The inwardly rectifying currents activated under both conditions showed a reversal potential near 0 mV. That is, ginsenoside did not shift the equilibrium potential of these currents. However, ginsenoside inhibited the inward current elicited at potentials more negative than −20 mV. Thus, these results indicated that the current inhibited by ginsenoside was an inward rectifier, presumably GIRK current. The ginsenoside-induced inhibition of the inward current (which will be referred to as GIRK current from now on) was dose dependent; EC_{50} was 4.9 ± 4.3 μg/ml (Fig. 2E).

3.2. Effects of pertussis toxin and Gα subunit expression on the ginsenoside inhibition of GIRK current

We examined whether the ginsenoside effect on the GIRK current was mediated by pertussis toxin-insensitive G proteins, since ginsenoside uses the pertussis toxin-insensitive $G\alpha_{q/11}$ signaling pathway to increase the Ca^{2+}-activated Cl^- current in the *Xenopus* oocytes (Choi et al., 2001a). Pertussis toxin pretreatment (2 μg/ml, for 16–24 h) reduced the high K^+ solution-elicited GIRK current by 42 ± 10% ($P<0.01$; compared with pertussis toxin-untreated control). These results are consistent with previous reports that pretreatment

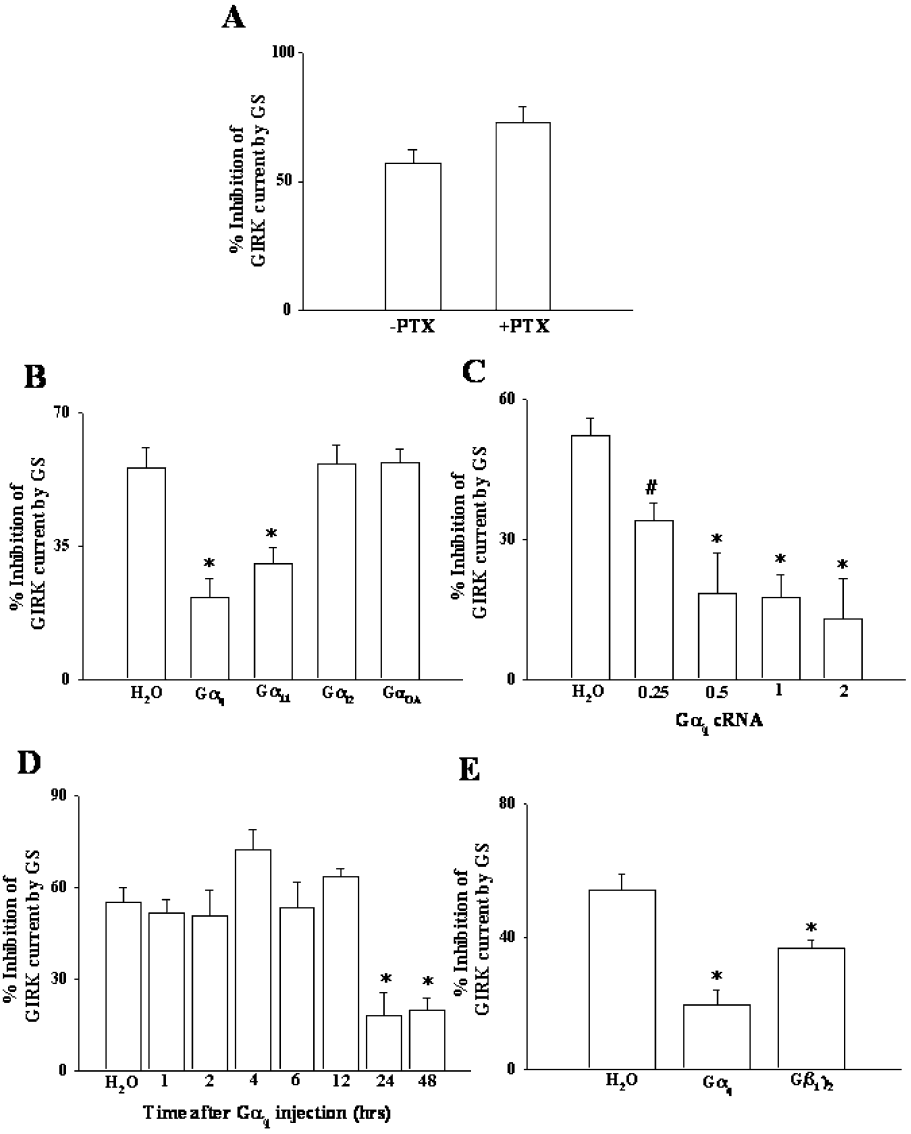


Fig. 3. Effects of pertussis toxin (PTX) and of expressing various G protein subunits on the ginsenoside (GS)-induced inhibition of the GIRK current. (A) PTX (2 μg/ml) was added for 16–24 h in some experiments (+PTX) ($n=12$ each). (B) Before testing the GS effect, oocytes were injected with vehicle (i.e. distilled water) or cRNA (2 ng/oocyte) coding either $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{i2}$ or $G\alpha_{oA}$ subunit ($n=10$ each). (C) Oocytes injected with different amounts of $G\alpha_q$ cRNA ($n=12$ each) before GS application. (D) GS effect was examined at different times after $G\alpha_q$ cRNA injection (2 ng/oocyte; $n=10$ each). (E) Prior to GS application, oocytes were injected with vehicle or cRNAs coding $G\beta_1\gamma_2$ (10 ng) ($n=10$ each). The data denoted with *'s and #'s were significantly different from the others; $*P<0.01$, $\#P<0.05$.

with pertussis toxin (2 μg/ml, for 16–24 h) inhibits the basal GIRK current (Chan et al., 1996; Sharon et al., 1997; Vivaudou et al., 1997; Kobrinsky et al., 1999). However, pertussis toxin pretreatment did not attenuate the inhibitory effect of ginsenoside on the GIRK current (−pertussis toxin/+pertussis toxin = 56.9 ± 5.2:72.6 ± 6.3% inhibition, Fig. 3A), indicating that the ginsenoside effect on the GIRK current is pertussis toxin insensitive. To further assess the type of G protein subunits involved in the ginsenoside effect on the GIRK current, we investigated the consequences of expressing various Gα subunits by prior intraoocyte injection of the corresponding cRNAs. The expression of all Gα subunits resulted in a significant decrease in the basal GIRK current (% inhibition: $G\alpha_q$: 55 ± 4, $G\alpha_{11}$: 63 ± 9, $G\alpha_{i2}$: 36 ± 5, $G\alpha_{oA}$: 31 ± 7). Furthermore, the expression of $G\alpha_q$ and $G\alpha_{11}$ (but not $G\alpha_{i2}$ and $G\alpha_{oA}$) subunits attenuated the inhibitory ginsenoside effect on the GIRK current (% ginsenoside inhibition of the GIRK current: H_2O: 55.6 ± 5.2, $G\alpha_q$: 21.4 ± 5.1*, $G\alpha_{11}$: 30.2 ± 4.3*, $G\alpha_{i2}$: 56.7 ± 4.7, $G\alpha_{oA}$: 57.0 ± 3.5%, *$P < 0.05$, compared with the H_2O-injected control; Fig. 3B).

The extent of the blockade of the ginsenoside effect by $G\alpha_q$ and $G\alpha_{11}$ cRNA injection was proportional to the amount of the cRNAs injected (Fig. 3C; data from $G\alpha_{11}$ cRNA injection experiments are not shown), and the time required for the injected cRNAs to work was >12 h (Fig. 3D). Taken together, the results from experiments conducted with pertussis toxin and cRNAs coding Gα subunits suggested that the inhibitory ginsenoside effect on the GIRK current was mediated by a protein of the pertussis toxin-insensitive $G\alpha_{q/11}$ family.

3.3. Effect of Gβγ expression on the ginsenoside inhibition of GIRK current

Previous studies have shown that Gβγ subunits enhance the GIRK current by increasing the chance that these subunits bind to the cytoplasmic domains of the GIRK channel (Reuveny et al., 1994; Chan et al., 1996; Velimirovic et al., 1996). In this study, we assessed whether Gβγ subunit expression affected the ginsenoside effect on the GIRK current. Injection of cRNAs coding $G\beta_1\gamma_2$ subunits attenuated the inhibitory effect of ginsenoside on the GIRK current ($H_2O/G\beta_1\gamma_2$ = 55.6 ± 5.1:36.7 ± 2.4% inhibition, $n = 12$, $P < 0.05$) (Fig. 3E). In addition, the cRNA injection increased the basal GIRK current (~ 30%; data not shown), opposite to the effect of injection of cRNA coding $G\alpha_q$ or $G\alpha_{11}$ subunit (see above). The effect of $\beta_1\gamma_2$ overexpression on the ginsenoside-induced inhibition of the GIRK current is not inconsistent with the idea that these G protein subunits mediate the ginsenoside effect. However, the effect on the basal GIRK current is hardly consistent with this idea. In other words, it does not make sense that βγ subunits enhance the GIRK current on one hand, and that they mediate the inhibitory ginsenoside effect on the GIRK current on the other.

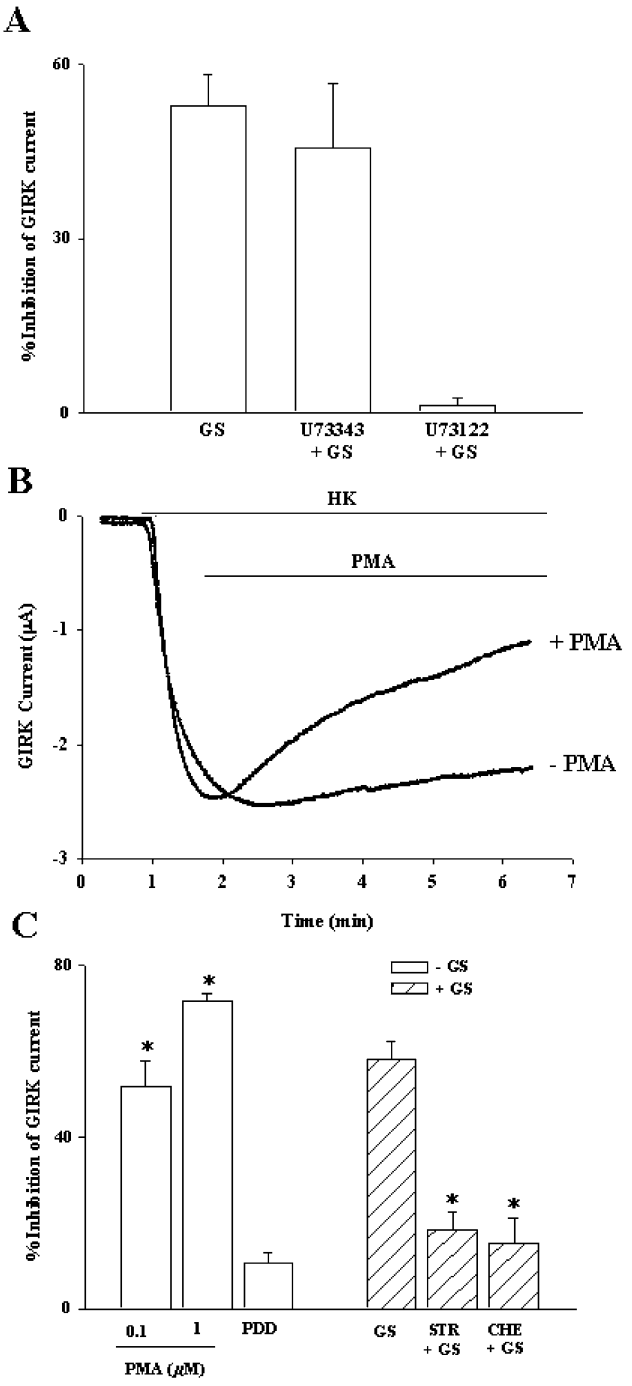


Fig. 4. Effects of phospholipase C and protein kinase C inhibitors on the ginsenoside (GS)-induced inhibition of the GIRK current. (A) Prior to GS application, oocytes were left untreated or incubated for 5 min with U73122 (3 μM; PLC inhibitor) or U73343 (3 μM; inactive analog). (B) Traces represent GIRK 1/4 currents in the absence or presence of 0.1 μM PMA. These figures show that incubation with PMA during electrophysiological recording directly inhibits the GIRK 1/4 current. Traces are representative of same batch of oocytes exhibiting a similar current amplitude. (C, left panel) Oocytes were treated for 5 min with PMA (0.1 or 1 μM; PKC activator) or PDD (1 μM; inactive PKC activator). (B, right panel) Oocytes were incubated for 5 min with the PKC inhibitors staurosporine (STR; 1 μM) and chelerythrine (CHE; 1 μM) before the application of GS. The data denoted with *'s were significantly different from the others ($P < 0.01$).

3.4. Effect of phospholipase C inhibitor on ginsenoside inhibition of GIRK current

To gain further insight into the downstream signaling pathway involved in the ginsenoside effect on the GIRK current, we determined whether phospholipase C inhibitor had any influence on the ginsenoside effect. For this study, we used the aminosteroid U73122, which is known to inhibit phospholipase C with half-maximal inhibition at 3.7 μM (Thompson et al., 1991). As shown in Fig. 4A, the phospholipase C inhibitor U73122 (3 μM) blocked almost completely the ginsenoside effect, whereas the inactive phospholipase C inhibitor U73343 (3 μM) had no effect. Thus, these results indicated that phospholipase C mediated the inhibitory ginsenoside effect on the GIRK current.

3.5. Effects of calcium chelator and protein kinase C inhibitor on ginsenoside inhibition of GIRK current

To see if the ginsenoside-induced inhibition of the GIRK current involved inositol 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} release from intracellular stores, we tested the effect of BAPTA. Intracellular injection of BAPTA (23-nl injection of 50-mM solution to give rise to 1-mM intracellular concentration), an effective way of chelating Ca^{2+} released from intracellular storage (Hartzell, 1996; Choi et al., 2001b), was ineffective in preventing ginsenoside from inhibiting the GIRK current (H_2O + ginsenoside/BAPTA + ginsenoside = 50.2 ± 4.3/41.7 ± 5.8%; P>0.05). This result indicated that intracellular Ca^{2+} release via the IP_3 pathway was not involved in the ginsenoside-induced inhibition of the GIRK current. Therefore, we assessed the role of protein kinase C, which is activated by 1,2-diacylglycerol produced along with IP_3 following phospholipase C activation. We examined the effects of PMA (a potent PKC activator) and PDD (inactive phorbol ester) on the GIRK current. Fig. 4 B shows that 0.1-μM PMA reduced the GIRK current by ~ 50%. The graph summarizing the results from the 'PMA' and 'PDD' experiments (Fig. 4C, left panel) further illustrates that treatment with PMA, but not PDD, inhibited the GIRK current significantly (n = 15, *P < 0.01; compared with PMA-untreated oocytes). These results are consistent

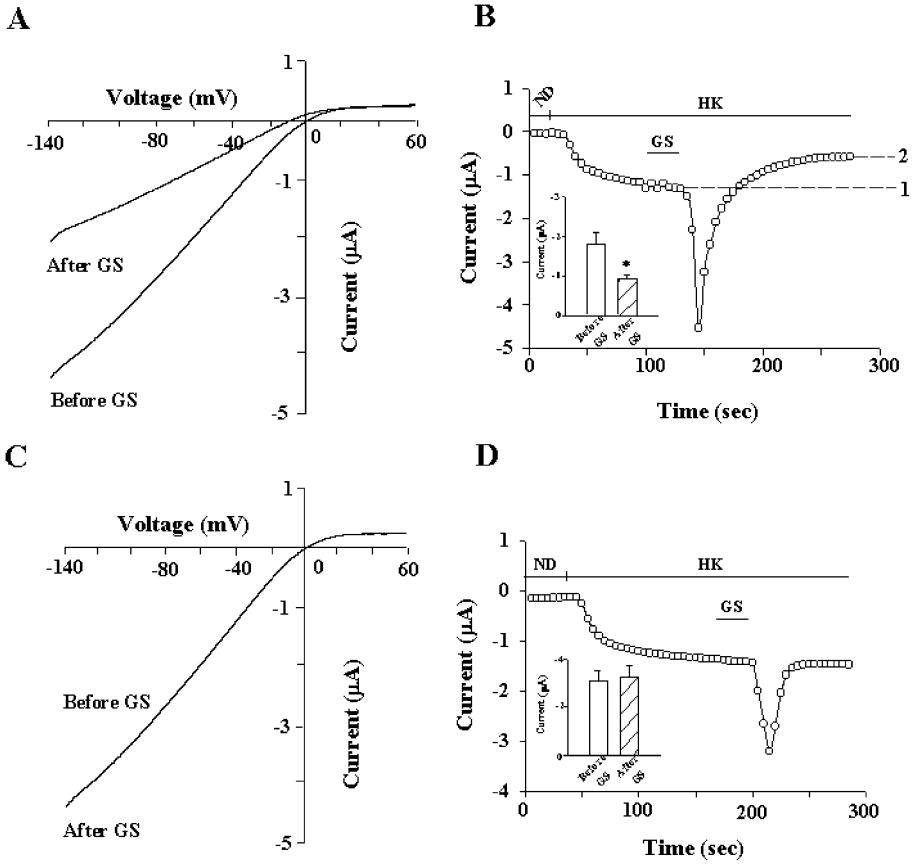


Fig. 5. Effects of ginsenoside (GS) on the activity of the mouse brain inwardly rectifying K^+ 2 (MB-IRK2) channel. (A and C) Current–voltage relationships in oocytes injected with GIRK1/4 (3 ng/oocyte) or IRK channel cRNAs (40 ng/oocyte). These were obtained using a voltage ramp protocol (− 140 to + 60 mV) before and after application of GS. (B and D) Oocytes were held at 0 mV and pulsed to − 80 mV for 300 ms at 10-s intervals. The application of GS induced a transient inward current in oocytes injected with GIRK 1/4 or IRK channel cRNA (n = 18 each). In addition, it reduced the high K^+ (HK) solution-elicited inward current in oocytes injected with GIRK 1/4 channel cRNA (B). Insets: bar graphs illustrating the effects of GS on HK solution-elicited inward currents in oocytes injected with GIRK 1/4 (B) and IRK channel cRNAs (D). The asterisk-denoted data were significantly different from the others (P < 0.05) by analysis of variance (ANOVA) and Dunnett's procedure.

with previous reports that protein kinase C activators inhibit the GIRK current in the *Xenopus* oocyte (DiMagno et al., 1996; Sharon et al., 1997; Stevens et al., 1999; Hill and Peralta, 2001). Next, we examined whether the protein kinase C inhibitors staurosporine (1 μM; DiMagno et al., 1996; Hill and Peralta, 2001) and chelerythrine (1 μM; Herbert et al., 1990) reduced the ginsenoside effect on the GIRK current. Preincubation of oocytes in staurosporine- or chelerythrine-containing solution for 5 min reduced the inhibitory effects of ginsenoside (ginsenoside/staurosporine + ginsenoside/chelerythrine + ginsenoside = 58.2 ± 4.2/18.6 ± 3.9/26.3 ± 6.0% inhibition; $P < 0.01$; compared with oocytes treated with ginsenoside alone) (Fig. 4C, right panel). These results suggested that protein kinase C mediated the ginsenoside-induced inhibition of the GIRK current. We further determined whether or not the ginsenoside inhibition of the GIRK current was related to the ginsenoside-induced increase in the Ca^{2+}-activated Cl^- current. For this, we tested the effect of the chloride channel blocker niflumic acid on the ginsenoside-induced inhibition of the GIRK current, since our earlier study showed that niflumic acid blocked the ginsenoside effect on the Cl^- current (Choi et al., 2001b). At 300 μM, niflumic acid failed to alter the inhibitory effect of ginsenoside on the GIRK current (ginsenoside/niflumic acid + ginsenoside = 50.2 ± 4.28/46.0 ± 3.7% inhibition; $P > 0.05$), whereas it blocked almost completely the ginsenoside effect on the Cl^- current (data not shown). These results suggested that the ginsenoside-induced inhibition of the GIRK current was unrelated to the increase in the Ca^{2+}-activated Cl^- current evoked by ginsenoside.

3.6. Ginsenoside does not inhibit the activity of mouse brain inwardly rectifying K^+ 2 (MB-IRK2) channel

To see if ginsenoside affects the inwardly rectifying potassium (IRK) current other than the GIRK current, we expressed the MB-IRK2 channel in the *Xenopus* oocyte and examined the effect of ginsenoside on the current flowing through this channel. The reason we chose this channel was that it has considerable homology with the GIRK 1/4 channels but is not coupled to $G\alpha_i/G\alpha_o$ proteins (Takahashi et al., 1994; Yamada et al., 1998). In oocytes injected with GIRK 1/4 channel cRNAs, ginsenoside inhibited GIRK current and caused a transient inward Cl^- current (Fig. 5A and B). However, in MB-IRK2 channel cRNA-injected cells, ginsenoside failed to inhibit the IRK current although it induced a transient Cl^- current (Fig. 5D). These results suggested that the target of ginsenoside signaling mediated via the $G\alpha_{q/11}$–protein kinase C pathway is the GIRK, not the IRK, channel.

4. Discussion

Previous studies have shown that a variety of agents inhibit GIRK channel activity through the activation of membrane receptors coupled to the pertussis toxin-insensitive $G\alpha_q$–protein kinase C signal transduction pathway (Stevens et al., 1999; Rogalski and Chavkin, 2001; DiMagno et al., 1996; Sharon et al., 1997; Hill and Peralta, 2001). Recently, we provided evidence that ginsenoside enhances the Ca^{2+}-activated Cl^- current by activating pertussis toxin-insensitive $G\alpha_{q/11}$ coupled to phospholipase C-β3 in the *Xenopus* oocyte (Choi et al., 2001a). In the present study, we sought to determine whether or not ginsenoside could alter GIRK channel activity, and if so, the underlying signal transduction mechanism. We found that ginsenoside inhibited the activity of GIRK 1/4 channels expressed in the *Xenopus* oocyte and that this inhibition might be through the pertussis toxin-insensitive $G\alpha_{q/11}$–phospholipase C–protein kinase C signaling cascade.

The evidence supporting the notion that the α_q family of G proteins mediated the ginsenoside effect on the GIRK current is 2-fold. Firstly, pertussis toxin treatment had no significant effect on the ginsenoside-induced inhibition of the GIRK current; it is well established that the α_q family of G proteins is pertussis toxin insensitive (Berman and Gilman, 1998). Secondly, the expression of $G\alpha_q$ and $G\alpha_{11}$ (but not $G\alpha_{i2}$ and $G\alpha_{oA}$) subunits significantly attenuated the ginsenoside effect on the GIRK current. This attenuation was probably due to the persistent activation of effectors such as phospholipase C by $G\alpha_q$ or $G\alpha_{11}$ expressed after cRNA injection, which led to their desensitization.

The present results suggest that Gβγ subunits can modulate the effect of ginsenoside on the GIRK channel, but they do not support the idea that these G protein subunits mediate effect of the ginsenoside (see Results). Then, why did $G\beta_1\gamma_2$ subunit expression attenuate the effect of ginsenoside on the GIRK current, as did the expression of the $G\alpha_q$ or $G\alpha_{11}$ subunit? This might be because the extra copies of $G\beta_1\gamma_2$ subunits derived from the cRNA injection chelated $G\alpha_{q/11}$ subunits mediating the inhibitory ginsenoside signal. Similar to this interpretation, Hill and Peralta (2001) argued that overexpressed Gβγ subunits competed with the $G\alpha_q$ signal which mediated the inhibitory effect of acetylcholine on basal and dopamine 2 receptor-activated GIRK 1/4 currents in *Xenopus* oocytes (Hill and Peralta, 2001).

The results obtained with active and inactive phospholipase C inhibitors indicate that phospholipase C mediates the ginsenoside effect on the GIRK current. The activation of phospholipase C produces both water-soluble IP_3 and lipid-soluble 1,2-diacylglycerol. IP_3 releases Ca^{2+} from internal stores. In an earlier communication, we reported that IP_3-mediated Ca^{2+} release was responsible for the ginsenoside enhancement of the Cl^- current (Choi et al., 2001b). In the present study, we assessed whether IP_3-mediated Ca^{2+} release was also responsible for the ginsenoside effect on the GIRK current. The results from experiments performed with the Ca^{2+} chelator BAPTA indicate that this was not the case. So, we investigated the other possibility. That is, we assessed whether 1,2-diacylglycerol mediated the ginsenoside-induced inhibition of the GIRK current by examining

the effects of one protein kinase C activator and two protein kinase C inhibitors. Previous studies have shown that protein kinase C activator inhibits both basal GIRK currents and receptor agonist-induced GIRK currents, suggesting that protein kinase C is involved in GIRK channel regulation (Stevens et al., 1999; Rogalski and Chavkin, 2001; DiMagno et al., 1996; Sharon et al., 1997; Hill and Peralta, 2001). In the present study, we observed that PMA, a potent protein kinase C activator, reduced the GIRK current, and that the protein kinase C inhibitors staurosporine and chelerythrine attenuated the inhibitory effect of ginsenoside on the GIRK current. On the basis of these results, we conclude that the ginsenoside effect on the GIRK current is mediated through the 1,2-diacylglycerol–protein kinase C pathway.

The data obtained with the use of niflumic acid indicate that the enhancement of the Ca^{2+}-activated Cl^- current by ginsenosides is not a prerequisite for the ginsenoside-induced inhibition of the GIRK current. This conclusion is consistent with the idea that separate signal transduction pathways mediate the effects of ginsenoside on the Cl^- and GIRK currents.

The results from experiments comparing the effects of ginsenoside on MB-IRK2 and GIRK currents indicated that ginsenoside affects specific K^+ channel(s). However, it still remains to be determined whether G protein coupling is mandatory for a given K^+ channel to be responsive to ginsenoside.

In the present study, using a *Xenopus* oocyte model that allows the expression of various foreign genes, we have provided evidence that ginsenoside inhibits the GIRK current through the $G\alpha_{q/11}$–phospholipase C–protein kinase C pathway. At the moment, we do not know whether the effect of ginsenoside seen in *Xenopus* cells is related to the effects of ginsenoside observed in mammals (e.g., antinociceptive effect) (Yoon et al., 1998; Nah et al., 2000). However, the fact that the in vivo and in vitro effects of ginsenoside are elicited by comparable concentrations of ginsenoside supports the above notion.

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