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Gintonin, a Ginseng-Derived Lysophosphatidic Acid Receptor Ligand, Potentiates ATP-Gated $P2X_1$ Receptor Channel Currents

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Ginseng, the root of *Panax ginseng* C.A. Meyer, is used as a general tonic. Recently, we isolated a novel ginseng-derived lysophosphatidic acid (LPA) receptor ligand, gintonin. Gintonin activates G protein-coupled LPA receptors with high affinity in cells endogenously expressing LPA receptors, e.g., *Xenopus* oocytes. P2X receptors are ligand-gated ion channels activated by extracellular ATP, and 7 receptor subtypes ($P2X_1$-$P2X_7$) have been identified. Most of the $P2X_1$ receptors are expressed in the smooth muscles of genitourinary organs involved in reproduction. A main characteristic of the $P2X_1$ receptor is rapid desensitization after repeated ATP treatment of cells or tissues expressing $P2X_1$ receptors. In the present study, we examined the effect of gintonin on $P2X_1$ receptor channel activity. $P2X_1$ receptors were heterologously expressed in *Xenopus* oocytes. ATP treatment of oocytes expressing $P2X_1$ receptors induced large inward currents (I_{ATP}), but repetitive ATP treatments induced a rapid desensitization of I_{ATP}. Gintonin treatment after $P2X_1$ receptor desensitization potentiated I_{ATP} in a concentration-dependent manner. We further examined the signaling transduction pathways involved in gintonin-mediated potentiation of I_{ATP}. Gintonin-mediated I_{ATP} potentiation was blocked by Ki16425, an LPA1/3 receptor antagonist, a PKC inhibitor, a PLC inhibitor, and a PI4-Kinase inhibitor but not by a calcium chelator. In addition, mutations of the phosphoinositide binding site of the $P2X_1$ receptor greatly attenuated the gintonin-mediated I_{ATP} potentiation. These results indicate that G protein-coupled LPA receptor activation by gintonin is coupled to the potentiation of the desensitized $P2X_1$ receptor through a phosphoinositide-dependent pathway.

INTRODUCTION

Ginseng, the root of *Panax ginseng* C.A. Meyer, is a general tonic consumed throughout the world. Ginseng shows multiple physiological and pharmacological effects (Nah et al., 1997). For example, ginseng affects male reproductive systems, including spermatogenesis, sperm motility, and sperm survival (Hwang et al., 2004; Jang et al., 2011; Park et al., 2006). However, the active ingredients and the underlying molecular mechanism by which ginseng exerts its diverse effects are not fully understood. Recently, we isolated a novel ligand of the ginseng-derived G protein-coupled lysophosphatidic acid (LPA) receptor, gintonin (Hwang et al., 2012; Pyo et al., 2011). We demonstrated that gintonin activates LPA receptors with high affinities in cells expressing LPA receptors endogenously or heterologously (Hwang et al., 2012). Activation of LPA receptors by gintonin affects cell survival, proliferation, migration, and morphological changes in neuronal and non-neuronal cells; LPA receptors are linked to multiple G proteins such as $G\alpha_{i/o}$, $G\alpha_{12/13}$, and $G\alpha_{q/11}$ (Hwang et al., 2012). LPA receptor activation by gintonin is also coupled to diverse downstream events, including stimulation of phospholipase C, mitogen-activated protein kinases, and phosphoinositide 4-kinase (PI4-kinase) (Hwang et al., 2012). However, it is unknown whether the activation of G protein-coupled LPA receptors by gintonin regulates $P2X_1$ receptor channel activity.

P2X receptors are ligand-gated cation channels activated by extracellular ATP (Burnstock, 1997; Valera et al., 1994). Seven P2X receptor subtypes, $P2X_1$ to $P2X_7$, have been identified (North et al., 2002). The P2X subtypes assemble to form either homo- or hetero-trimeric channels, leading to a great variety of phenotypes. Each P2X subunit has 2 putative membrane-spanning segments, TM1 and TM2 (Fig. 1A)-hydrophobic regions that cross the plasma membrane-and a large extracellular loop as well as intracellular N- and C- termini (Javis et al., 2009; North et al., 1996; Surprenant et al., 1995). P2X receptors are associated with diverse physiology and pathophysiology such as immune response, neuropathic pain by sensory transduction of the central nervous system, and control of smooth muscle contraction in genitourinary systems (Burnstock et al., 2007; Khakh and North, 2006). $P2X_1$ receptors are expressed in organs containing smooth muscle cells, such as the urinary bladder, vas deferens, and other genitourinary systems,

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where the receptors are involved in the regulation of smooth muscle contraction (Mulryan et al., 2000; North et al., 2002). In particular, mice lacking $P2X_1$ receptors exhibit a reduced vas deferens smooth muscle contraction and subsequent infertility, indicating that the $P2X_1$ receptor plays an important role in male fertility (Mulryan et al., 2000). Another characteristic of the $P2X_1$ receptor channel activity is fast desensitization, resulting in a decreased response to repeated agonist application (North et al., 2002).

Xenopus oocytes express endogenous LPA1 receptors (Kimura et al., 2001). In the present study, we examined whether activation of the endogenous G protein-coupled LPA receptor by gintonin in *Xenopus* oocytes affects $P2X_1$ receptor channel activity. $P2X_1$ receptors were heterologously expressed in *Xenopus* oocytes. We observed that when $P2X_1$ receptors were stimulated by repeated treatments with ATP, the ATP-mediated inward current (I_{ATP}) dramatically decreased compared with the initial I_{ATP}. Interestingly, gintonin treatment, after induction of desensitization by repetitive application of ATP, greatly potentiated I_{ATP}. The potentiating effect of gintonin on I_{ATP} observed after desensitization was blocked by the protein-kinase C, PI4-kinase, and phospholipase C signaling pathway but not the Ca^{2+} signaling pathway. Site-directed mutagenesis of the phosphoinositide (PIP_2)-binding site of the $P2X_1$ receptor also attenuated the gintonin-mediated I_{ATP} potentiation. We discuss the signaling transduction pathways and the role of LPA receptors in the genitourinary systems involved in the gintonin-mediated potentiation of I_{ATP} after $P2X_1$ receptor desensitization. LPA receptor activation by gintonin is coupled to the regulation of $P2X_1$ receptor channel activity, and gintonin-mediated I_{ATP} potentiation might be the molecular basis of the beneficial effects of ginseng in the genitourinary systems.

MATERIALS AND METHODS

Materials

Gintonin devoid of ginseng saponins was prepared from *Panax* ginseng according to method of Pyo et al. (2011). Gintonin used in this study was dissolved in dimethyl sulfoxide (DMSO) and then diluted with bath medium before use. The final DMSO concentration was less than 0.01%. The cDNA of the human $P2X_1$ receptor (GenBank accession no. NM_002558.2) was purchased from Missouri S&T cDNA Resource Center (USA). The phospholipid diC8-PI(4,5)P_2 was purchased from Echelon Biosciences Inc., (USA). All other agents were purchased from Sigma-Aldrich (USA).

Preparation of *Xenopus* oocytes

Xenopus laevis frogs were purchased from *Xenopus* I (USA). The care and handling of the frogs were in accordance with institutional guidelines. The frogs underwent surgery twice, and the 2 surgeries were separated by at least 3 weeks. For isolation of oocytes, frogs were anesthetized with an aerated solution of 3-amino benzoic acid ethyl ester, and the ovarian follicles were removed. Oocytes were separated by treatment with collagenase by gentle shaking for 2 h in $CaCl_2$-free medium containing 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM *N*-(2-hydroxyethyl)piperazine-*N*'-2 ethanesulfonic acid (HEPES), 2.5 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin. Stage V-VI oocytes were collected and stored in ND96 medium (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 gentamycin. The oocyte-containing solution was maintained at 18°C with gentle continuous shaking, and the supplemented MD96 medium was replaced daily. All electrophysiological experiments were performed within 5 to 6 days following isolation of the oocytes, with chemicals applied to the bath.

cRNA preparation of $P2X_1$ receptor and microinjection

A recombinant plasmid (from the Missouri S&T cDNA Resource Center), containing a human $P2X_1$ receptor cDNA insert, was linearized by digestion with the appropriate restriction enzymes. The cRNAs were transcribed from linearized templates with an *in vitro* transcription kit (mMessage mMachine, Ambion, USA) using T7 polymerase. The cRNA was dissolved in RNase-free water at a final concentration of approximately 1 μg/μl, aliquoted, and stored at -70°C until used. $P2X_1$ receptor cRNA (40 nl) was injected into the animal or vegetal pole of each oocyte using a 10 μl VWR microdispenser (VWR Scientific, USA). The injection pipette was pulled from glass capillary tubing and used for recording electrodes; the tip had a diameter of 15-20 μm.

Site-directed mutagenesis of the $P2X_1$ receptor, and *in vitro* transcription of $P2X_1$ receptor cDNA

Single or double amino acid substitutions were prepared using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, USA) along with *Pyrococcus furiosus* DNA polymerase and sense and antisense primers encoding the desired mutations. Overlap extension of the target domain by sequential polymerase chain reaction (PCR) was conducted according to the manufacturer's protocol. The final PCR products were transformed into *Escherichia coil* strain DH5α, screened by PCR, and confirmed by sequencing of the target regions. The mutant DNA constructs were linearized at the 3′ ends by digestion with *Sma* I, and run-off transcripts were prepared using the methylated cap analog, $m^7G(5')ppp(5')G$. The cRNAs were prepared using the mMessage mMachine transcription kit (Ambion) 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 $P2X_1$ receptor cDNA inserts were linearized by digestion with the appropriate restriction enzymes, and cRNAs were obtained using the mMessage mMachine *in vitro* transcription kit with T7 polymerase. The final cRNA products were resuspended at a concentration of 1 μg/μl in RNase-free water and stored at -80°C.

Data recording

A custom-made Plexiglas net chamber was used for the 2-electrode voltage-clamp recordings. The chamber was constructed by milling of 2 concentric wells to the chamber bottom (diameter/height: upper well: 8/3 mm, lower well: 6/5 mm) and by gluing plastic meshes (*ca*. 0.4 mm grid diameter) to the bottom of the upper well. A perfusion inlet (*ca*. 1 mm in diameter) was added in the wall of the lower well, and a suction tube was placed on the edge of the upper well. The oocyte was then placed on the net separating the upper and lower wells; the grids of the net served as dimples to keep the oocyte in place during the electrophysiological recording. The oocytes were impaled with 2 microelectrodes filled with 3 M KCl (0.2-0.7 MΩ). The recordings were performed at a flow rate of 2 ml/min with Ca^{2+}-free ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5). ND96 solution without $CaCl_2$ was used to exclude any contributions of Ca^{2+}-activated Cl^- currents by gintonin. The electrophysiological experiments were performed at room temperature with an Oocyte Clamp (OC-725C,

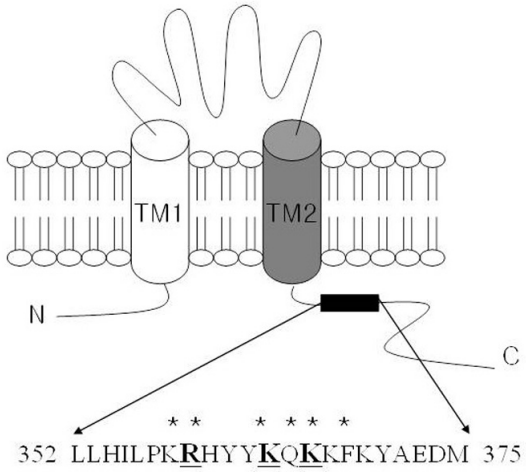

Fig. 1. Schematic of the transmembrane regions and the basic residues of the C-terminal domain of the P2X$_1$ receptor. P2X receptors likely possess 2 transmembrane segments (TM1 and TM2) and a long extracellular loop. The N- and C-termini comprise a cytosolic domain. The proximal C-terminal region after the TM2 segment of the P2X$_1$ receptor includes the putative phosphoinositide binding site. Amino acid residues mutated in the present study are underlined.

Warner Instrument, USA), and the stimulation and data acquisition were controlled with a pClamp 8 (Molecular Devices, USA). For most of the electrophysiological ex-periments, the oocytes were clamped at a holding potential of -70 mV, and 500 ms voltage steps were applied from -100 to +50 mV to assess the current-voltage relationship. In the indicated experiments, stimulation with 1 μM ATP (Sigma-Aldrich), dissolved in Ca^{2+}-free ND96 solution, was performed 5 times with 5 min intervals. Because the ATP-induced P2X$_1$ responses were stable after the second application of ATP, 3 μg/ml gintonin was applied for 30 s between the third and fourth ATP applications. Normalization of potentiation of the P2X$_1$ receptor was defined as the ratio of the fourth over the third I_{ATP} performed by calculating the ratio of the third and fourth I_{ATP}. The phospholipid diC8-PI(4,5)P_2 was injected (20 nl; 10 mM) in the cytoplasm of oocytes 30 min before recording. For all the calculations of final diC8-PI(4,5)P_2 concentration, estimated the oocyte cell volume at 1 μl (Bernier et al., 2008).

Data analysis

To obtain concentration-response curves of the effects of gintonin on the ATP-mediated inward currents, the peak amplitudes at different concentrations of gintonin were plotted, and the Origin software (OriginLab, USA) was used to fit the data to the Hill equation: $I/I_{max} = [A]^{nH}/([A]^{nH} + [EC_{50}]^{nH})$, where I is the peak current at a given concentration of ATP, I_{max} is the maximal peak current, EC_{50} is the concentration of gintonin producing a half-maximal effect, [A] is the concentration of gintonin, and nH is the Hill coefficient. All values are presented as the mean ± S.E.M. The significance of differences between control and treatment values was determined using Student's *t*-test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Human P2X$_1$ receptors desensitize rapidly, and gintonin potentiates P2X$_1$ receptor channel activity

We examined the effects of gintonin on P2X$_1$ receptor channel activity. Thus, we first expressed the human P2X$_1$ receptor subunits in *Xenopus* oocytes (North et al., 2002). As shown in Fig. 2A, the addition of ATP (100 μM) to the bathing solution induced a large inward current (I_{ATP}) in oocytes injected with human P2X$_1$ receptor subunit cRNAs at a holding potential of -70 mV, as reported previously (North et al., 2002; Rettinger and Schmalzing, 2003). When ATP was re-applied after 5 min, I_{ATP} was reduced by approximately 50-80%, corresponding to a rapid desensitization of the P2X$_1$ receptor. Subsequent applications of ATP at 5 min intervals produced reproducible but desensitized responses (Fig. 2A). In H_2O-injected control oocytes, the application of ATP did not induce inward currents (data not shown). Next, we examined whether activation of endogenous LPA receptors by gintonin in *Xenopus* oocytes affected P2X$_1$ receptor-mediated channel activity. Gintonin was applied between the third and fourth ATP applications. Gintonin elicited an endogenous Ca^{2+}-activated Cl^- channel (CaCC) current (Pyo et al., 2011). Gintonin also significantly potentiated the amplitude of subsequent I_{ATP} responses by 2-3-fold, as compared with the third I_{ATP} (Fig. 2B). The potentiating effect of gintonin on I_{ATP} was concentration dependent, with saturation observed at 3 μg/ml, and the EC_{50} value was 0.37 ± 0.19 μg/ml (Fig. 2C).

Suramin is a broad-spectrum antagonist of the P2X receptor,

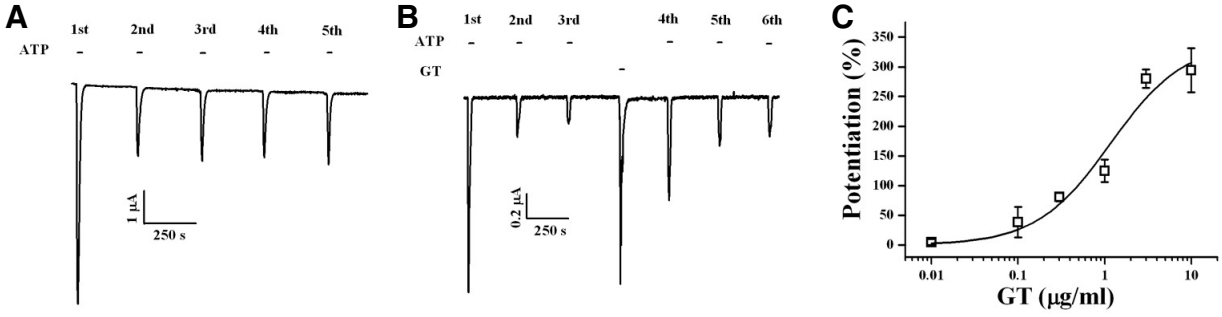

Fig. 2. Concentration effect of gintonin (GT) on P2X$_1$ receptor channel activity. (A) Representative ATP-evoked I_{ATP} in *Xenopus* oocytes expressing human P2X$_1$ receptors (1 μM ATP application at 5 min intervals). (B) Representative trace demonstrating that gintonin (3 μg/ml) activates endogenous CaCC and potentiates P2X$_1$ receptor channel activity by ATP. (C) The stimulatory effects of gintonin on I_{ATP} are concentration dependent. Gintonin was added between the third and fourth ATP applications. The percentage potentiation of I_{ATP} by gintonin is defined as the ratio of the fourth I_{ATP} to the third I_{ATP} amplitude measured after gintonin stimulation. Each point represents the mean ± SEM (n = 5-7).

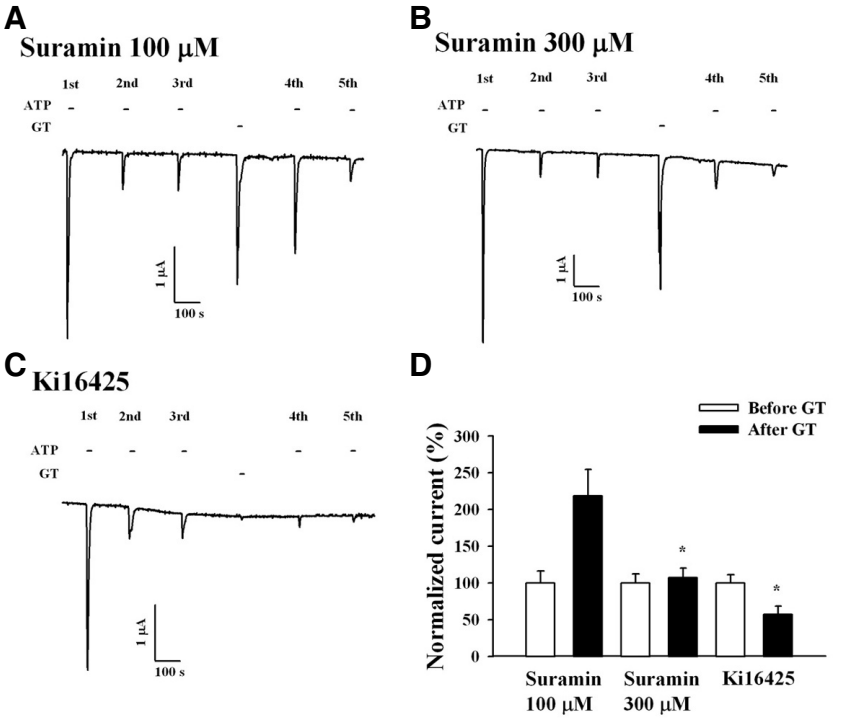

Fig. 3. P2X and LPA receptor antagonists block gintonin-mediated I_{ATP} potentiation of human P2X$_1$ receptor channel activity. (A, B) Representative trace showing 2 different concentrations of suramin, a P2X receptor antagonist, on the gintonin-mediated I_{ATP} potentiation. (C) Representative trace showing the gintonin-mediated I_{ATP} potentiation in the absence or presence of Ki16425, an LPA1/3 receptor antagonist, in *Xenopus* oocytes expressing human P2X$_1$ receptors. (D) Histograms summarizing the effects of suramin and Ki16425 on the gintonin-mediated I_{ATP} potentiation (*p < 0.001, compared to the absence of suramin). Data represent the mean ± SEM (n = 4-5/group).

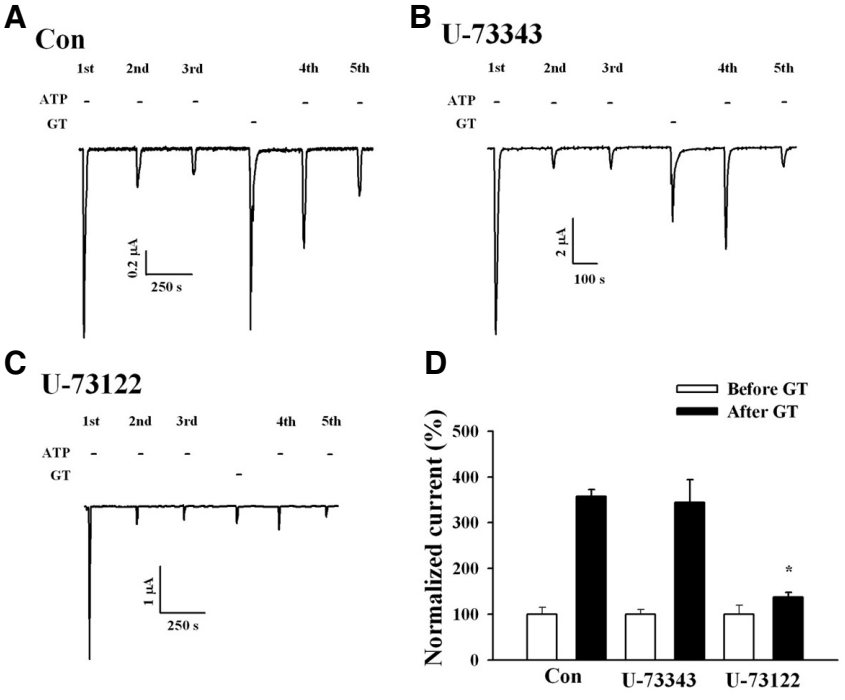

Fig. 4. Active PLC inhibitor U-73122 blocking the gintonin action on P2X$_1$ receptor desensitization. (A) Representative trace showing the gintonin-mediated I_{ATP} potentiation in *Xenopus* oocytes expressing human P2X$_1$ receptors. (B, C) Representative trace showing the effects of active (U73122) and inactive (U73343) PLC inhibitors (each 1 μM) on the gintonin-mediated I_{ATP} potentiation. The active PLC inhibitor (1 μM) blocks both gintonin-evoked CaCC and I_{ATP} potentiation. (D) Histograms summarizing the effects of the active or inactive PLC inhibitors on gintonin-mediated I_{ATP} potentiation (*p < 0.001, compared to control). Data represent the mean ± SEM (n = 5/group).

and human P2X$_1$ receptors are effectively blocked by suramin (Sim et al., 2008). We examined the effect of suramin to determine whether gintonin-mediated I_{ATP} potentiation is achieved through P2X receptor activation. Co-application of gintonin (3 μg/ml) and suramin had no effect on the gintonin-induced CaCC current at a suramin concentration of 100 and 300 μM. Thus, although no effect was observed on the gintonin-induced CaCC current, suramin (300 μM) abolished the gintonin-mediated potentiation of I_{ATP} (Figs. 3A, 3B, and 3D). These results indicate that the gintonin-mediated potentiation of I_{ATP} was achieved through P2X$_1$ receptor activation.

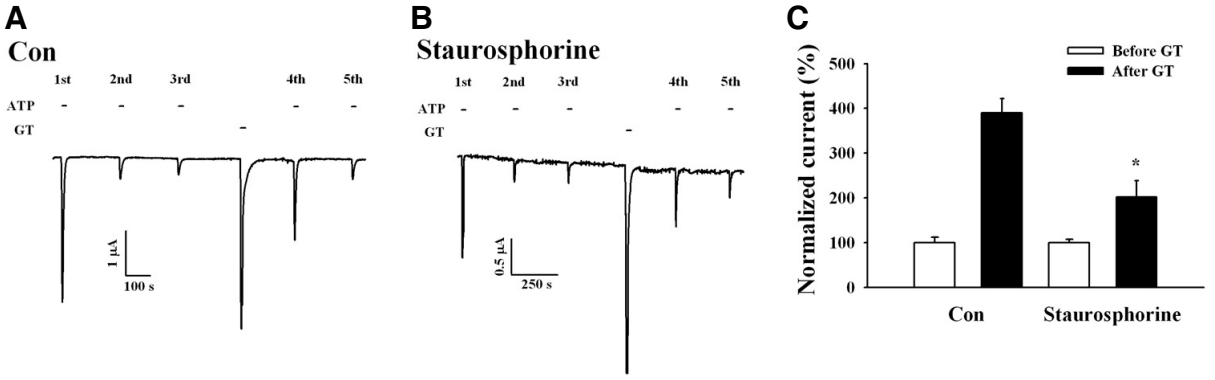

Fig. 5. Effects of PKC inhibitor on gintonin-mediated I_{ATP} potentiation of the human P2X$_1$ receptor. (A) Representative trace showing the prevention of gintonin-mediated P2X$_1$ receptor desensitization in *Xenopus* oocytes expressed the P2X$_1$ receptor. (B) Representative traces showing that the prevention of P2X$_1$ receptor desensitization by gintonin is affected by the PKC inhibitor, staurosporine. (C) Histograms summarizing the effects of PKC inhibitor on gintonin-mediated I_{ATP} potentiation (*p < 0.005, compared to control). Data represent the mean ± SEM (n = 4-5/group).

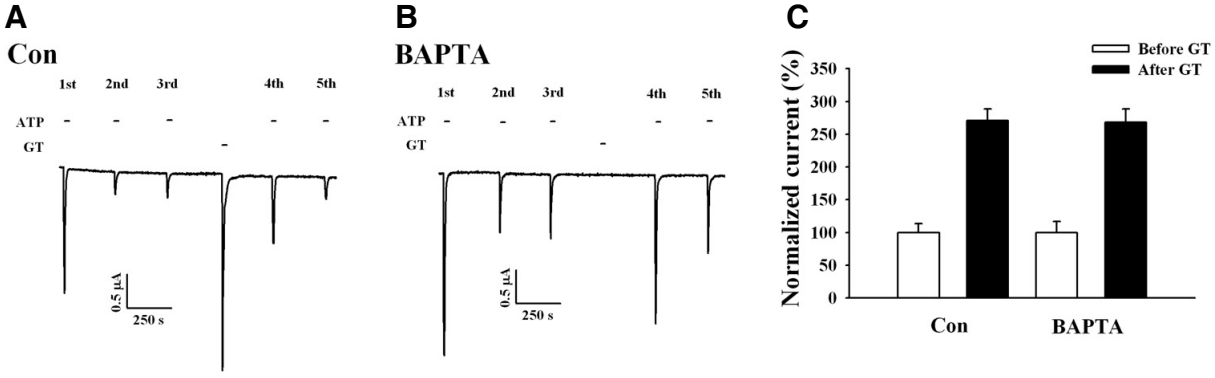

Fig. 6. Gintonin-mediated I_{ATP} potentiation does not involve intracellular calcium. (A) Representative trace showing the gintonin-mediated I_{ATP} potentiation in *Xenopus* oocytes expressing human P2X$_1$ receptors. (B) Representative trace showing that although the treatment with the calcium chelator BAPTA-AM (100 μM) abolished gintonin-mediated CaCC activation, BAPTA-MA had no effect on I_{ATP} potentiation by gintonin. (C) Histograms summarizing the gintonin-mediated I_{ATP} potentiation in the absence or presence of BAPTA-AM. Data represent the mean ± SEM (n = 4-5/group).

Potentiation of P2X$_1$ receptor channel activity by gintonin involves the LPA receptor-PLC-PKC pathway but not the Ca^{2+} pathway

Xenopus oocytes express endogenous LPA1 receptors (Kimura et al., 2001). We demonstrated that gintonin induces $[Ca^{2+}]_i$ transients and enhances CaCC currents *via* LPA receptor activation (Hwang et al., 2012). We examined the effect of the LPA1/3 receptor antagonist, Ki16425, on the gintonin-mediated I_{ATP} potentiation. In the absence of Ki16425, gintonin treatment enhanced I_{ATP}. However, treatment of Ki16425 (10 μM) not only abolished gintonin-mediated CaCC activation, but also attenuated gintonin-mediated I_{ATP} potentiation. This result indicates that the gintonin-mediated potentiation of I_{ATP} was achieved through the activation of endogenously expressed LPA1 receptors in *Xenopus* oocytes (Figs. 3C and 3D).

We next examined the signaling pathways involved in the gintonin-mediated I_{ATP} potentiation. Application of an inactive PLC inhibitor, U-73343, had no effect on gintonin action (Fig. 4B), whereas application of an active PLC inhibitor, U-73122, nearly abolished the gintonin-mediated P2X$_1$ receptor potentiation (Figs. 4C and 4D). These results indicate that gintonin-mediated potentiation of I_{ATP} involves PLC activation.

Because the above-described results suggest that gintonin-mediated I_{ATP} potentiation may involve PKC activation, we next examined the effects of the PKC inhibitor, staurosporine, on gintonin-mediated potentiation of I_{ATP}. Pre-incubation with 1 μM staurosporine for 1 h attenuated the gintonin-mediated I_{ATP} potentiation by 75% (Fig. 5). These results indicate that gintonin-mediated I_{ATP} potentiation after P2X$_1$ receptor desensitization is achieved through PKC activation. Because gintonin-mediated I_{ATP} potentiation after P2X$_1$ receptor desensitization was observed after the CaCC current enhancements (induced after the mobilization of intracellular Ca^{2+}), we examined whether gintonin-mediated I_{ATP} potentiation also requires transient $[Ca^{2+}]_i$ mobilization (Fig. 2), using the membrane-permeable calcium chelator, BAPTA-AM [1,2-bis(*O*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid]. Oocytes expressing human P2X$_1$ receptors were first pre-incubated with 100 μM BAPTA-AM for 3 h, and the gintonin-mediated I_{ATP} potentiation was then examined. Interestingly, although BAPTA-AM treatment abolished the gintonin-mediated CaCC activation (Fig. 6B), BAPTA-AM treatment had no effect on the gintonin-mediated I_{ATP} potentiation

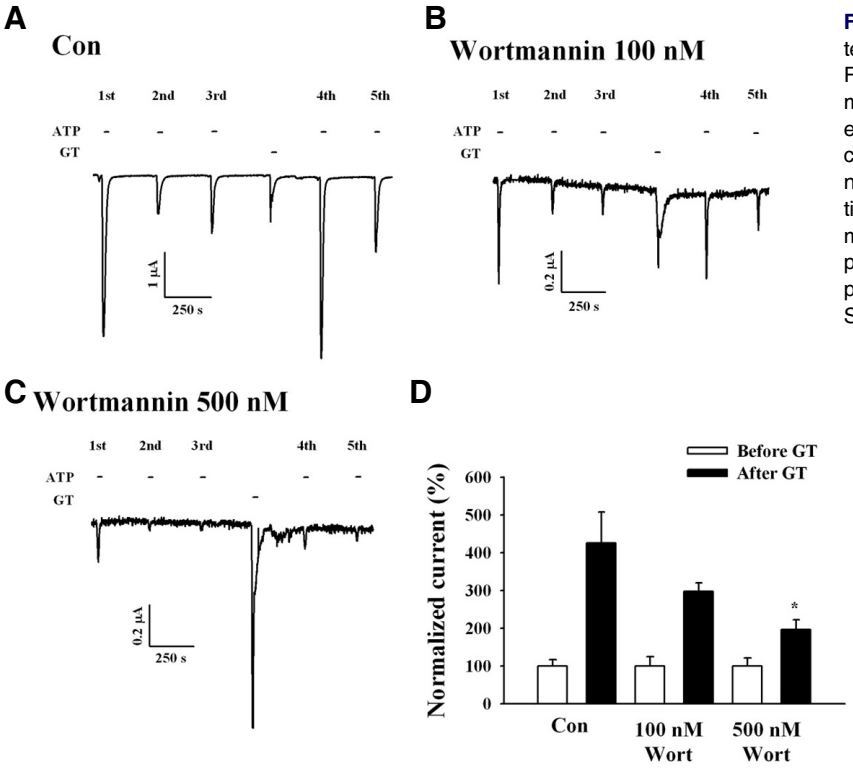

Fig. 7. Wortmannin, a PI4-kinase inhibitor, attenuates gintonin-mediated I_{ATP} potentiation. (A) Representative trace showing the gintonin-mediated I_{ATP} potentiation in *Xenopus* oocytes expressing human $P2X_1$ receptors. (B, C) Traces showing that wortmannin (500 but not 100 nM) attenuates gintonin-mediated I_{ATP} potentiation. (D) Histograms summarizing the gintonin-mediated I_{ATP} potentiation in the absence or presence of wortmannin (WT). (*$p < 0.05$, compared to control). Data represent the mean ± SEM (n = 6-7/group).

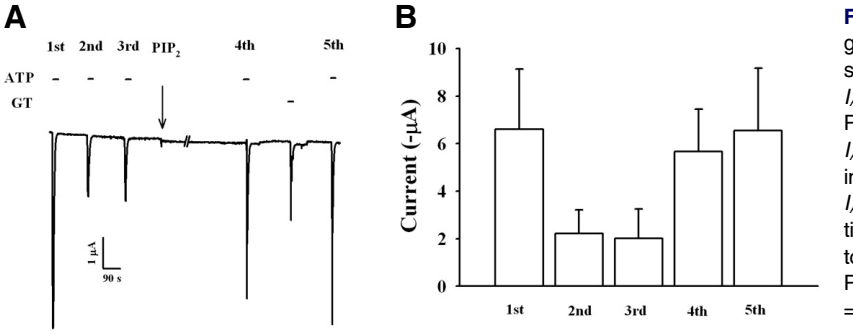

Fig. 8. Effects of PIP_2 injected into oocytes on gintonin-mediated I_{ATP} potentiation. (A) Representative trace showing the gintonin-mediated I_{ATP} potentiation in oocytes injected with PIP_2. PIP_2 was injected into oocytes 30 min before 4th I_{ATP} recording. Treatment of gintonin to ooytes injected with PIP_2 did not induce potentiation of I_{ATP}, although PIP_2 rescued of I_{ATP} from desensitization. (B) Histograms summarizing the gintonin-mediated I_{ATP} potentiation before or after PIP_2 injection. Data represent the mean ± SEM (n = 5/group).

(Fig. 6C). These results indicate that the gintonin-mediated I_{ATP} potentiation is independent of the intracellular calcium level.

Effects of wortmannin, an inhibitor of PI4-kinase, phosphatidylinositol 4,5-bisphosphate (PIP_2), or mutations of the phosphoinositide binding sites on gintonin-mediated I_{ATP} potentiation

Phosphoinositides are membrane lipids involved in many cellular signaling processes. Phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$ or PIP_2) is abundant phosphoinositide in the cell membrane and regulates numerous ion channels and transporters (Suh et al, 2005; Zhao et al., 2007b). To study the relationship between gintonin-mediated I_{ATP} potentiation and the role of phosphoinositides, we used wortmannin. Wortmannin inhibits phosphoinositide phosphatidylinositol 4-kinase (PI4-kinase) (Balla et al., 1998). Wortmannin also attenuates $PI(4,5)P_2$ synthesis (Zhao et al., 2007a). Oocytes expressing $P2X_1$ receptors were pre-incubated with 100 or 500 nM wortmannin for 2 h. In oocytes pre-incubated with 100 nM wortmannin, the gintonin-mediated I_{ATP} potentiation decreased by 30% (Figs. 7B and 7D), and in oocytes pre-incubated with 500 nM wortmannin, the potentiation decreased by 55% (Figs. 7C and 7D). Interestingly, we observed that the presence of wortmannin itself diminished I_{ATP} compared with the control. These results indicate that gintonin-mediated I_{ATP} potentiation is achieved through the activation of PI4-kinase.

To confirm the involvement of PIP_2 in gintonin-mediated I_{ATP} potentiation, we injected PIP_2 into oocytes and examined gintonin-mediated I_{ATP} potentiation. As shown in Fig. 8, although PIP_2 injection rescued I_{ATP} from desensitization induced by repeated treatment, treatment of gintonin did not further potentiate I_{ATP} in oocytes injected with PIP_2. These results indicate that gintonin-mediated I_{ATP} potentiation might be achieved *via* the stimulation of PIP_2 production.

Interactions between PIP_2 and ion channels are mainly electrostatic, and anionic PIP_2 sites interact with a cluster of posi-

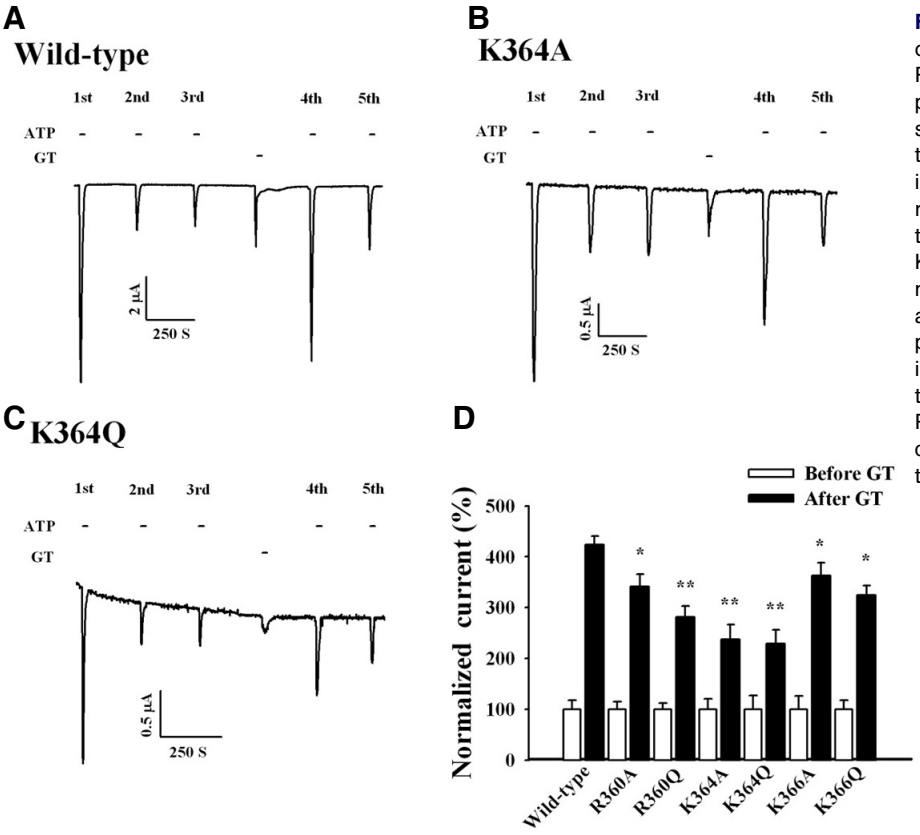

Fig. 9. Effects of site-directed mutations of the C-terminal domain of the human P2X$_1$ receptor on gintonin-mediated I_{ATP} potentiation. (A) Representative trace showing the gintonin-mediated I_{ATP} potentiation in *Xenopus* oocytes expressing human P2X$_1$ receptors. (B, C) Representative traces showing that mutations of the basic amino acid residue K364 in the C-terminal domain to alanine (A) or glutamine (Q) significantly attenuated the gintonin-mediated I_{ATP} potentiation. (D) Histograms summarizing the gintonin-mediated I_{ATP} potentiation in wild-type and various mutant P2X$_1$ receptors (*$p < 0.05$, **$p < 0.005$, compared to control). Data represent the mean ± SEM (n = 6-7/group).

tively charged amino acid residues on the ion channels (Lopes et al., 2002). In several families of ion channels that are regulated by PIP_2, the region proximal to the cytoplasmic C-terminal domain contains basic residues that interact with PIP_2 (Fujiwara and Kubo, 2006). Previous reports showed that P2X$_1$ receptors have phosphoinositide binding sites, and site-directed mutations of PIP_2 binding sites attenuate I_{ATP} potentiation by $G\alpha_{q/11}$-mediated receptor activation (Bernier et al., 2008). To further investigate the direct interaction between P2X$_1$ receptors and phosphoinositides, we constructed several P2X$_1$ receptor mutants that have been reported to disrupt the interaction with phosphoinositides (Bernier et al., 2008). We selected R360, K364, and K366 in the C-terminal domain of the P2X$_1$ receptor and mutated each of these basic residues, lysine (K) and arginine (R), to glutamine (Q) or alanine (A). We then examined the effects of each mutant receptor on the gintonin-mediated I_{ATP} potentiation. The K364A and K364Q mutant receptors significantly attenuated the I_{ATP} potentiation by gintonin (Figs. 9B and 9C). In addition, the R360Q mutant receptor caused a slight attenuation of gintonin-mediated I_{ATP} potentiation (Fig. 9D). These results again indicate that gintonin induces the production of phosphoinositides *via* PI4-kinase activation, and the produced phosphoinositides might interact with lysine residues in the C-terminal domain of the P2X$_1$ receptor, further potentiating I_{ATP}.

DISCUSSION

P2X receptors are ligand-gated cation channels activated by extracellular ATP (Burnstock et al., 1997). P2X receptors are widely distributed in the brain, peripheral nervous system, smooth muscles, and blood cells and have physiological and pathophysiological roles (Jarvis et al., 2009; North et al., 2002). P2X$_1$ receptors are expressed in smooth muscle cells, e.g., in the urinary bladder, vas deferens, and genitourinary systems, regulating smooth muscle contraction in rodents (Mulryan et al., 2000) and humans (Banks et al., 2006). A main characteristic of the P2X$_1$ receptor is rapid desensitization after repeated treatment with receptor agonists (North et al., 2002).

In the present sturdy, we found that gintonin, a novel ginseng-derived LPA receptor ligand, potentiates P2X$_1$ receptor channel activity. We further investigated how activation of G protein-coupled LPA receptor by gintonin is coupled to potentiation of ligand-gated P2X$_1$ receptor channel activity. Three key observations indicate that gintonin regulates P2X$_1$ receptor channel activity *via* phosphoinositides. First, gintonin-mediated I_{ATP} potentiation was achieved through PLC and PKC activation. Second, gintonin-mediated I_{ATP} potentiation was independent of the intracellular Ca^{2+} level. Third, gintonin-mediated I_{ATP} potentiation was blocked by a PI4-kinase inhibitor, and mutations of the phosphoinositide binding site of the P2X$_1$ receptor significantly attenuated the action of gintonin on the P2X$_1$ receptor channel activity. These results indicate that activation of the LPA receptor-signaling pathway by gintonin causes PI4-kinase activation and the production of PIP_2. The produced PIP_2 might interact with the P2X$_1$ receptor C-terminal domain and hence potentiate I_{ATP} even after P2X$_1$ receptor desensitization by ATP.

We previously demonstrated that gintonin evoked a $[Ca^{2+}]_i$ transient and activated endogenous CaCC in *Xenopus* oocytes *via* LPA receptor activation (Hwang et al., 2012; Pyo et al., 2011). Thus, the main action of gintonin is to induce a transient elevation of Ca^{2+} from the endoplasmic reticulum, and the re-

leased Ca^{2+} can subsequently influence diverse Ca^{2+}-dependent ion channel and receptor activities (Hwang et al., 2012; Pyo et al., 2011). However, in the present study, although we observed that gintonin-mediated Ca^{2+} release is coupled to endogenous CaCC activation, we found that gintonin-mediated Ca^{2+} release did not mediate I_{ATP} potentiation because the membrane-permeable Ca^{2+} chelator, BAPTA-AM, had no effect on the gintonin-mediated I_{ATP} potentiation. Thus, the present study showed that LPA receptor activation by gintonin might be coupled to the production of PIP_2, because wortmannin, a PI4-kinase inhibitor, attenuated the gintonin-mediated I_{ATP} potentiation. Supporting this notion is that direct injection of PIP_2 into oocytes expressing $P2X_1$ receptor also caused a rescue of I_{ATP} from desensitization but gintonin did not further potentiate I_{ATP}.

In previous reports, the activation of G protein-coupled receptors, such as the $5\text{-}HT_{2A}$ and mGlu receptors coupled to the $G\alpha_{q/11}$ signaling pathway, has been shown to potentiate I_{ATP} after $P2X_1$ receptor desensitization (Ase et al., 2005; Bernier et al., 2008; Vial et al., 2004). These reports showed that phosphoinositides (and not calcium) are involved in $5\text{-}HT_{2A}$ or mGlu receptor-mediated potentiation of I_{ATP} after $P2X_1$ receptor desensitization and further showed that site-directed mutagenesis of the phosphoinositide binding site greatly attenuates the receptor agonist-mediated I_{ATP} potentiation of the $P2X_1$ receptor. In the present study, we also showed that the gintonin-mediated I_{ATP} potentiation of the $P2X_1$ receptor through LPA receptor activation (coupled to the $G\alpha_{q/11}$ signaling pathway) was attenuated in the presence of wortmannin, a PI4-kinase inhibitor. Furthermore, mutations of the phosphoinositide binding site, such as K364A and K364Q, also attenuated the gintonin-mediated I_{ATP} potentiation. However, we could observe that mutations of phosphoinositide binding sites did not completely abolish gintonin-mediated I_{ATP} potentiation. These results are well consistent with previous report that mutations of phosphoinositide binding sites did not completely abolish $5\text{-}HT_{2A}$ receptor-mediated I_{ATP} potentiation (Bernier et al., 2008). These results indicate that LPA receptor activation by gintonin utilizes phosphoinositides in addition to Ca^{2+} as signaling mediators.

As described above, $P2X_1$ receptors are mainly expressed in reproductive organs and are involved in smooth muscle contraction in reproductive organs such as the prostate (Banks et al., 2006; Dunn et al., 2000; Mulryan et al., 2000). The rapid desensitization of the $P2X_1$ receptor is associated with reduced contractive activity (Banks et al., 2007; Burnstock et al., 2007; Sneddon et al., 2000). Thus, $P2X_1$ receptors are essential for reproductive functions, and selective $P2X_1$ receptor antagonists and agonists that potentiate the ATP-action on $P2X_1$ receptors are under development (Dunn et al., 2000). LPA receptors, especially LPA3 receptors, are also abundantly expressed in female and male reproductive organs, e.g., the uterus, and are important during embryo implantation (Diao et al., 2010). Currently, it is unknown whether LPA receptors are co-expressed with $P2X_1$ receptors in the same cells or organs. In the present study, we found that gintonin potentiates I_{ATP} *via* LPA receptor activation after $P2X_1$ receptor desensitization; however, it is unclear whether gintonin-mediated potentiation of I_{ATP} is beneficial to smooth muscles in reproductive organs. Further studies are therefore required for clinical evaluation of gintonin for reproductive smooth muscle function in organs such as vas deferens.

In summary, we found that gintonin potentiates I_{ATP} after $P2X_1$ receptor desensitization *via* the LPA receptor-PLC-PKC-PI4-kinase signaling pathways and that mutations of the phosphoinositide binding site of the $P2X_1$ receptor attenuate the effect of gintonin on $P2X_1$ receptor channel activity. These results suggest that gintonin, *via* LPA receptor activation, stimulates phosphoinositide turnover, and the gintonin-mediated production of phosphoinositides contributes to the potentiation of $P2X_1$ receptor channel activity.

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