The Signal Transduciton of Ginsenosides, Active Ingredients of *Panax ginseng*, in *Xenopus oocyte*: A Model System for Ginseng Study

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Abstract

Recently, we have provided evidence that ginsenosides, the active components of*Panax* ginseng, utilize pertussis toxin (PTX)-insensitive $G\alpha_{q/11}$-phospholipase C-β3 (PLC-β3) signal transduction pathway for the enhancement of Ca^{2+}-activated Cl^- current in the *Xenopus* oocyte (British J. Pharmacol. 132, 641-647, 2001; JBC 276, 48797-48802, 2001). Other investigators have shown that stimulation of receptors linked to $G\alpha_q$-PLC pathway inhibits the activity of G protein-coupled inwardly rectifying K^+ (GIRK) channel. In the present study, we sought to determine whether ginsenosides 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 oocyte injected with GIRK 1/4 channel cRNAs, bath-applied ginsenosides inhibited high potassium (HK) solution-elicited GIRK current (EC_{50}: 4.9 ± 4.3 μg/ml). Pretreatment of the oocyte with PTX reduced the HK solution-elicited GIRK current by 49%, but it did not alter the inhibitory ginsenoside effect on 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. Similarly, injection of the cRNAs coding regulators of G protein signaling 1, 2 and 4 (RGS1, RGS2 and RGS4), which interact with $G\alpha_i$ and/or $G\alpha_{q/11}$ and stimulates the hydrolysis of GTP to GDP in active GTP-bound Gα subunit, resulted in a significant reduction of ginsenoside effect on GIRK current. Preincubation of GIRK channel-expressing oocyte in PLC inhibitor (U73122) or protein kinase C (PKC) inhibitor (staurosporine or chelerythrine) blocked the inhibitory ginsenoside effect on GIRK current. On the other hand, intraoocyte injection of BAPTA, a free Ca^{2+} chelator, had no significant effect on the ginsenoside action. Taken together, these results suggest that ginsenosides inhibit the activity of GIRK 1/4 channel expressed in the *Xenopus* oocyte through a PTX-insensitive and $G\alpha_{q/11}$-, PLC- and PKC-mediated signal transduction pathway.

Introduction

Ginseng, the root of *Panax ginseng* C.A. Meyer, is a well-known folk medicine and has been used as a tonic agent. The main molecular components responsible for the actions of ginseng are ginsenosides, which are also known as ginseng saponins. Ginsenoside is one of the derivatives of triterpenoid dammarane consisting of thirty carbon atoms (1). 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*.

Ginsenosides have been shown to exert various effects on diverse living cells and tissues (1). They increase the intracellular Ca^{2+} concentration in macrophages, NIH3T3 and endothelial cells (2-4). Also, ginsenosides inhibit high-threshold voltage-gated Ca^{2+} channels in chromaffin cells (5) and sensory neurons (6,7) and activate Ca^{2+}-activated K^+ channels in vascular smooth muscle cells (8). Further, ginsenosides inhibit acetylcholine-stimulated catecholamine release from chromaffin cells (9,10), and when treated chronically in the mouse, it increases phosphate incorporation into inositol phospholipids in the liver (11). Recent studies have suggested that G proteins mediate some ginsenoside effects. Nah and McCleskey (6) and Nah *et al.* (7) showed that the inhibitory effect of ginsenosides on voltage-dependent Ca^{2+} current in sensory neurons was through the activation of PTX-sensitive G protein. Further, Choi *et al.* (12, 13) provided evidence that ginsenosides increase Ca^{2+}-activated Cl^- current by activating PTX-insensitive $G\alpha_{q/11}$ coupled to PLC-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 nervous system (14, 15). In the nervous system, GIRK channels are linked to a variety of PTX-sensitive, G protein coupled receptors (GPCRs), including cannabinoid, $GABA_B$, muscarinic, opioid, $serotonin_{1A}$ and somatostatin receptors (16-20). 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 (21) with consequent channel activation. GIRK channels are also linked to PTX-insensitive GPCRs such as bombesin, endothelin, $5\text{-}HT_{2C}$, metabotropic glutamate1a and m1 muscarinic receptors. Agonist stimulation of these receptors activates $G\alpha_q$-PLC signal transduction pathway, leading to the inhibition of GIRK channel activity (22-26). In the present study, we examined whether ginsenosides influenced the activity of GIRK channel expressed in the *Xenopus oocyte*. Finding this to be the case, we further investigated the underlying signal trans-

duction mechanism. Here we present results suggesting that ginsenosides inhibit GIRK channel activity in a PTX-insensitive fashion through the $G\alpha_{q/11}$-PLC-PKC pathway.

Materials and Methods

Materials

cDNAs coding Gβ1, Gγ2, $G\alpha_{oA}$, $G\alpha_{11}$, $G\alpha_{q}$, RGS1, RGS2 and RGS4 were purchased from Guthrie Research Institute. 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). U73122 (active PLC inhibitor) and U73343 (inactive PLC inhibitor) were obtained from Calbiochem (La Jolla, CA), and pertussis toxin (PTX) was from List Biological Laboratories (Campbell, CA). Ginsenosides were kindly provided by Korea Ginseng and Tobacco Research Institute (Taejon, Korea). The stock solutions of ginsenosides and other drugs were prepared and used as described in previous experiment (12).

In Vitro Synthesis of cRNA

Recombinant plasmids containing cDNA inserts for various G protein subunits, various RGSs, 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.

Oocyte Preparation

Xenopus laevis were obtained from Xenopus I (Ann Arbor, MI, USA). Their care and handling were in accordance with the highest standards of institutional guidelines. To isolate oocytes, frogs were operated on under anesthesia with an aerated solution of 3-amino benzoic acid ethyl ester. Oocytes were separated by treatment with collagenase and agitation for 2 hours 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.

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 performed 2 days after. The intraoocyte injections were with glass micropipettes pulled from capillary tubing whose tips were broken to an outer diameter of about 20 μm.

Oocyte recording

Two-electrode voltage-clamp recordings were obtained from single oocytes placed in a small Plexiglas net chamber (0.5 ml), which was continuously superfused with the bathing medium (i.e., ND96). Oocytes were impaled with two microelectrodes filled with 3 M KCl (0.2-0.7MΩ) and voltage-clamped at −80 mV. After stabilization of oocytes with ND96, oocytes were then changed 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^+ channel at negative holding potentials. Recordings of IRK currents and some of GIRK currents were performed in solution (composition in mM; KCl 90, $MgCl_2$ 3, HEPES 5, pH 7.4) as described previously (27). The electrophysiological experiments were done at room temperature with Oocyte Clamp (OC-725C, Warner Instrument, CT). Linear leak and capacitance currents were corrected with leak subtraction procedure.

Data analysis

All numerical values are presented as mean ± SEM. The differences between control and experimental data were analyzed using unpaired *t* test. $P<0.05$ was considered significant.

Results

Effect of Ginsenosides on the Activity of GIRK 1, 4 or 1/4 Channel Expressed in the Xenopus oocytes

In oocytes injected with vehicle (i.e. distilled water), superfusion of a high-K^+ (HK) solution

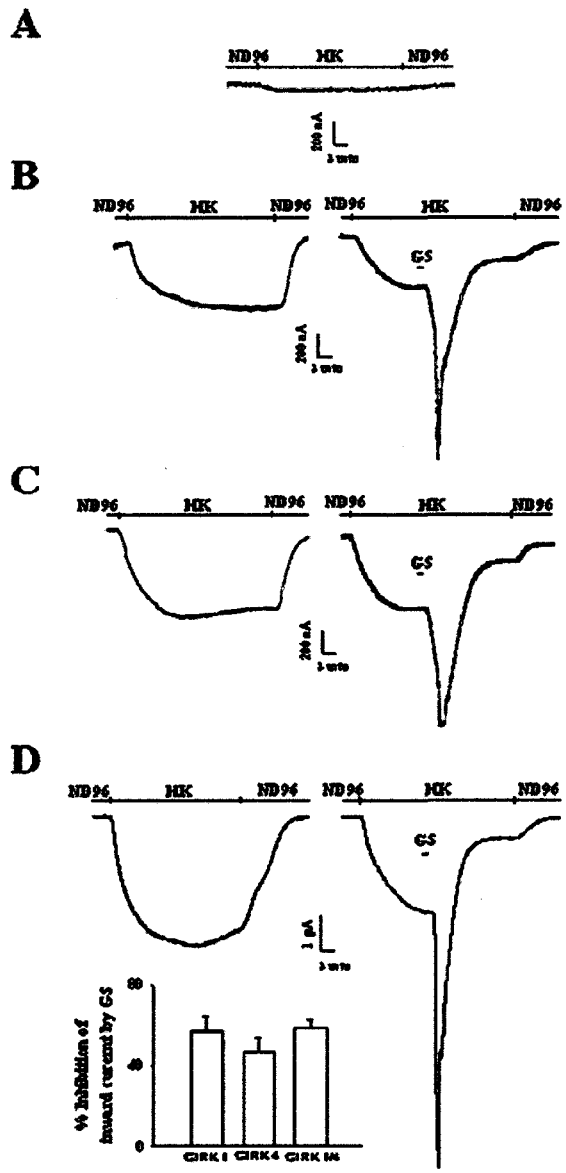

Fig. 1. Effect of Ginsenosides (GS) on GIRK1, GIRK4, or GIRK1/4 current. *A-D*, Representative current traces from individual oocytes (voltage-clamped at −80 mV for *A* and *D* and at −100 mV for *B* and *C*) that were injected with vehicle (*i.e.* distilled water) (*A*), 4 ng of GIRK 1 (*B*), GIRK4 (*C*) or GIRK 1/4 channel cRNA(s) (*D*). Application of high K^+ (HK) solution evoked slight (*A*), moderate (*B* & *C*), and large inward currents (*D*). Application of GS (50 μg/ml for 60 sec in this and following experiments, unless stated otherwise) induced a transient inward current and caused a decline of the HK solution-elicited inward current (*B-D*). *Inset*; graphic representation of the % inhibition of HK solution-elicited inward currents by GS in oocytes injected with GIRK1, GIRK4 and GIRK1/4 cRNAs. Each data point in the graph is the mean (± S.E.M) of 10-13 independent experiments performed using different batches of oocytes.

(96 mM KCl) elicited only a slight inward current (Fig. 1A). In contrast, it elicited significant inward current in oocytes injected with GIRK 1 channel cRNA, GIRK 4 channel cRNA or both (Fig. 1B-D). The HK solution-elicited inward current was much larger in oocytes injected with both GIRK 1 and 4 channel cRNAs than in those injected with either of the cRNAs. These results are very consistent with previous reports that heterologous expression of GIRK 1/4 channel was needed to give rise to functionally active GIRK channel (28-30). Ginsenoside (50 μg/ml) application during the superfusion of HK solution induced a large transient inward current (which was sensitive to niflumic acid, a Cl^- channel blocker; data not shown) (Fig. 1B-D). In addition, ginsenoside application resulted in a significant decrease in HK solution-elicited inward current (Fig. 1B-D). The degree of the ginsenoside inhibition of the inward current was not significantly different among oocytes injected with GIRK 1, GIRK 4 and GIRK 1/4 channel cRNA(s) (57 ± 7.1, 47 ± 6.8 and 58 ± 4.3% inhibition, respectively; n=38 each, $p<0.01$, Fig. 1D *inset*). In the following experiments, we used only those oocytes co-injected with GIRK 1 and 4 channel cRNAs, since the signals (i.e., HK solution-elicited inward currents) were large, and GIRK1/4 channel is close to native GIRK channel in terms of ensemble membrane currents and single-channel properties as well as receptor and G-protein regulation (28-30). HK solution-elicited inward current disappeared almost completely when Ba^{2+} (300 μM) was applied (Fig. 2A). Ginsenosides had no effect on the residual inward current that was insensitive to Ba^{2+} (Fig. 2A, *inset*), indicating that ginsenosides inhibit Ba^{2+}-sensitive inward currents. As a positive control, we also tested the effect of acetylcholine (ACh) in oocytes injected with cRNAs coding GIRK 1/4 channel and m1 muscarinic ACh receptor, which is known to utilize $G\alpha_q$-PLC pathway (26). ACh (10 μM) induced a large transient inward current as ginsenosides and other receptor agonists (22-25) and inhibited HK solution-elicited inward current (27.3 ± 5.3% inhibition; n=12, $p<0.01$, Fig. 2A *inset*). Figure 2B shows the representative current-voltage relationships of the HK solution-elicited inward currents before and after the application of ginsenosides (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, ginsenosides did not shift the equilibrium potential of these currents. However, ginsenosides inhibited the inward current elicited at potentials more negative than –20 mV. Thus, these results indicated that the current inhibited by ginsenosides was an inward rectifier, presumably GIRK current. The ginsenoside 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. 2C).

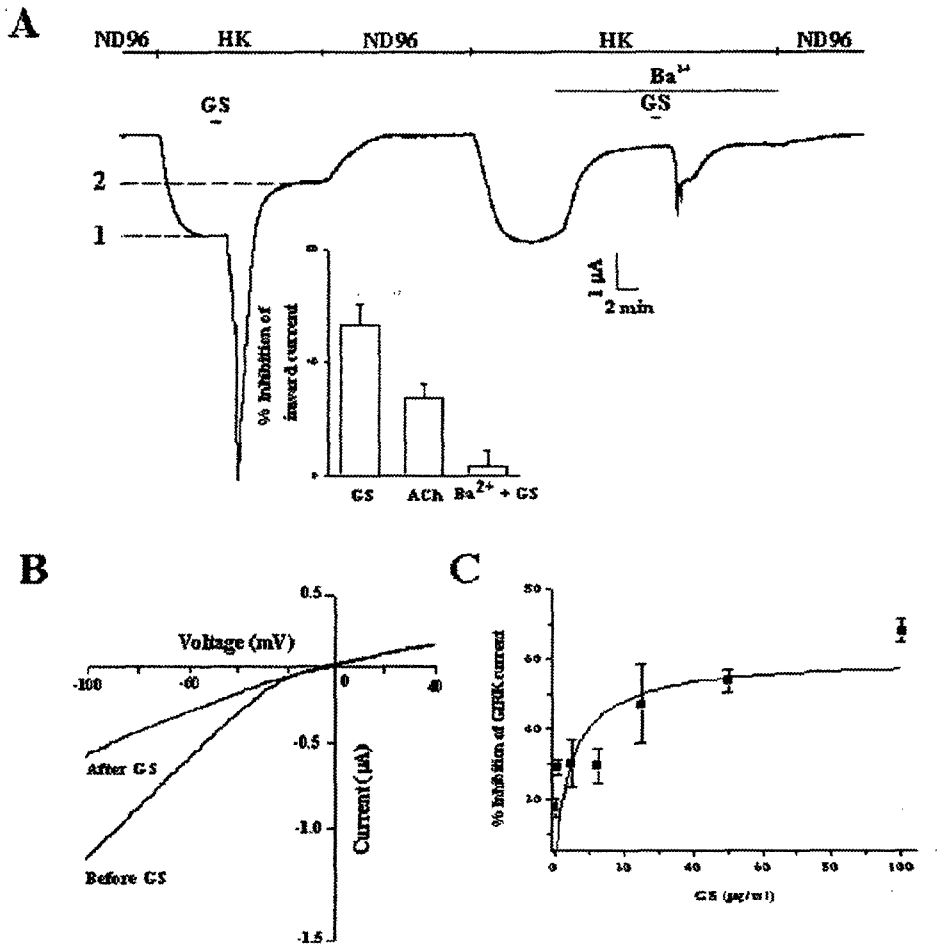

Fig. 2. Ginsenosides (GS) inhibit GIRK1/4 current in a dose-dependent and Ba^{2+}-sensitive manner. *A*. Application of GS induced a transient inward current and caused a decline of the HK solution-elicited inward current (from dashed line 1 to 2). Application of Ba^{2+} (300 μM) almost completely blocked the HK solution-elicited inward current, and GS had no effect on residual inward current. *Inset*; graphic representation of the inhibition of HK solution-induced inward current by GS, ACh and GS in the presence of Ba^{2+} (10 μM for 60 sec; 5 ng/oocyte injection of m1 muscarinic acetylcholine receptor cRNA in ACh experiments). *B*. Current-voltage relationships in oocytes injected with GIRK 1/4 channel cRNAs before and after GS treatment. These were obtained using a voltage ramp protocol (−100 to + 40 mV). *C*. Dose dependence of the inhibitory GS effect on HK 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.

Effects of PTX and Gα Subunit Expression on the Ginsenoside Inhibition of GIRK Current

We examined whether the ginsenoside effect on GIRK current was mediated by PTX-insensi-

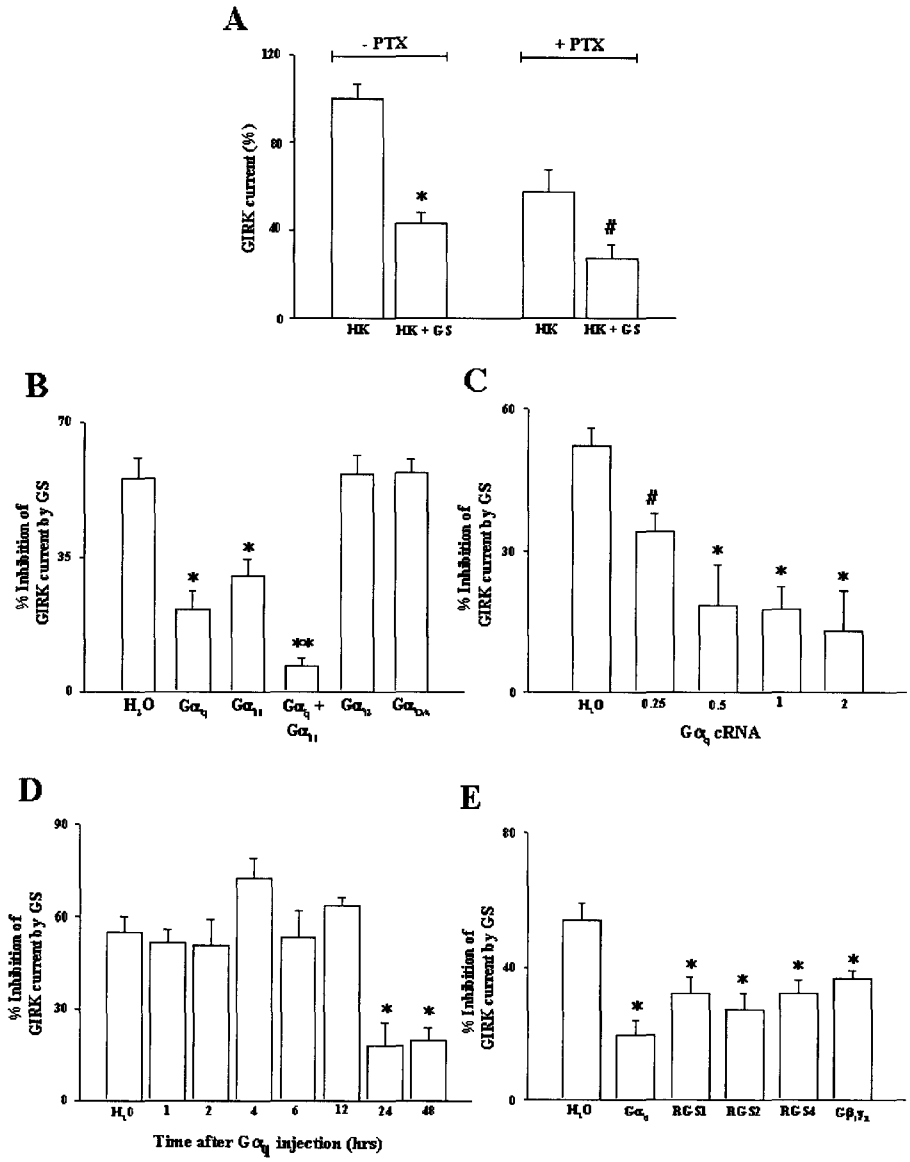

Fig. 3. Effects of PTX and of expressing various G protein subunits and RGS isoforms on the ginsenoside (GS) inhibition of GIRK current. *A.* PTX (2 μg/ml) was pretreated for 16-24h in some experiments (+ PTX). *B.* Before testing the GS effect, oocytes were injected with vehicle (i.e., distilled water) 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 RGS1 (4 ng), RGS2 (4 ng), RGS4 (4 ng) or $G\beta_1\gamma_2$ (10 ng) (n=10 each). The data denoted with *'s and #'s were significantly different from the others; *: $p < 0.01$, **: $p < 0.001$, #: $p < 0.05$.

tive G proteins, since ginsenosides utilize PTX-insensitive $G\alpha_{q/11}$ signaling pathway for the enhancement of Ca^{2+}-activated Cl^- current in the *Xenopus* oocyte (12). PTX pretreatment (2 μg/ml, for 16-24 h) reduced HK solution-elicited GIRK current by 49% ($p<0.01$; compared with PTX-untreated control) (Fig. 3A). However, it did not alter the inhibitory ginsenoside effect on GIRK current (HK : HK+GS = 57.48 ± 10.1 : 27.4 ± 6.4% inhibition in PTX-pretreated oocytes; $p<0.05$) (Fig. 3A), indicating that the ginsenoside effect on GIRK current is PTX-insensitive. To further assess the type of G protein subunits involved in the ginsenosides effect on 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 basal GIRK current (% inhibition: $G\alpha_q$: 55 ± 4, $G\alpha_{11}$: 63v9, $G\alpha_{i2}$: 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 GIRK current (% GS inhibition of 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). Interestingly, co-expression of $G\alpha_q$ and $G\alpha_{11}$ subunits produced a synergistic effect against the ginsenoside inhibition of GIRK current (% GS inhibition of GIRK current: H_2O : 55.6 ± 5.2, $G\alpha_q$+$G\alpha_{11}$: 6.7 ± 2.1**, $p<0.001$, compared with the H_2O-injected control; Fig. 3B).

The degree of the blockade of the ginsenoside effect by $G\alpha_q$ and $G\alpha_{11}$ cRNA injection was proportional to the amounts 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 hrs (Fig. 3D). Taken together, the results from experiments conducted with PTX and cRNAs coding Gα subunits suggested that the inhibitory ginsenoside effect on GIRK current was mediated by PTX-insensitive $G\alpha_{q/11}$ family protein.

Effects of RGS1, RGS2 and RGS4 expression on the Ginsenoside Inhibition of GIRK Current

These RGS isoforms are intrinsic GTPase activating proteins that limit the life time of active GTP-Gα complex (31); RGS1 binds with Gαi family with high affinity (32, 33). On the other hand, RGS2 binds mainly with Gαq family (34, 35), and RGS4 with Gαi and/or Gαq family (36-38). The RGS isoforms also act as effector antagonists inhibiting the interaction between Gα-GTP complex and effector proteins like MAP kinase or PLC (35-37). In this study, we examined whether or not RGS1, RGS2 and RGS4 expressed in oocytes by prior injection of corresponding cRNAs altered the ginsenoside effect on GIRK current elicited in these cells. As illustrated in

Figure 3E, the expression of RGS significantly blocked the ginsenoside effect, and the degree of this blockade was not significantly different among different RGS isoforms (% GS inhibition of GIRK current: H_2O : 54.1 ± 4.7, RGS1 : 32.3 ± 4.1*, RGS2 : 28.1 ± 5.4*, RGS4 : 31.9 ± 4.4* % inhibition; $p<0.05$, n=15-20 each, from three different batches of oocytes).

Effect of Gβγ Expression on the Ginsenoside Inhibition of GIRK Current

Previous studies have shown that Gβγ subunits enhance GIRK current by increasing the chance for these subunits to bind to the cytoplasmic domains of GIRK channel (21, 30, 39). In this study, we assessed whether Gβγ subunit expression affected the ginsenoside effect on GIRK current. Injection of cRNAs coding $G\beta_1\gamma_2$ subunits attenuated the inhibitory effect of ginsenosides on 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 injection of cRNA coding $G\alpha_q$ or $G\alpha_{11}$ subunit (see above). These results combined with the ones presented above suggested that $G\beta_1\gamma_2$ subunits were somehow involved in the ginsenoside action, but they did not mediate the ginsenoside effect.

Effect of PLC Inhibitor on Ginsenosides Inhibition of GIRK Current

To gain insight further into the downstream signaling pathway involved in the ginsenoside effect on GIRK current, we determined whether PLC inhibitor had any influence on the ginsenoside effect. As shown in Figure 4A, the PLC inhibitor U73122 blocked almost completely the ginsenoside effect, whereas the inactive PLC inhibitor U73343 had no effect. Thus, these results indicated that PLC mediated the inhibitory ginsenoside effect on GIRK current.

Effects of Calcium Chelator and Protein Kinase C Inhibitor on Ginsenosides Inhibition of GIRK current

To see if the ginsenoside inhibition of GIRK current involved IP_3-mediated Ca^{2+} release from intracellular stores, we tested the effect of BAPTA, a calcium chelator. Intracellular injection of BAPTA (final intracellular concentration, 1 mM) was ineffective in preventing ginsenosides from inhibiting GIRK current (H_2O+GS : BAPTA+GS = 50.2 ± 4.3% : 41.7 ± 5.8%; $p>0.05$). This result indicated that intracellular Ca^{2+} release via IP_3 pathway was not involved in the ginsenoside inhibition of GIRK current. Therefore, we assessed the role of PKC, which is activated by 1,2-diacylglycerol (DAG) produced along with IP_3 following PLC activation. First, we examined

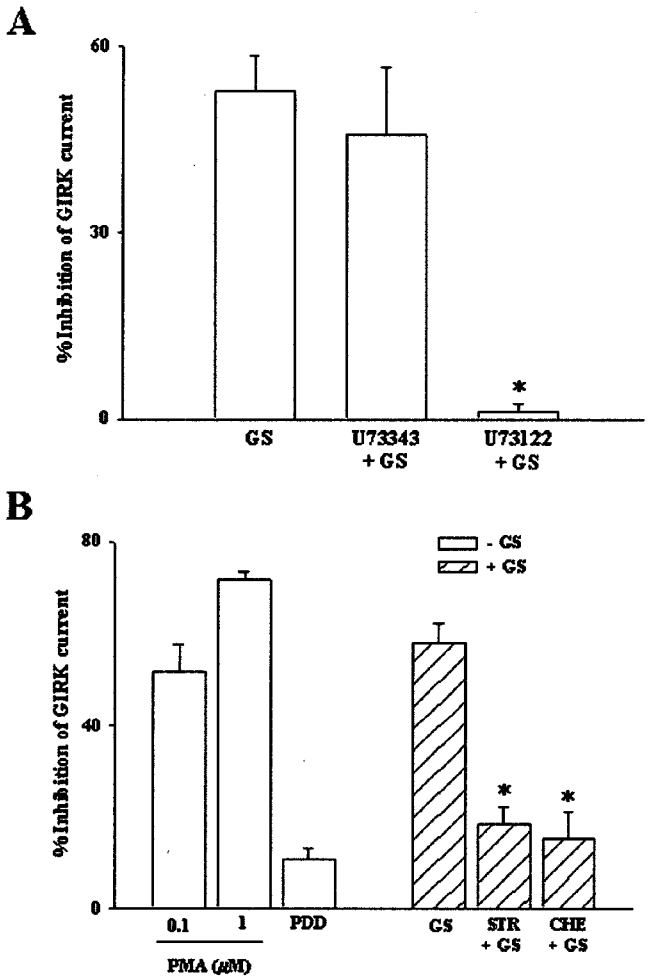

Fig. 4. Effects of PLC and PKC inhibitors on the ginsenoside (GS) inhibition of GIRK current. *A.* Prior to GS application, oocytes were left untreated or incubated for 5 minutes in U73122 (3 μM; PLC inhibitor) or U73343 (3 μM; inactive analog). *B, left panel.* Oocytes were treated for 5 minutes with PMA (0.1 or 1 μM; PKC activator) or PDD (1 μM; inactive PKC activator). *B, right panel.* Oocytes were incubated for 5 minutes in the PKC inhibitors staurosporine (STR) and chelerythrine (CHE) before the application of GS. The data denoted with *'s significantly different from the others ($p < 0.01$).

the effects of PMA (a potent PKC activator) and PDD (inactive phorbol ester) on GIRK current. As shown in Figure 4B, treatment of PMA, but not PDD, reduced GIRK current by 50-70% at concentrations of 0.1 and 1 μM (n=15, $p<0.01$; compared with PMA-untreated oocytes). These results are consistent with previous reports that PKC activators inhibit GIRK current in the *Xenopus* oocyte (22, 24-26). Next, we examined whether PKC inhibitors such as staurosporine and

chelerythrine reduced the ginsenoside effect on GIRK current. Preincubation of oocyte in staurosporine or chelerythrine-containing solution for 5 min reduced the inhibitory ginsenoside effect (GS: STR+GS : CHE+GS = 58.2 ± 4.2 : 18.6 ± 3.9 : 26.3 ± 6.0% inhibition; $p<0.01$; compared with oocytes treated with ginsenosides alone) (Fig. 4B). These results suggested that PKC mediated the ginsenoside inhibition of GIRK current.

Effect of Anionic Channel Blocker on the Ginsenoside Inhibition of GIRK current

We further determined whether or not the ginsenoside inhibition of GIRK current was related to ginsenosides enhancement of Ca^{2+}-activated Cl^- current. For this, we tested the effect of the chloride channel blocker niflumic acid on the ginsenoside inhibition of GIRK current, since our earlier study showed that niflumic acid blocked the ginsenoside effect on the Cl^- current (12, 13). At 300-μM concentration, niflumic acid failed to alter the inhibitory ginsenoside effect on GIRK current (GS : NFA+GS=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 inhibition of GIRK current was unrelated to the enhancement of Ca^{2+}-activated Cl^- current by ginsenosides.

Ginsenosides do not Inhibit the Activity of Mouse Brain Inwardly Rectifying K^+ 2 (MB-IRK2) Channel

To see if ginsenosides affect inwardly rectifying potassium (IRK) current other than GIRK current, we expressed MB-IRK2 channel in the *Xenopus* oocyte and examined the effect of ginsenosides on the current flowing through this channel. The reason we chose this channel was that it has considerable homology with GIRK 1/4 channels but is not coupled to $G\alpha_i/G\alpha_o$ proteins (40, 41). In oocytes injected with GIRK 1/4 channel cRNAs, ginsenosides inhibited GIRK current and caused a transient inward Cl^- current to flow (Fig. 5A and 5B). On the other hand, in MB-IRK2 channel cRNA-injected cells, ginsenosides failed to inhibit IRK current although it induced a transient Cl^- current (Fig. 5D). These results suggested that the target of ginsenosides signaling mediated via $G\alpha_{q/11}$-PKC pathway was GIRK, not IRK, channel.

Discussion

Previous studies have shown that a variety of agents inhibit GIRK channel activity through the

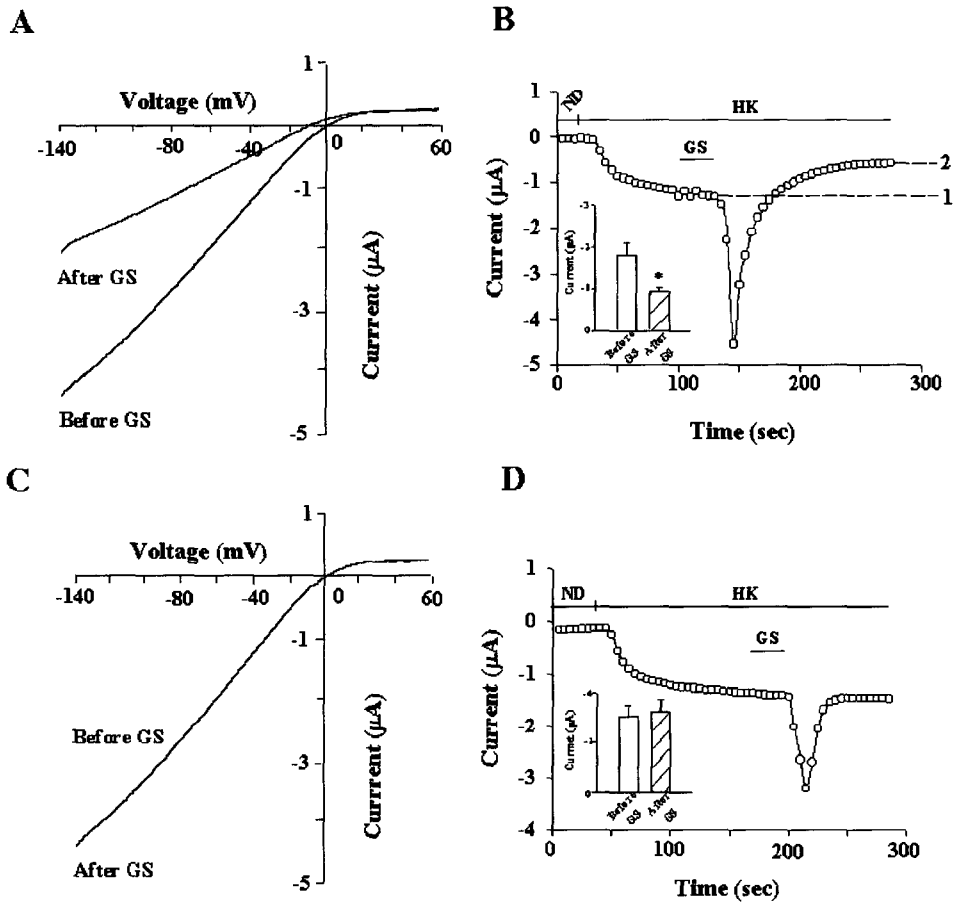

Fig. 5. Effects of ginsenoside (GS) on the activity of mouse brain inwardly rectifying K^+ 2 (MB-IRK2) channel. *A & C.* Current-voltage relationships in oocytes injected with GIRK1/4 (3 ng/oocyte) or IRK channel cRNAs (40 ng/oocyte). These were obtained using a voltage ramp protocol (−140 to + 60 mV) before and after application of GS. *B & 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 currents in both oocytes injected with GIRK 1/4 and IRK channel cRNAs (n=18 each). In addition, it reduced the HK solution-elicited inward current in oocytes injected with GIRK 1/4 channel cRNAs (*B*). *Insets*; histograms 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 asterisks-denoted data were significantly different from the others ($p<0.01$).

activation of membrane receptor coupled to the PTX-insensitive $G\alpha_q$-PLC signal transduction pathway (22-26). Recently, we have provided evidence that ginsenosides enhance Ca^{2+}-activated Cl^- current by activating PTX-insensitive $G\alpha_{q/11}$ coupled to PLC-β3 in the *Xenopus* oocyte (12, 13). In the present study, we sought to determine whether or not ginsenosides could alter the GIRK channel activity, and if so, the underlying signal transduction mechanism. We found that

ginsenosides inhibited the activity of GIRK 1/4 channel expressed in the *Xenopus oocyte* and this inhibition might be through the PTX-insensitive $G\alpha_{q/11}$-PLC-PKC signaling cascade.

The evidence supporting the notion that the α_q family of G proteins mediated the ginsenoside effect on GIRK current is two-fold. Firstly, PTX treatment had no significant effect on the ginsenoside inhibition of GIRK current; it is well established that the α_q family of G proteins is PTX-insensitive (31). 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 GIRK current. This attenuation was probably due to the persistent activation of the effectors like PLC by $G\alpha_q$ or $G\alpha_{11}$ expressed after the cRNA injection, which led to their desensitization.

The results from experiments involving intraoocyte injection of cRNAs coding different RGS isoforms do not provide support for the idea that α_q family of G proteins mediate the ginsenoside effect, because the degree of reduction of ginsenoside effect was not much different among oocytes injected with different RGS cRNAs. We had thought that the expression of RGS2, which, among various identified RGS isoforms, interacts more selectively with $G\alpha_{q/11}$ (34), would be most effective in blocking the ginsenoside inhibition of GIRK current. The lack of difference of the three different RGS isoforms in blocking ginsenoside effect might be due to non-selective interaction of RGS1 and RGS4 with αi and αq families of G proteins (37, 42).

The present results suggest that Gβγ subunits can modulate the ginsenoside effect on GIRK channel, but they do not support the idea that these G protein subunits mediate the ginsenoside effect. Then, why $G\beta_1\gamma_2$ subunit expression attenuated the ginsenoside effect on GIRK current as the expression of $G\alpha_q$ or $G\alpha_{11}$ subunit? This might be because of that 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 $G\alpha_q$ signal which mediated the inhibitory acetylcholine effect on basal and dopamine 2 receptor-activated GIRK 1/4 currents in the *Xenopus oocyte* (26).

The results obtained with active and inactive PLC inhibitors indicate that PLC mediates the ginsenoside effect on GIRK current. The activation of PLC produces both water-soluble IP_3 and lipid-soluble DAG. 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 Cl^- current (12). In the present study, we assessed whether IP_3-mediated Ca^{2+} release was also responsible for the ginsenoside effect on GIRK current. The results from experiments performed with the calcium chelator BAPTA indicate that this was not the case. So, we investigated the

other possibility. That is, we assessed whether DAG mediated the ginsenoside inhibition of GIRK current, by examining the effects of one PKC activator and two PKC inhibitors. Previous studies have shown that PKC activator inhibited both basal GIRK current and receptor agonist-induced GIRK current, suggesting that PKC is involved in GIRK channel regulation (22-26). In the present study, we observed that PMA, a potent PKC activator, reduced GIRK current, and the PKC inhibitors staurosporine and chelerythrine attenuated the inhibitory ginsenoside effect on GIRK current. On the basis of these results, we conclude that the ginsenoside effect on GIRK current is mediated through the DAG-PKC pathway.

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

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

In the present study, using a *Xenopus oocyte* model that allows various foreign gene expression, we have provided evidence that ginsenoside inhibits GIRK current through the $G\alpha_{q/11}$-PLC-PKC pathway. At the moment, we do not know that the effect of ginsenoside seen with *Xenopus* cells is related to the ginsenoside effects observed in mammals (e.g., antinociceptive effect) (43, 44). However, the fact that the *in vivo* and *in vitro* effects of ginsenosides are elicited by comparable concentrations of ginsenosides supports the notion above.

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Abbreviations

[b]The abbreviations used are: GIRK 1/4 channel, G protein coupled inwardly rectifying K^+ 1 and

4 channel; G protein, guanine nucleotide-binding protein; IP_3, inositol (1,4,5) trisphosphate; IRK channel, inwardly rectifying K^+ channel; PLC, phospholipase C; PTX, pertussis toxin; RGS, regulator of G protein signaling

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