

# Ginseng Gintonin Activates the Human Cardiac Delayed Rectifier K<sup>+</sup> Channel: Involvement of Ca<sup>2+</sup>/Calmodulin Binding Sites

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Gintonin, a novel, ginseng-derived G protein-coupled lysophosphatidic acid (LPA) receptor ligand, elicits [Ca<sup>2+</sup>]<sub>i</sub> transients in neuronal and non-neuronal cells via pertussis toxin-sensitive and pertussis toxin-insensitive G proteins. The slowly activating delayed rectifier K<sup>+</sup> (*I*<sub>KS</sub>) channel is a cardiac K<sup>+</sup> channel composed of KCNQ1 and KCNE1 subunits. The C terminus of the KCNQ1 channel protein has two calmodulin-binding sites that are involved in regulating *I*<sub>KS</sub> channels. In this study, we investigated the molecular mechanisms of gintonin-mediated activation of human *I*<sub>KS</sub> channel activity by expressing human *I*<sub>KS</sub> channels in *Xenopus* oocytes. We found that gintonin enhances *I*<sub>KS</sub> channel currents in concentration- and voltage-dependent manners. The EC<sub>50</sub> for the *I*<sub>KS</sub> channel was 0.05 ± 0.01 μg/ml. Gintonin-mediated activation of the *I*<sub>KS</sub> channels was blocked by an LPA1/3 receptor antagonist, an active phospholipase C inhibitor, an IP<sub>3</sub> receptor antagonist, and the calcium chelator BAPTA. Gintonin-mediated activation of both the *I*<sub>KS</sub> channel was also blocked by the calmodulin (CaM) blocker calmidazolium. Mutations in the KCNQ1 [Ca<sup>2+</sup>]/CaM-binding IQ motif sites (S373P, W392R, or R539W) blocked the action of gintonin on *I*<sub>KS</sub> channel. However, gintonin had no effect on hERG K<sup>+</sup> channel activity.

These results show that gintonin-mediated enhancement of *I*<sub>KS</sub> channel currents is achieved through binding of the [Ca<sup>2+</sup>]/CaM complex to the C terminus of KCNQ1 subunit.

## INTRODUCTION

The KCNQ family of channel proteins (also known as Kv7) form K<sup>+</sup>-selective, voltage-gated channels (Hille, 2001) that are slowly activating delayed rectifier K<sup>+</sup> (*I*<sub>KS</sub>) channels. Four members of the KCNQ family are neuronal (KCNQ2-5), and one (KCNQ1) is expressed in cardiac tissue. KCNQ1 as well as the hERG K<sup>+</sup> (*I*<sub>Kr</sub>) channel is responsible for repolarization in the heart, and both are involved in cardiac diseases such as arrhythmia (Sanguinetti et al., 1996). Channels formed from KCNQ proteins consist of homomeric tetramers or heteromeric tetramers containing KCNQ as the α-subunit, and each KCNQ subunit is composed of six α-helical transmembrane segments (S1-S6). KCNQ channel proteins also co-assemble with KCNE1-4 subunits (McCrossan and Abbott, 2004). It has been shown that cells expressing KCNQ1 in the absence of KCNE1 exhibit outward K<sup>+</sup> currents with unique activation kinetics (Salata et al., 1998). In addition, the C terminus of KCNQ1 contains binding sites for the intracellular Ca<sup>2+</sup>/calmodulin (CaM) complex, which regulates KCNQ1 channel activity (Ghosh et al., 2006; Tohse, 1990). It has been reported that LQT syndrome in humans is caused by defective interaction of the Ca<sup>2+</sup>/CaM complex with the KCNQ1 C terminus (Ghosh et al., 2006; Shamgar et al., 2006). Thus, the Ca<sup>2+</sup>/CaM interaction sites in KCNQ1 are important for normal cardiac activity.

*Panax ginseng* root has been commonly used for centuries as a tonic that has pharmacological effects on multiple organs (Attelet et al., 1999). For example, ginseng extract has been shown to protect against cardiac ischemia-reperfusion injury (Furukawa et al., 2006) and to shorten action potential duration by enhancing the *I*<sub>KS</sub> current (Bai et al., 2003). Bai et al. (2003) also showed that ginsenoside Re, a ginseng saponin, might protect against cardiac ischemia-reperfusion injury; however, the molecular mechanisms for this activity at the level of the cell membrane were not well explained. Recent reports have shown that ginseng also contains a ligand for the G protein-coupled lysophosphatidic acid (LPA) receptor called gintonin (Hwang et

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al., 2012; Pyo et al., 2011). Gintonin exerts its effects through induction of  $[Ca^{2+}]_i$  transients, resulting in the regulation of Kv1.2 channel activity (Lee et al., 2013). In the present study, we used the *Xenopus* oocyte gene expression system to investigate the molecular mechanisms underlying how gintonin-mediated  $[Ca^{2+}]_i$  transients are coupled to the regulation of  $I_{Ks}$  channel activity. Next, we also examined gintonin effect on  $I_{Ks}$  in guinea pig cardiac myocytes to know whether gintonin could directly regulate the intrinsic  $I_{Ks}$  in mammalian cardiac myocytes. The results show that gintonin enhances  $I_{Ks}$  channel currents through the interaction of the  $Ca^{2+}/CaM$  complex with KCNQ1 IQ motifs and that gintonin also increases  $I_{Ks}$  currents in guinea pig cardiac myocytes. We further discuss the pharmacological roles and applications of gintonin in the regulation of KCNQ1 channel activity in the heart.

## MATERIALS AND METHODS

### Materials

Gintonin was prepared from *Panaxginseng* according to the method described in Pyo et al. (2011). Prior to use, gintonin was dissolved in dimethyl sulfoxide (DMSO), the final concentration of which was less than 0.01%. This stock was then added to the bath medium buffer. The cDNAs for the human KCNQ1 and KCNE1 channels (GenBank ID: NM\_000218) were kindly provided by Dr. Pongs (University of Hamburg, Germany). All other agents were purchased from Sigma-Aldrich (USA).

### Preparation of *Xenopus* oocytes and microinjection

*Xenopus laevis* frogs were purchased from Xenopus I (USA). Their care and handling were in accordance with the highest standards of the institutional guidelines of Konkuk University. For isolation of oocytes, frogs were anesthetized with an aerated solution of 3-amino benzoic acid ethyl ester, after which the ovarian follicles were removed. The oocytes were treated with collagenase and then agitated for 2 h in  $Ca^{2+}$ -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  $\mu$ g/ml streptomycin. Stage V-VI oocytes were collected and stored in ND96 medium (96 mM NaCl, 2 mM KCl, 1 mM  $MgCl_2$ , 1.8 mM  $CaCl_2$ , and 5 mM 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 every day. Electrophysiological experiments were performed within 5-6 days of oocyte isolation, with gintonin applied to the bath. For  $K^+$  channel experiments, cRNAs encoding KCNQ1 + KCNE1 (40 nl) were injected into the animal or vegetal pole of each oocyte one day after isolation, using a 10  $\mu$ l microdispenser (VWR Scientific, USA) fitted with a tapered glass pipette tip (15-20  $\mu$ m in diameter) (Lee et al., 2005).

### Site-directed mutagenesis of human KCNQ1

Single amino acid substitutions were made using the QuikChange™ XL Site-Directed Mutagenesis Kit (Stratagene, USA), along with Pfu DNA polymerase and sense and antisense primers encoding the desired mutations. Overlap extension of the target domain by sequential polymerase chain reaction (PCR) was carried out according to the manufacturer's protocol. The final PCR products were transformed into *E. coli* DH5 $\alpha$ , screened by PCR, and confirmed by sequencing of the target regions. The mutant DNA constructs were linearized at their 3' ends by digestion with *Xho*I, and run-off transcripts were prepared using the methylated cap analog m<sup>7</sup>G(5')ppp(5')G. The cRNAs were prepared using the mMessageMachine *in vitro* transcription kit (Ambion, USA) with T7 RNA polymerase. The absence of

degraded RNA was confirmed by denaturing agarose gel electrophoresis followed by ethidium bromide staining. Similarly, recombinant plasmids containing human KCNQ cDNA inserts were linearized by digestion with the appropriate restriction enzymes, and cRNAs were obtained using the mMessageMachine transcription kit with SP6 RNA polymerase or T7 polymerase. The final cRNA products were resuspended at a concentration of 1  $\mu$ g/ $\mu$ l in RNase-free water and stored at -80°C (Lee et al., 2008).

### Guinea pig ventricular myocyte isolation

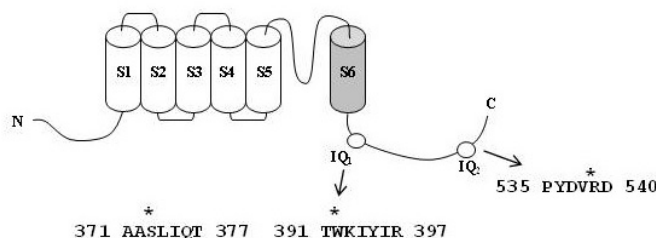
Ventricular cells were isolated from hearts of guinea pigs (body weights of 250-300 g) using the enzymatic dissociation technique (Fujisawa et al., 2000). All experimental procedures were conducted in accordance with the guidelines of the Sungkyunkwan University School of Medicine Institutional Animal Care and Use Committee (Approval No. IACUC-11-39). Briefly, guinea pigs were injected with heparin (1.0 units/kg) and euthanized by stunning-induced coma with loss of all reflex-responses, followed by cardiac excision. The heart was cannulated by a 18-gauge needle and then retrogradely perfused via the aorta on a Langendorff apparatus. During coronary perfusion all perfusates were maintained at 37°C and equilibrated with 100% O<sub>2</sub>. Initially the heart was perfused with normal Tyrode solution for 2-3 min to clear the blood. The heart was then perfused with  $Ca^{2+}$ -free solution for 2 min. Finally the heart was perfused with enzyme solution for 14-16 min. Enzyme solution contains 1 mg/ml collagenase (Worthington Type 2) and 0.1mg/ml protease (Sigma) in  $Ca^{2+}$ -free solution. After perfusion with enzyme solution, the ventricles were separated with the atria and chopped into small pieces. Single cells were dissociated in high  $K^+$ , low  $Cl^-$  solution from these small pieces using blunt-tip glass pipette and stored in the same solution at 4°C until use.

### Voltage-clamp recording

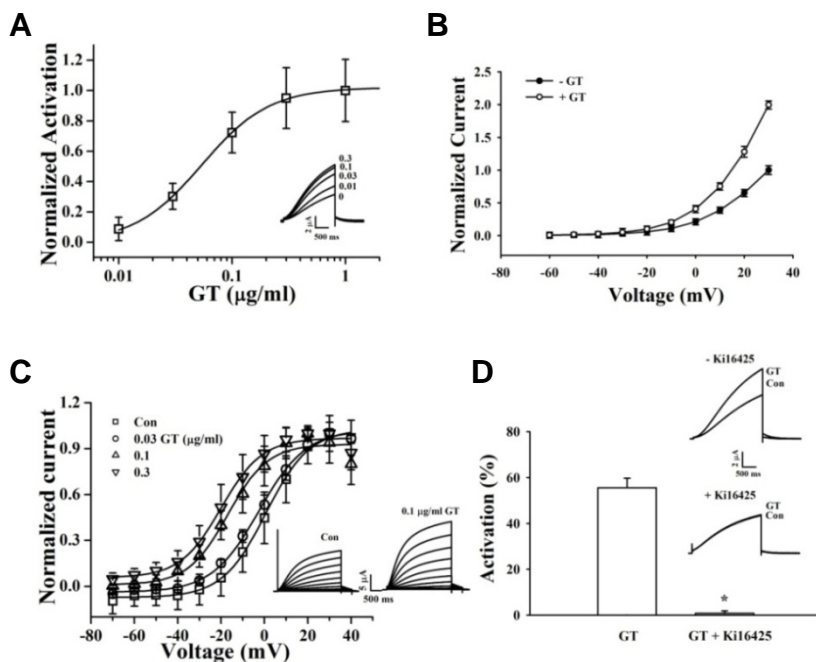
Membrane currents were recorded from single isolated myocytes in a perforated patch configuration by using nystatin (200  $\mu$ g/ml; ICN) at 35  $\pm$  1°C. Voltage clamp was performed by using an EPS-8 amplifier (HEKA Instruments) and filtered at 5 kHz. The patch pipettes (World Precision Instruments) were made by a Narishige puller (PP-830; Narishige, Japan). The patch pipettes used had a resistance of 2-3 mega ohms when filled with the below pipette solutions. The bath solution (or normal Tyrode solution) contained (mM): NaCl 140, KCl 5.4,  $MgCl_2$  0.5,  $CaCl_2$  1.8, glucose 10, HEPES 5, titrated to pH 7.4 with NaOH.  $Ca^{2+}$  free solution contained (mM): NaCl 140, KCl 5.4,  $MgCl_2$  0.5, glucose 10, HEPES 5, titrated to pH 7.4 with NaOH. The high- $K^+$  and low- $Cl^-$  solution contained (mM): KOH 70, KCl 40, L-glutamic acid 50, taurine 20,  $KH_2PO_4$  20,  $MgCl_2$  3, glucose 10, HEPES 10, EGTA 0.5. The pipette solution for perforated patches contained (mM): KCl 140, HEPES 10,  $MgCl_2$  1, EGTA 5, titrated to pH 7.2 with KOH.

### Two-electrode voltage clamp recording

A custom-made Plexiglas net chamber was used for two-electrode voltage-clamp recordings as previously reported (Lee et al., 2005). The oocytes were impaled with two 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, USA). Stimulation and data acquisition were controlled with a pClamp 8 (Axon Instruments, USA). For most electrophysiological experiments, oocytes were perfused initially with a  $Cl^-$  and  $Ca^{2+}$ -free solution (96 mM NaOH, 2 mM KOH, 8 mM Mg-gluconate, 5 mM HEPES, and 5 mM EGTA, pH 7.4 with methanesulfonic acid) in the pres-



**Fig. 1.** Schematic of the human KCNQ1 subunit topology showing amino acid mutations. (A) A sequence alignment of the KCNQ1 channel protein and the amino acid residues that were mutated CaM-binding sites IQ<sub>1</sub> (373, 392) and IQ<sub>2</sub> (539).



**Fig. 2.** Effects of gintonin on  $I_{K_S}$  channel activity. (A) Gintonin concentration-response curves for  $I_{K_S}$  channels (mean  $\pm$  S.E.M;  $n = 7-10$  oocytes each). *Inset*, representative traces of gintonin-mediated  $I_{K_S}$  (KCNQ1 + KCNE1) channel activation at various gintonin concentrations. The representative peak outward current amplitude at +30 mV from a holding potential of -80 mV was measured in the absence or presence of gintonin. (B) Effects of gintonin (0.1 and 0.3  $\mu\text{g/ml}$  each) on the current-voltage (I-V) relationship of the  $I_{K_S}$  channels (mean  $\pm$  S.E.M;  $n = 10-12$  oocytes each). (C) Voltage-dependence activation curves for the  $I_{K_S}$  channel. *Inset*, Left and right current traces are before and after application of 0.1  $\mu\text{g/ml}$  gintonin to  $I_{K_S}$  channels, respectively. Currents recorded during 3 s depolarizing pulses to membrane potentials of -60 to +50 mV, applied from a holding potential of -80 mV. Tail currents were measured at -70 mV. I-V relationships for normalized  $I_{K_S}$  tail currents. Data were fitted to a Boltzmann function. (D) Attenuation of gintonin-induced  $I_{K_S}$  channel activity after treatment with Ki16425. The

histogram shows blockage of gintonin-mediated  $I_{K_S}$  channel activation by the LPA1/3 receptor antagonist, Ki16425. Application of 0.1 and 1  $\mu\text{g/ml}$  gintonin to  $I_{K_S}$  channels, respectively (mean  $\pm$  S.E.M;  $n = 10-12$  each) ( $*P < 0.001$ , compared to gintonin treatment only). *Inset*, Currents recorded in the absence and presence of 1  $\mu\text{M}$  Ki16425 in oocytes expressing  $I_{K_S}$  channels; currents were recorded with a 3-s voltage step to +30 mV from a holding potential of -80 mV.

ence of a  $\text{Cl}^-$  channel blocker (500  $\mu\text{M}$  anthracene-9-carboxylic acid) to inhibit endogenous  $\text{Cl}^-$  channels (Choi et al., 2011). The oocytes were then clamped at a holding potential of -80 mV, the membrane potential was depolarized to +30 mV for 2.5 s at 10-s intervals, and currents were recorded.

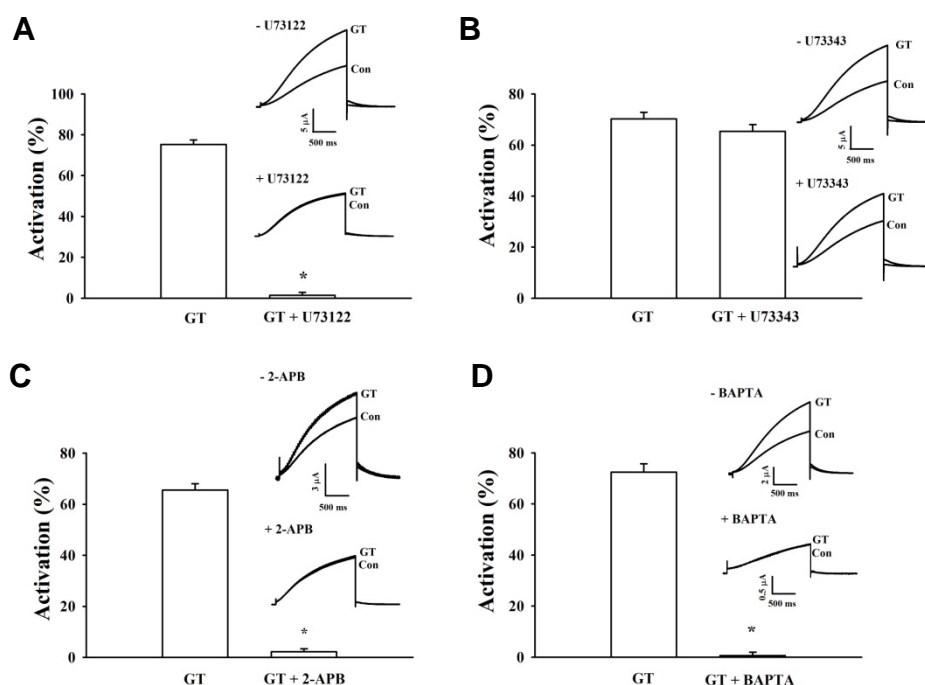
#### Data analysis

To obtain the concentration-response curves showing the effect of gintonin on  $I_{K_S}$  currents, the peak amplitudes at different concentrations of gintonin were plotted, and Origin software (Origin, USA) was used to fit the plot to the Hill equation  $y / y_{\text{max}} = [A]^{\text{nH}} / ([A]^{\text{nH}} + [\text{IC}_{50}]^{\text{nH}})$ , where  $y$  is the peak current at a given concentration of gintonin,  $y_{\text{max}}$  is the maximal peak current,  $\text{EC}_{50}$  is the concentration of gintonin producing a half-maximal effect,  $[A]$  is the concentration of gintonin, and  $\text{nH}$  is the Hill coefficient. All values are presented as mean  $\pm$  S.E.M. The significance of differences between mean control and treatment values was determined using Student's  $t$ -test.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Gintonin increases $I_{K_S}$ channel currents in a concentration- and voltage-dependent manner and shifted the steady-state activation curves leftward

We examined the effect of gintonin on the activities of  $I_{K_S}$  channels expressed in *Xenopus* oocytes. The channel currents were elicited by a 2.5-s depolarization to a test potential of +30 mV at 10-s intervals from a holding potential of -80 mV. Gintonin activates  $I_{K_S}$  channels in a concentration-dependent and reversible manner. The maximum mean activation values for  $I_{K_S}$  are  $10.89\% \pm 8.71\%$ ,  $32.76\% \pm 9.64\%$ ,  $78.78\% \pm 26.26\%$ ,  $104.3\% \pm 22.49\%$ , and  $112.12\% \pm 24.1\%$  at 0.01, 0.03, 0.1, 0.3, and 1  $\mu\text{g/ml}$ , respectively (Fig. 2A). The  $\text{EC}_{50}$  for gintonin on the  $I_{K_S}$  channel is  $0.05 \pm 0.01$   $\mu\text{g/ml}$  (Fig. 2A). The current-voltage relationship curves (10 mV increments from -60 mV to +30 mV) show that the enhancement of  $I_{K_S}$  channels by gintonin is voltage-dependent (Fig. 2B). To study steady-state activation of these channels, cells were held at -80 mV and then pulsed with voltages ranging from -70 mV to +30 mV. The voltage-activation relationships for the  $I_{K_S}$  channels were determined from tail cur-



**Fig. 3.** Signal transduction pathways of gintonin-mediated  $I_{K_S}$  channel activation. (A, B) Representative recordings of  $I_{K_S}$  (A) channel currents following application of gintonin (GT) for 30 s in the presence of U73122, an active PLC inhibitor. U73343, an inactive PLC inhibitor, in oocytes expressing  $I_{K_S}$  channels. *Inset*, the representative peak outward current amplitude at +30 mV from a holding potential of -80 mV was measured in the presence of gintonin. The active or inactive PLC inhibitor was pretreated for 5 min before gintonin application. (C, D) Time-current relationship after application of gintonin (GT) for 30 s in the presence of 2-APB, an  $IP_3$  receptor antagonist, or BAPTA-AM, a membrane permeable  $Ca^{2+}$  chelator, in oocytes expressing  $I_{K_S}$  channels. *Inset*, the representative peak outward current amplitude at +30 mV from a holding potential of -80 mV was measured in the presence of gintonin. The application of 2-APB or BAPTA preceded the gintonin application by 2 h. Summary histograms show the peak outward  $I_{K_S}$  channel currents (mean  $\pm$  S.E.M.;  $n = 13-14$  oocytes each) recorded in oocytes expressing the  $I_{K_S}$  channel in the absence or presence of the indicated agents (\* $P < 0.001$ , compared to gintonin alone).

rent analysis. Tail currents were normalized to the maximal currents and fitted with a Boltzmann equation. A representative trace is shown in Fig. 2C. The steady-state activation  $V_{1/2}$  for  $I_{K_S}$  is  $0.64 \pm 0.78$  mV in the control, and  $k$  for this relationship is  $10.03 \pm 0.70$  mV (Fig. 2C). The higher the gintonin concentration used, the more the activation curves of the  $I_{K_S}$  channel shift leftward (control:  $V_{1/2} = 0.64 \pm 0.78$  mV,  $n = 6$ ;  $0.03 \mu\text{g/ml}$  gintonin:  $-2.61 \pm 0.82$  mV,  $n = 7$ ;  $0.1 \mu\text{g/ml}$  gintonin:  $-16.90 \pm 2.08$  mV,  $n = 6$ ;  $0.3 \mu\text{g/ml}$  gintonin:  $-19.96 \pm 1.47$  mV,  $n = 10$ ).

#### Ki16425 attenuates gintonin-mediated activation of the $I_{K_S}$ channels

Because *Xenopus* oocytes express endogenous LPA receptors (Kimura et al., 2001), oocytes expressing  $I_{K_S}$  channels were first pretreated with the LPA1/3 antagonist Ki16425 ( $1 \mu\text{M}$ , 30 min) and then challenged with gintonin ( $0.1$  or  $1 \mu\text{g/ml}$ ). As shown in Fig. 2D,  $0.1 \mu\text{g/ml}$  gintonin activates  $I_{K_S}$  channels by 55.5%; however, Ki16425 abolishes gintonin-mediated activation of  $I_{K_S}$  channels ( $n = 6$ ,  $P < 0.001$ ). Thus, gintonin activates these potassium channels via endogenous LPA receptors (Fig. 2D).

#### Activation of the G protein-coupled LPA receptor by gintonin up-regulates $I_{K_S}$ channel currents by activating the PLC- $IP_3$ - $[Ca^{2+}]_i$ pathway

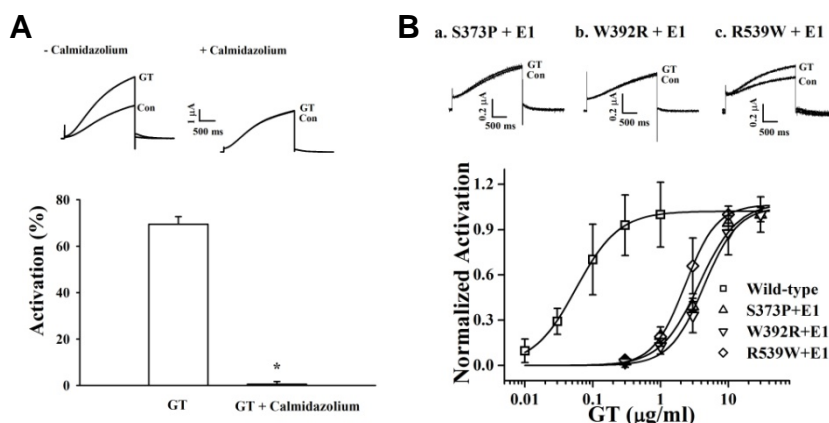
Next, we pretreated cells with U73122, an active inhibitor of phospholipase C. As a result of this treatment, gintonin-mediated

activation of  $I_{K_S}$  channels is completely eliminated. In contrast, when we pretreated cells with U73343, an inactive analog, gintonin-mediated activation is not significantly affected (Figs. 3A and 3B). These data indicate that the  $G_{\alpha_{q11}}$ -PLC pathway is involved in gintonin-mediated activation of  $I_{K_S}$  channels.

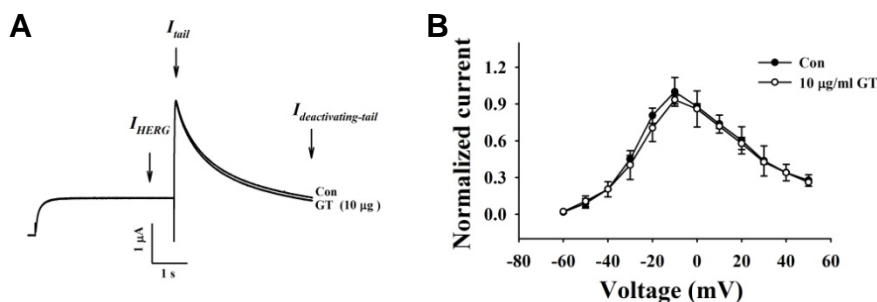
To investigate the role of  $IP_3$  receptors on gintonin action, the effect of the  $IP_3$  receptor inhibitor 2-APB on gintonin-mediated activation of  $I_{K_S}$  channels was examined (Fig. 3C). We found that gintonin-mediated activation of  $I_{K_S}$  channels is blocked completely by 2-APB. These data also demonstrate that gintonin-mediated activation of  $I_{K_S}$  channels requires signaling through the  $IP_3$  receptor. To determine whether  $[Ca^{2+}]_i$  transients from intracellular  $Ca^{2+}$  stores play a role in gintonin-mediated activation of  $I_{K_S}$  channels, we used the calcium chelator BAPTA. As shown in Fig. 3D, we treated oocytes with  $10 \mu\text{M}$  BAPTA-AM, a membrane permeable  $Ca^{2+}$  chelator, to chelate free cytosolic  $Ca^{2+}$  and almost abolishes gintonin action on  $I_{K_S}$  channels. These results indicate that gintonin-mediated  $[Ca^{2+}]_i$  transients are required for activation of  $I_{K_S}$  channels.

#### Involvement of $Ca^{2+}$ /CaM in gintonin-mediated activation of $I_{K_S}$ channels

KCNQ1 has two CaM-binding IQ motifs (Fig. 1; Yus-Najera et al., 2002). CaM regulates the activity of  $I_{K_S}$  channels through interactions with two CaM-binding IQ motifs (Ghosh et al., 2006; Shamgar et al., 2006). To examine the role of CaM in gintoninmedi-



**Fig. 4.** Involvement of CaM in gintonin-mediated  $I_{K_S}$  channel activation. (A) Oocytes expressing  $I_{K_S}$  channels were incubated in the absence or presence of calmidazolium (1.5  $\mu\text{M}$ ) for 10 min. *Insets*, the representative gintonin-mediated peak outward current amplitude at +30 mV from a holding potential of -80 mV was measured in the absence or presence of calmidazolium. Summary histograms show peak outward  $I_{K_S}$  channel currents recorded in the absence or presence of calmidazolium (mean  $\pm$  S.E.M.;  $n = 13$ -14 oocytes each;  $*P < 0.001$ , compared to gintonin alone). (B) Oocytes expressing  $I_{K_S}$  channels mutated at the  $\text{Ca}^{2+}$ /CaM-binding sites (S373P, W392R, or R539W) were treated with gintonin for 60 s. Mutation of  $\text{Ca}^{2+}$ /CaM-binding sites resulted in a rightward shift of the gintonin concentration-response curve (mean  $\pm$  S.E.M.;  $n = 10$ -12 oocytes each). *Insets*, the representative peak outward current amplitude at +30 mV from a holding potential of -80 mV was measured in the presence of gintonin. Gintonin-mediated peak outward  $I_{K_S}$  channel currents recorded in oocytes expressing mutant channels were significantly attenuated (mean  $\pm$  S.E.M.;  $n = 10$ -12 oocytes each;  $*P < 0.001$ , compared to the wild type).



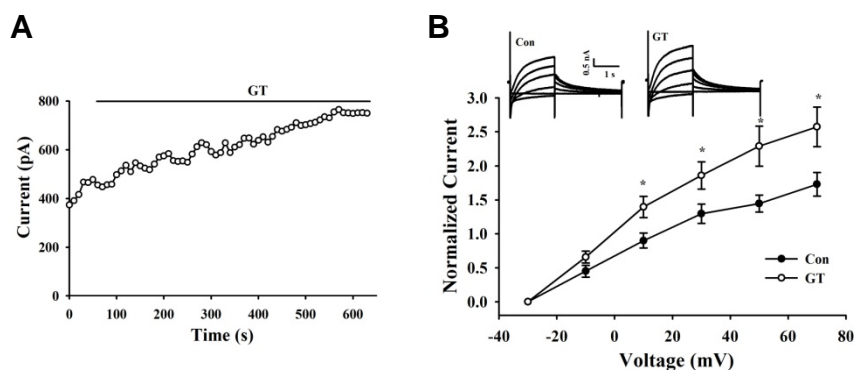
**Fig. 5.** Effects of gintonin on  $I_{\text{HERG}}$ ,  $I_{\text{tail}}$ , and slow  $I_{\text{deactivating-tail}}$ . (A) Representative current trace showing hERG  $\text{K}^+$  channel enhancement by gintonin (10  $\mu\text{g/ml}$ ). Currents were in response to 4-s voltage steps to 0 mV from a holding potential of -90 mV, followed by repolarization to -60 mV. (B) I-V relationship for hERG  $\text{K}^+$  currents measured at the end of the 4-s test pulse before and after application of 10  $\mu\text{g/ml}$  gintonin ( $n = 5$ ). Currents were normalized to the control current at 0 mV for each oocyte. Data are represented as mean  $\pm$  S.E.M. ( $n = 7$ ).

ated activation of  $I_{K_S}$  channels, we first treated cells with calmidazolium, a CaM inhibitor. As shown in Fig. 4A, gintonin has almost no effect on either channel current in the presence of calmidazolium. Next, we constructed channels using KCNQ1 subunits that had been mutated in the C-terminal CaM-binding IQ motifs: S373P or W392R in the IQ<sub>1</sub> region and R539W in the IQ<sub>2</sub> region. Mutations of CaM-binding IQ motifs reduced the amplitude of both the  $I_{K_S}$  channel currents. Gintonin-mediated activation of the  $I_{K_S}$  channels is also attenuated (Fig. 4B, *insets*). The EC<sub>50</sub> values for the wild-type, S373P, W392R, and R539W  $I_{K_S}$  channels are  $0.06 \pm 0.01$ ,  $3.66 \pm 0.41$ ,  $4.36 \pm 0.67$ , and  $2.30 \pm 0.05$   $\mu\text{g/ml}$ , respectively (Fig. 4B). Mutations in the KCNQ1 C terminus cause a rightward shift in the concentration-response curves, indicating that gintonin-mediated activation of  $I_{K_S}$  channels is achieved through  $\text{Ca}^{2+}$ /CaM binding to the IQ motifs in KCNQ1. Interestingly, gintonin has no effect on hERG  $\text{K}^+$  ( $I_{K_r}$ ) channel activity in  $I_{\text{HERG}}$  and  $I_{\text{tail}}$ , although gintonin slightly inhibits  $I_{\text{deactivating-tail}}$  compared to the control (Fig. 5). Thus, gintonin selectively regulates  $I_{K_S}$  but not  $I_{K_r}$  channels.

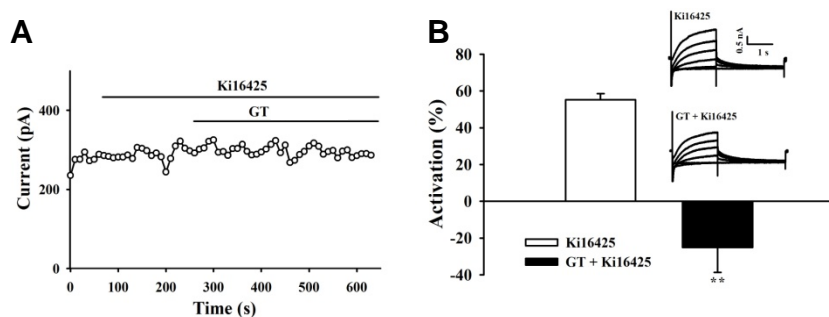
#### $I_{K_S}$ of guinea-pig ventricular myocytes is modulated by gintonin

Finally, we investigated whether gintonin could modulate  $I_{K_S}$  in guinea-pig ventricular myocytes. Myocytes were depolarized from -50 to +50 mV for 500 ms at 0.05 Hz. Under these experimental conditions, the time-dependent outward current developed during depolarization and the outward tail current that deactivated during repolarization was almost exclusively composed of  $I_{K_S}$  (Missan et al., 2006). We confirmed that the amplitude of the tail current was unaffected by acute application of the rapidly activating  $\text{K}^+$  channel blocker E4031 (50  $\mu\text{M}$ , data not shown). As indicated by the representative data in the time plot of Fig. 6A, the tail  $I_{K_S}$  current was significantly increased by gintonin (3  $\mu\text{g/ml}$ ). In the present study, the average of gintonin-mediated amplitude increase of the tail current was  $55.34 \pm 3.21\%$  ( $n = 10$ ). We applied a family of voltage steps from a holding potential of -50 mV, and measured the amplitude of the tail current at -30 mV following each test current. As indicated by the current records obtained during the example gintonin experiment and mean I-V relationships, gintonin increased  $I_{K_S}$  without altering I-V relationships (Fig. 6B).

To confirm that the LPA receptor was involved in gintonin ef-



**Fig. 6.** Gintonin increases  $I_{Ks}$  of guinea pig ventricular myocytes. (A) Time course of changes in the amplitude of the  $I_{Ks}$  tail during applications of gintonin (3  $\mu\text{g/ml}$ ). (B) I-V relationships obtained before and after application of gintonin. Currents were elicited by voltage steps from -30 mV to +70 mV with a subsequent step to -30 mV for the tail current. Representative recordings are shown in the *inset*. Data are represented as mean  $\pm$  S.E. ( $n = 8$ ).  $*p < 0.05$  versus control condition (paired t test)



**Fig. 7.** Effects of gintonin on ventricular  $I_{Ks}$  depend on LPA receptor. (A) LPA1/3 antagonist, Ki16425 (10  $\mu\text{M}$ ) significantly blocked the activation of  $I_{Ks}$  by gintonin (3  $\mu\text{g/ml}$ ). (B) Summary of the percent activation of  $I_{Ks}$ . Values are expressed as means  $\pm$  S.E.  $**p < 0.01$  versus control condition ( $n = 3$ ). Representative recordings obtained before and after gintonin in Ki16425-pretreated cells are shown in the *inset*.

fecton  $I_{Ks}$  in guinea-pig ventricular myocytes, we repeated similar experiments in the presence of LPA1/3 antagonist Ki16425 (10  $\mu\text{M}$ ). As shown in Fig. 7A, Ki16425 almost completely blocked gintonin-mediated modulation of  $I_{Ks}$  in guinea-pig ventricular myocytes. Summarized data in Fig. 7B showed that in the presence of Ki16425, the  $I_{Ks}$  was not enhanced but rather reduced ( $25.13 \pm 13.64\%$ ,  $n = 3$ ) by subsequent bath application of gintonin. Taken together, these data suggest that gintonin modulates  $I_{Ks}$  in native cardiac myocytes possibly via endogenous LPA receptors.

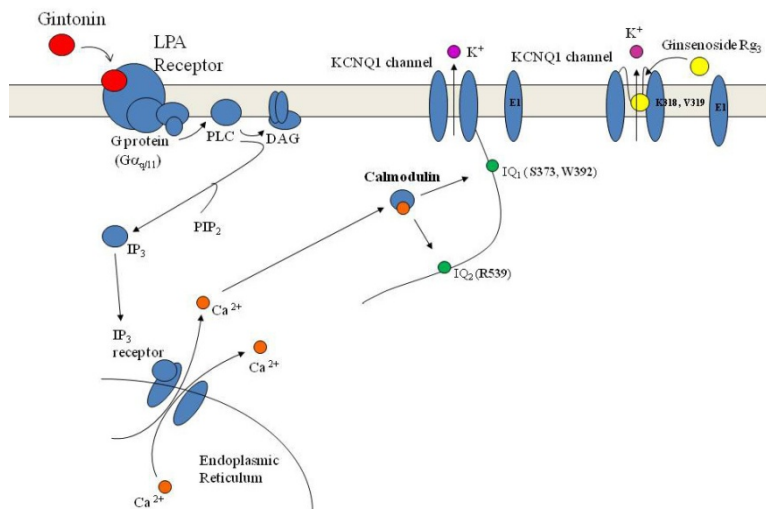
## DISCUSSION

KCNQ1 ( $I_{Ks}$ ) and hERG  $K^+$  ( $I_{Kr}$ ) channels are the main  $K^+$  channels in cardiac myocytes and both contribute to heart repolarization after cardiac action potential and shorten the action potential duration (Robbins, 2001). Although both channels are closely associated with cardiac activities, regulation of the two channels differs. For example, the C terminus of KCNQ1 contains  $\text{Ca}^{2+}/\text{CaM}$  complex interaction sites whereas the hERG  $K^+$  ( $I_{Kr}$ ) channel subunit does not (Ghosh et al., 2006; Tohse, 1990); it has been shown that mutations that disrupt the KCNQ1- $\text{Ca}^{2+}/\text{CaM}$  complex interactions cause LQT syndrome (Ghosh et al., 2006; Shamgar et al., 2006). It has been reported that ginseng root has a beneficial effect on a variety of cardiovascular diseases, including hypertension (Atteleat et al., 1999). In previous reports, we have shown that gintonin but not ginsenosides, another class of active ingredient in ginseng, elicits  $[\text{Ca}^{2+}]_i$  transients via G protein-coupled LPA receptors (Hwang et al., 2012) and that gintonin regulates  $\text{Ca}^{2+}$ -dependent  $K^+$  ( $\text{BK}_{\text{Ca}}$ ) and  $\text{Kv}1.2$  channels (Choi et al., 2013; Lee et al., 2013). However, thus far, little was known about the molecular mechanisms through which gintonin regulates  $I_{Ks}$  activity.

In this study, we have demonstrated that gintonin activates  $I_{Ks}$  channels through the LPA receptor- $\text{G}\alpha_{q11}$ -PLC- $\text{IP}_3$ - $\text{Ca}^{2+}/\text{CaM}$

signal pathway using human  $I_{Ks}$  channels in *Xenopus* oocytes. We also demonstrated a similar gintonin/LPA receptor-dependent regulation of  $I_{Ks}$  in guinea-pig ventricular myocytes (Figs. 6 and 7). Gintonin-mediated activation of  $I_{Ks}$  channels is concentration- and voltage-dependent. In addition, gintonin treatment causes the steady-state activation curves for these channels to shift leftward in a dose-dependent manner. Interestingly, the  $\text{EC}_{50}$  for the effect of gintonin on  $I_{Ks}$  activation was about nine times less than for KCNQ1-only channels (data not shown). Thus, the KCNE1 subunit might be necessary for full activation of  $I_{Ks}$  induced by  $\text{Ca}^{2+}/\text{CaM}$  complex after gintonin treatment. We also examined the effect of gintonin on hERG ( $I_{Kr}$ ) channel activity. However, gintonin only has a slight inhibitory effect on  $I_{\text{deactivating-tail}}$  (Fig. 5). Consequently, our study shows a possibility that gintonin-mediated  $[\text{Ca}^{2+}]_i$  transients via LPA receptor activation might govern  $I_{Ks}$  rather than  $I_{Kr}$  in the regulation of  $K^+$  channels in cardiac myocyte repolarization.

The presence of BAPTA, a calcium chelator, or calmidazolium, a CaM inhibitor, attenuated the gintonin-mediated activation of  $I_{Ks}$  channels. In addition, mutation of the amino acid residues in the  $\text{Ca}^{2+}/\text{CaM}$ -binding sites (IQ<sub>1</sub> and IQ<sub>2</sub>) in the C terminus of KCNQ1 also attenuated gintonin-mediated activation of  $I_{Ks}$  channels (Fig. 4). These results show that  $\text{Ca}^{2+}$ , CaM, or binding of  $\text{Ca}^{2+}/\text{CaM}$  complex to the C terminus of KCNQ1 is required to activate  $I_{Ks}$ . In further studies on the role of these  $\text{Ca}^{2+}/\text{CaM}$ -binding sites, mutations of IQ motifs exhibited differential effects on gintonin-mediated activation of  $I_{Ks}$  channels. The  $\text{EC}_{50}$  in the W392R, S373P, and R539R mutant  $I_{Ks}$  channels was increased by 72-, 61-, and 38-fold as compared to the wild-type channels, respectively. The  $\text{EC}_{50}$  for IQ<sub>1</sub> (W393R and S373P) and IQ<sub>2</sub> (R539W) mutants was about 1.5-fold less than that of the IQ<sub>2</sub> site (Fig. 4B). These results indicate that mutations in individual residues in the  $\text{Ca}^{2+}/\text{CaM}$ -binding sites might affect the conformation of the three-dimensional structure of the KCNQ1 C terminus and



**Fig. 8.** Diagram comparing gintonin- and ginsenoside Rg<sub>3</sub>-mediated activation of the  $I_{K_S}$  channel. Gintonin-mediated  $I_{K_S}$  activation proceeds via  $Ca^{2+}$ /CaM binding to IQ motifs via G protein-coupled LPA1 receptors, whereas ginsenoside Rg<sub>3</sub> activates  $I_{K_S}$  through a direct interaction with specific amino acids located at the pore entryway of channel proteins following depolarization (Choi et al., 2010).

change  $Ca^{2+}$ /CaM binding in a differential manner.

In a previous study, we demonstrated that ginsenoside Rg<sub>3</sub>, a ginseng saponin, also enhances  $I_{K_S}$  channel currents following depolarization (Choi et al., 2010). We found that gintonin differs from ginsenoside Rg<sub>3</sub> in the regulatory patterns for  $I_{K_S}$  channel activation. Ginsenoside Rg<sub>3</sub>-induced enhancement of  $I_{K_S}$  channel currents is not achieved through receptor-mediated  $[Ca^{2+}]_i$  transients as shown in schematic diagram in Fig. 6. Thus, ginsenoside Rg<sub>3</sub>-induced  $I_{K_S}$  current enhancement does not act via a membrane receptor signaling transduction pathway. Instead, ginsenoside Rg<sub>3</sub>-induced enhancement of  $I_{K_S}$  channels currents was abolished by substitution of the K318 and V319 residues located at the channel pore entrance (Choi et al., 2010). Thus, ginsenoside Rg<sub>3</sub> regulates  $I_{K_S}$  activity through direct interaction with channel proteins at the channel pore entrance. In contrast, gintonin amplifies  $I_{K_S}$  activation through a signaling pathway mediated by a membrane-bound G protein-coupled LPA receptors (Fig. 6). Supporting this observation is that fact that gintonin, even at much lower concentrations than ginsenoside Rg<sub>3</sub>, induces greater amplitudes of outward  $I_{K_S}$  channel currents (by 4- to 5-fold) than does ginsenoside Rg<sub>3</sub> (Fig. 6). The  $EC_{50}$  of gintonin is approximately 35 nM (under the assumption that the molecular weight of gintonin is 20 kDa) (Hwang et al., 2012), whereas that of the ginsenoside Rg<sub>3</sub> was about 15  $\mu$ M for  $I_{K_S}$  activation (Choi et al., 2010). In addition, interruption of the receptor signaling pathway by inhibitors or mutations abolished or attenuated gintonin-mediated but not ginsenoside Rg<sub>3</sub>-mediated  $I_{K_S}$  channel activation (data not shown). These results indicate that ginseng contains two agents with two different action modes for the regulation of  $I_{K_S}$  activity. However, gintonin is more efficient for  $I_{K_S}$  activation than ginsenoside Rg<sub>3</sub> because gintonin exerts its effects through LPA receptors whereas but ginsenoside Rg<sub>3</sub> does not (Fig. 6).

Gintonin comprises about 0.2% of ginseng (Pyo et al., 2011). Gintonin is shown to contain two proteins, ginseng major latex-like protein 151 (GLP151) and ginseng ribonuclease-like storage proteins (Hwang et al., 2012). GLP 151 belongs to the Bet v1 family of proteins and exhibits similar properties to other members of Bet v1 families (Hwang et al., 2012). GLP151 has hydrophobic ligand-binding sites (Hwang et al., 2012). GLP151 contains hydrophobic ligand-binding sites and a glycine-rich region that binds to phosphate groups (Hwang et al., 2012). Currently,

we are investigating the possibility that GLP151 could be a main candidate for LPA binding protein of gintonin.

The previous reports have shown that ginseng extract or ginseng components, including ginsenosides, exhibit beneficial effects against cardiovascular diseases by relaxation of blood vessels constricted by adrenergic receptor stimulations (Chen and Zhang, 2009; Kang et al., 1995; Kim et al., 1999). In a study using a single cell, ginsenosides inhibit L-type  $Ca^{2+}$  channel currents in cardiac myocytes (Bai et al., 2003; 2004). We have also shown in a previous study that ginsenoside Rg<sub>3</sub> inhibits L-type  $Ca^{2+}$  channel currents through interactions with the amino acid residues L427, N428, and L431 in the transmembrane domain I segment 6 (Choi et al., 2009). In addition to L-type  $Ca^{2+}$  channel regulation by ginsenosides, recent reports showed that ginsenoside Re regulates  $I_{K_S}$  in cardiac myocytes (Bai et al., 2004), and we have also demonstrated that ginsenoside Rg<sub>3</sub> enhances  $I_{K_S}$  through interaction with K318 and V319 residues (Choi et al., 2010). In the present study, we have demonstrated that gintonin activates  $I_{K_S}$  channels in cardiac myocytes through LPA receptors. Thus, although gintonin and ginsenosides differ from each other for the activation of  $I_{K_S}$  channel, the enhancing effect of both gintonin and ginsenosides on  $I_{K_S}$  channel currents might contribute to a facilitation of repolarization of cardiac action potential and shorten action potential duration. These results show the possibility that both ginsenosides and gintonin could be candidates against cardiovascular diseases. However, we do not know currently how the actions of gintonin on  $I_{K_S}$  currents might be coupled to alleviation of arrhythmia, which is caused by  $I_{K_S}$  and  $I_{K_r}$  channel dysfunctions. More investigations might be required for application of gintonin to heart dysfunction.

In summary, we found that gintonin induces activation of  $I_{K_S}$  channels via a membrane G protein-coupled LPA receptor signaling pathway. Using site-directed mutagenesis, we further confirmed the role of the  $Ca^{2+}$ /CaM complex in gintonin-mediated  $I_{K_S}$  channel regulation. These novel findings provide insight into the molecular basis of the pharmacological effects of ginseng in cardiovascular systems.

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