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Novel Glycolipoproteins from Ginseng

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Ginseng has been used as a general tonic agent to invigorate human body. In the present study, we isolated novel glycolipoproteins from ginseng that activate Ca^{2+}-activated Cl^- channel (CaCC) in *Xenopus* oocytes and transiently increase intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in mouse Ehrlich ascites tumor cells. We named the active ingredients as gintonin. Gintonin exists in at least six different forms. The native molecular weight of gintonin is about 67 kDa but its apparent molecular weight is about 13 kDa, indicating that gintonin might be a pentamer. Gintonin is rich in hydrophobic amino acids. Its main carbohydrates are glucose and glucosamine. Its lipid components are linoleic, palmitic, oleic, and stearic acids. Gintonin actions were blocked by U73122, a phospholipase C inhibitor, 2-aminoethxydiphenyl borate, an inositol 1,4,5-trisphosphate receptor antagonist, or bis (o-aminophenoxy) ethane-N,N,N0,N0-tetracetic acid acetoxymethyl ester, a membrane permeable Ca^{2+} chelator. In the present study, we for the first time isolated novel gintonin and showed the signaling pathways on gintonin-mediated CaCC activations and transient increase of $[Ca^{2+}]_i$. Since $[Ca^{2+}]_i$ as a second messenger plays a pivotal role in the regulation of diverse Ca^{2+}-dependent intracellular signal pathways, gintonin-mediated regulations of $[Ca^{2+}]_i$ might contribute to biological actions of ginseng.

Keywords: *Panax ginseng*, Korean red ginseng, Glycolipoproteins, Gintonin, $[Ca^{2+}]_i$ mobilization

INTRODUCTION

Ginseng has been used as a general tonic or an adaptogen to promote longevity and enhance bodily functions against stress, fatigue, diseases, cancer and diabetes mellitus [1]. Ginseng is one of the most widely used and precious herbal medicines consumed in around the world [1]. Ginsenosides (also called ginseng saponins) were first isolated from ginseng in the beginning of 1960s and well characterized [2,3]. Ginsenosides have been used and reported as the representative ingredients

in most ginseng-related biochemical studies [1]. Since the content of ginsenoside within ginseng is low, ginsenoside isolation from ginseng is complicated, and as a result pure ginsenosides are expensive, thus we and others have used crude ginseng total saponin fraction (CGSF) that has been prepared from butanol extraction of ginseng root to explain various *in vitro* and *in vivo* physiological or pharmacological actions of ginseng [4-10].

Regarding to intracellular Ca^{2+}-related actions of CGSF,

we have shown that CGSF achieves its action through well-known membrane signaling pathways such as guanosine triphosphate (GTP)-binding protein-coupled receptors. For example, Choi *et al.* [5] demonstrated that CGSF treatment activated endogenous Ca^{2+}-activated Cl^- channel (CaCC) through the signaling pathway that activates pertussis toxin-insensitive $G\alpha_{q/11}$ proteins coupled to phospholipase C (PLC)β-inositol 1,4,5-trisphosphate (IP_3) in *Xenopus* oocytes. Lee *et al.* [11] also demonstrated that CGSF-mediated CaCC activation was desensitized through activation of G protein kinase 2 and β- arrestin I in *Xenopus* oocytes. CGSF treatment induced store-operated Ca^{2+} entry in *Xenopus* oocytes [12]. These results indicate that CGSF treatment induces an increase of $[Ca^{2+}]_i$ from intracellular Ca^{2+} stores or from outside the cell and that the elevated $[Ca^{2+}]_i$ is coupled to CaCC activations in *Xenopus* oocytes [13].

During purification processes for isolating pure ginsenosides from CGSF to identify which ginsenoside(s) are responsible for CaCC activations, we have obtained evidences that ginsenosides in CGSF are not responsible for CaCC activations in *Xenopus* oocytes. In the present study, to further identify and characterize the novel ingredients underlying CaCC activations in CGSF, we have for the first time, isolated the active ingredients. We found that the active ingredients are a type of glycolipoproteins. We named the gintonin; transient intracellular Ca^{2+} elevating agents isolated from ginseng. Since Ca^{2+} is a second messenger and is involved in a variety of cellular functions such as the regulation of contraction, plasticity, secretion, synaptic transmission, and gene expression [14], gintonin-mediated elevation of $[Ca^{2+}]_i$ may play an important role in the regulation of a variety of Ca^{2+}-dependent intracellular signaling pathways.

MATERIALS AND METHODS

Materials

Six-year-old Korea red ginseng (Korea Ginseng Co-operation, Daejeon, Korea) were purchased from local ginseng market and identified by adjunct professor NJ Kim, College of Pharmacy, Kyung Hee University and the specimen (CVMKU 200607-14) were deposited at the herbarium of the laboratory. American ginseng (*Panax quinquefolis*) roots were obtained from Hsu's Ginseng Enterprise & Hsu Ginseng Farm (Wausau, WI, USA). BioLogic DuoFlow™ chromatography systems were purchased from Bio-Rad (Hercules, CA, USA). Ehrlich ascites tumor (EAT) cells were kindly provided from Riken BRC Cell Bank (Tsukuba, Japan). The

prepacked columns (Superdex 75 10/30/300 GL and HiTrap™ DEAE FF) and media (DEAE Sepharose CL-6B) were purchased from GE Healthcare (Uppsala, Sweden). Fura 2-AM was obtained from Molecular Probes (Eugene, OR, USA). Schiff's reagent, tubing cellulose membrane with molecular weight cut off at 13,000, and other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Male ICR mice (20-30 g) were obtained from Orient Bio, Inc. (Seongnam, Korea). Ginsenoside Ra, Rb_1, Rb_2, Rc, Rg_1, and other ginsenosides were purchased from LKT institute (St. Paul, MN, USA).

Preparation of non-saponin fraction from methanol and butanol extractions

Twenty kilograms of 6-year-old Korean red ginseng roots were grinded into small pieces (>3 mm) and refluxed with 80% methanol (MeOH) three times for 8 h at 80°C each. The MeOH extracts (6.2 kg) concentrated *in vacuo* was partitioned between *n*-butanol (*n*-BuOH) and water. *n*-BuOH fraction (908 g), after concentration, was applied to a silica gel column and was eluted with chloroform ($CHCl_3$):MeOH:water (H_2O)=13:7:2. Each fraction was tested on endogenous CaCC activations in *Xenopus* oocytes. The only active fraction was further separated through a silica gel column and was eluted with ethyl acetate (EtOAc):ethanol (EtOH):H_2O=1:3:0.5. The active fraction which activates CaCC was dialyzed at 4°C for 8 h with 1,000-fold excess distilled water using Spectra/Por dialysis membrane (molecular weight cut off 6,000-8,000) (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) to remove small molecular components such as ginsenosides and other components that might remain in the fraction. We named this fraction as crude gintonins, which does not contain ginsenosides.

Isolation of individual gintonins from crude gintonins

Crude gintonins from butanol extract of Korean red ginseng dissolved in phosphate buffer saline (PBS) (pH 7.2) was loaded onto a column packed with DEAE sepharose CL-6B (GE Healthcare) and equilibrated with PBS (pH 7.2). The unbound materials were eluted with the same buffer and the bound materials were eluted with a linear gradient of 0 to 2 M NaCl in PBS (pH 7.2). Fractions (10 mL) were collected with a flow rate 1 mL/min and monitored at 280 nm. Each fraction was assayed for CaCC activity of *Xenopus* oocytes. The active fractions were selected and pooled into two groups, early eluted unbound active fraction (gintonin E) and late eluted bound active fraction (gintonin L). Each pooled group was concentrated by CentriVap DNA vacuum

concentrator (Labconco, Kansas City, MO, USA) and chromatographed on Superdex 75 gel filtration column (10×300 mm) using PBS (pH 7.2) as an eluent. Fractions were collected with a flow rate 0.5 mL/min and monitored at 280 nm. Each pooled gintonin E and L fraction was dialyzed against H_2O and tested for CaCC activation. The pooled gintonin E fractions were again loaded onto a DEAE anion exchange column (HiTrap™ DEAE FF, 1 mL) and first equilibrated with 20 mM Tris-HCl (pH 8.0). The bound substances were eluted by four step gradients of 20 mM Tris-HCl (pH 8.0) containing 100, 150, 200, and 250 mM NaCl at a flow rate of 1 mL/min. Each peak containing 100, 150, and 200 mM NaCl eluent, after extensive dialysis and concentration, were again applied to the DEAE anion exchange column with a linear gradient of 0-1 M NaCl in 20 mM Tris-HCl (pH 8.0) and were finally applied to Superdex 75 gel filtration chromatography to confirm the single peak. Three single peaks obtained from DEAE anion exchange and Superdex 75 gel filtration chromatographies were named as gintonin E1 (3.7 mg), E2 (26.7 mg), and E3 (13.5 mg). Similarly, the pooled gintonin L fractions were also applied to the DEAE anion exchange column (HiTrap™ DEAE FF, 1 mL) and equilibrated with PBS (pH 7.2). The bound gintonin L fractions were eluted by five step gradients with PBS (pH 7.2) containing 150, 200, 250, 300, and 400 mM NaCl at flow rate 1 mL/min. Three peaks obtained from PBS (pH 7.2) containing only 150, 200, and 250 mM NaCl eluent were collected and were subjected to extensive dialysis and concentration. These peaks were again applied to linear gradient DEAE anion exchange chromatography with a gradient of 0-1 M NaCl in PBS (pH 7.2) to confirm the single peak. Finally, these peaks were applied onto Superdex 75 gel filtration chromatography to obtain a final single peak. These single peaks obtained from DEAE anion exchange and Superdex 75 gel filtration chromatographies were named as gintonin L1 (7.4 mg), L2 (17.6 mg), and L3 (6.6 mg) (Fig. 1).

Molecular mass determination of gintonin by gel filtration

The molecular weights of gintonins were determined by gel filtration chromatography using the method from Yamamoto *et al.* [15]. Gel filtration chromatography was carried out using a Superdex 75 column (10×300 mm) equilibrated with PBS (pH 7.2) on BioLogic DuoFlow™ chromatography systems (Bio-Rad). Fractions were collected with a flow rate of 0.5 mL/min and monitored at 280 nm. The molecular weights of gintonins were determined using a calibration curve consisting of standard

proteins such as IgG (160,000 Da), bovine serum albumin (BSA; 67,000 Da), β-lactoglobulin (35,000 Da), cytochrome C (12,327 Da), and aprotinin (6,512 Da).

Electrophoresis

The individual purified gintonins was subjected to SDS-PAGE using 12.0% separating gel [16]. Crude gintonins, gintonin E1-E3, or L1-L3 (100 μg each) was loaded in each lane. After electrophoresis, gintonin bands were visualized by Coomassie Brilliant Blue R-250 staining. For glycoprotein detection, gels were stained using periodic acid-Schiff base staining (PAS). The gel was incubated in 40% EtOH-7% acetic acid in H_2O for 30 min and then with 1% periodic acid-3% acetic acid for 1 h. The periodic acid solution was removed and Schiff's reagent was added and incubated for 1 h. Bands of stained glycoproteins developed a pink color and the PAS-stained gels were stored in water after soaking in 7.5% acetic acid.

Gintonin amino acid composition analysis

Gintonin (30 μg) was hydrolyzed *in vacuo* in 6 N HCl for 24 h at 110°C for general amino acid analysis. For the analysis of cysteine, gintonin was hydrolyzed in 6 N HCl for 24 h at 110°C after peroxidation treatment with formic acid:hydrogen peroxide=10:1. For the analysis of tryptophan, the sample was hydrolyzed in 4 M methanesulfonic acid and 4 M potassium hydroxide was also added. Amino acids converted to phenylisothiocyanate derivates were analyzed by high performance liquid chromatography (HP 1100; Hewlett-Packard, Palo Alto, CA, USA) with Waters Nova-Pak C18 column (3.9×300 mm) at the Korea Basic Science Institute (Seoul, Korea). Protein contents were determined by the Bradford method using BSA as a standard [17].

Partial amino acid sequence of gintonins

N-terminal amino acid sequencing of gintonin was performed by automated Edman degradation on a gas-phase protein sequencer model 477A (Applied Biosystems, Foster City, CA, USA) and on a MilliGen 6600 solid-phase sequencer (Milligen, Millford, MA, USA). Individual gintonin (100 μg each) was applied to a 12% SDS-PAGE and protein band was visualized by Coomassie Brilliant Blue R-250 staining. The band was cut and subjected to in-gel digestion with trypsin. The digestion products were desalted and concentrated. All mass spectrometer (MS/MS) of peptides generated by the in-gel digestion was performed by nano-electrosprary on a MS (QTOP II; Micromass, Manchester, UK) at the Korea Basic Science Institute. Product ions were analyzed us-
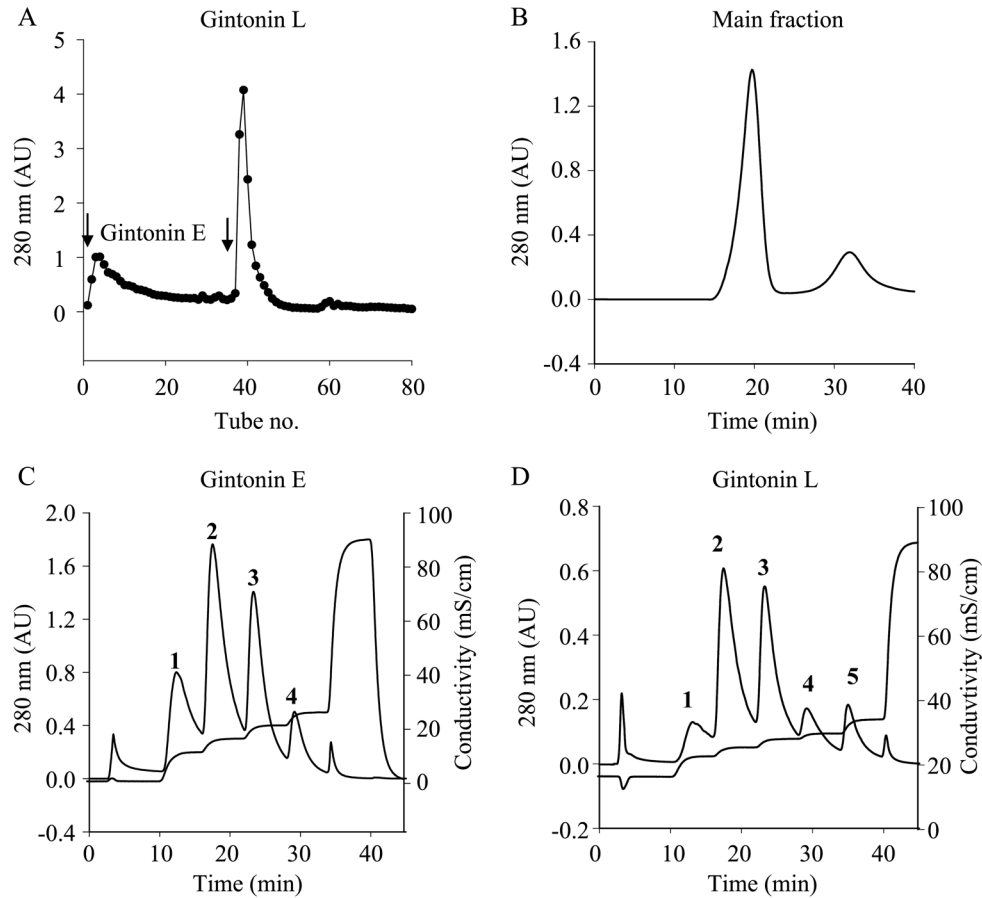
Fig. 1. Crude gintonin includes six individual gintonins with Ca²⁺-activated Cl⁻ channel activation in *Xenopus* oocytes. (A) Crude gintonin was separated into gintonin E and L fractions using DEAE sepharose CL-6B anion exchange chromatogram. Gintonin E fraction (first arrow) was eluted with phosphate buffer saline (PBS) (pH 7.2) and gintonin L fraction (second arrow) was eluted with a linear gradient of 0 to 2 M NaCl in PBS (pH 7.2). (B) The representative Superdex 75 gel filtration chromatogram with PBS (pH 7.2) of gintonin L fraction obtained from (A). The gel filtration chromatograms of gintonin E also exhibited a similar elution pattern with gintonin L. (C,D) Stepwise gradient DEAE anion exchange chromatogram of gintonin E and L fraction.

ing an orthogonal time-of-flight (TOF) analyzer fitted with a reflector, a microchannel plate detector, and a time-to-digital converter. The data were processed using a personal computer running Mass Lynx software on the Window NT environment. The sequence homologues were searched for using the BLAST program (http://www.ncbi.mlm.nih.gov/BLAST.cgi).

Carbohydrate composition

Individual gintonins (2.0 mg each) were hydrolyzed in 2 M trifluoroacetic acid for 4 h at 100°C for neutral sugar and hydrolyzed in 6 N HCl for 4 h at 100°C for amino sugar and acid sugar in glass. Carbohydrate compositions of gintonins were analyzed by high performance anion exchange chromatography-pulsed amperometric detection system (HPAEC-PAD system; Dionex, Sunnyvale, CA, USA) with a CarboPac™ PA1 column at the Carbohydrate Bioproduct Research Cen-

ter, Sejong University (Seoul, Korea). The molar ratios of monosaccharides were calculated from peak areas. The carbohydrate contents were also determined by phenol-sulfuric acid method for neutral sugar [18] and anthrone method for acid sugar [19].

Lipid composition analysis

Individual gintonins (1.0 mg each) were hydrolyzed in 6 N HCl for 4 h at 100°C or digested by lipoprotein lipase to confirm lipid and hydrophobic moiety. Acid hydrolyzed or digested gintonins were partitioned between distilled water and *n*-BuOH. The *n*-BuOH layer, after concentration, was further partitioned between distilled water and *n*-hexane. The *n*-hexane layer was prepared for lipid and hydrophobic moiety analysis by an Agilent 6890N gas chromatography (GC)-MS system (Agilent Technologies, Palo Alto, CA, USA) with a DB5-MS capillary column (30 cm×250 μm×0.25 μm) at the Korea Ba-

sic Science Institute and by GC (Agilent 6890N) equipped with flame ionization detector and a split injection system and fitted with a supelco SPB-1 capillary column (15 m×0.32 mm inside diameter, 0.25 mm thickness).

Oocyte preparation and measurements of endogenous CaCC in *Xenopus* oocytes

Collection of *Xenopus laevis* oocytes and recording of endogenous CaCC in *Xenopus laevis* oocytes were performed as described in previous reports [11].

EAT cell preparation, loading of EAT cells with fura 2-AM, and fluorescence measurements of $[Ca^{2+}]_i$ in cell suspensions

EAT cells were maintained in male mice by weekly intraperitoneal transplantation into fresh mice. The Ehrlich cells were harvested as described by Jorgensen *et al*. [20]. EAT cell preparation, loading of EAT cells with fura 2-AM, and fluorescence measurements of $[Ca^{2+}]_i$ in cell suspensions were performed as described by Jorgensen *et al*. [20]. $[Ca^{2+}]_i$ was estimated in fura 2-loaded cells in suspension using a RF-5300PC intracellular ion measurement system (Shimadzu Corporation, Kyoto, Japan). Briefly, the fura 2-AM loaded cells were diluted with experimental medium to a final 2-4×10^6/mL and transferred to polystyrene cuvettes (Elkay Ultra-VU; Elkay Products, Shrewsbury, MA, USA). The cells were stirred using Teflon-coated magnets, and the cuvette housing was thermostatically controlled at 37°C. The excitation wavelengths were alternated between 340 and 380 nm under computer control. Emission was detected at 510 nm. Excitation and emission slit widths were 5 nm. Background correction was performed as described in Jorgensen *et al*. [20]. Digitonin and ethylene glycol tetraacetic acid were used as concentration adjustment reagents to make states in which fura 2 completely combines with and disassociates from Ca^{2+}. All experiments were conducted at 37°C. Finally, estimation of $[Ca^{2+}]_i$ from fura 2 measurements was performed as described in previous report [21].

Data analysis

To obtain the concentration-response curve in the presence of crude gintonin or purified individual gintonins the observed peak amplitudes were normalized and plotted using Origin software (MicroCal, Northampton, MA, USA). The EC_{50} is the concentration of crude gintonin or individual gintonins producing half-maximum effect of the control response to crude gintonin or individual gintonins and was calculated as described in previous

report [11]. All values are presented as means±SEM.

RESULTS AND DISCUSSION

Separation of crude and individual gintonins from CGSF of ginseng

We first prepared large amounts of CGSF through butanol extraction and performed the massive silica gel chromatography with composition of solvents ($CHCl_3$:MeOH:H_2O=13:7:2). The fraction causing CaCC activations in *Xenopus* oocytes was further subjected to silica gel chromatography with different solvent compositions (EtOH:EtOAc:H_2O=3:1:0.5). We found that the fraction causing CaCC activations was high molecular ingredients. So, we performed further extensive dialysis (pore size, 6,000-8,000) of the active fraction to remove the remaining low molecular components and named as a crude gintonin fraction, which was devoid of ginsenosides and other small molecules in ginseng.

Crude gintonin fraction was first subjected to DEAE Sepharose anion-exchange chromatography using PBS (pH 7.2) containing 0 to 2 M NaCl as an eluent and this procedure produced two major peaks with CaCC activations (Fig. 1A). We named major peak 1 as early eluted gintonin (gintonin E), unbound active fraction and major peak 2 as late eluted gintonin (gintonin L), bound active fraction. We further performed gel filtration chromatography using the pooled gintonin E and L fractions and obtained one main peak with higher molecular weights and minor peaks in gintonin E and L fractions (Fig. 1B). The pooled main peaks causing CaCC activations of gintonin E fractions were again eluted by stepwise gradient anion exchange chromatography with a buffer of 20 mM Tris-HCl (pH 8.0) containing 100, 150, 200, and 250 mM NaCl, and this procedure produced four peaks (Fig. 1C). In these peaks, the first, second, and third peaks but not the fourth peak mainly caused CaCC activations (data not shown). The pooled main peaks of gintonin L fractions were also eluted by stepwise gradient anion exchange chromatography with PBS (pH 7.2) containing 150, 200, 250, 300, and 400 mM NaCl (Fig. 1D). In these peaks, the first, second, and third peaks, but not the fourth and fifth peaks, also mainly caused CaCC activations (data not shown). Thus, we were able to obtain six different individual gintonins from gintonin E and L fractions associated with CaCC activation in *Xenopus* oocytes. We named them as gintonin E1, E2 and E3 or gintonin L1, L2 and L3, depending on their elution patterns after DEAE-anion exchange chromatography (Fig. 1). The purity of these individual gintonins

was confirmed by the following gel filtration chromatography and further linear anion exchange chromatography and SDS-PAGE (Figs. 2 and 3). The final yields of gintonin E1 (3.7 mg), E2 (26.7 mg), E3 (13.5 mg), L1 (7.4 mg), L2 (17.6 mg), and L3 (6.6 mg) from CGSF (908 g) were 0.0004%, 0.0029%, 0.0015%, 0.0008%, 0.0019%, and 0.0007%, respectively.

Determination of molecular weight of gintonin

Although most of individual gintonins were eluted at the same time in gel filtration chromatography (Fig. 2A, C), they showed slightly different elution patterns in NaCl gradient application (Fig. 2B, D), revealing the possibility that their molecular weights are almost same but the charges that they carry might differ between them. We determined the molecular weight of gintonin L2 as a representative gintonin using standard proteins and found that the molecular weight of gintonin L2 was about 67 kDa (Fig. 3A). The native molecular weights of

other gintonins were also about 67 kDa (data not shown). Interestingly, in SDS–PAGE six individual gintonins showed a broad but single major band and its apparent molecular weight was about 13 kDa, showing a possibility that gintonins are of a pentameric nature (Fig. 3B).

De novo sequence and amino acid composition of individual gintonins

Purified gintonins were subjected to N-terminal amino acid sequencing by automated Edman degradation. We could not obtain full amino acid sequences of gintonin because of N-terminal blocking. We could only obtain several partial internal peptides sequences from trypsinized gintonins using MALDI-TOF-MS/MS. The amino acid sequences did not show any homologies with known plant proteins (data not shown). The contents of total proteins in gintonins by Bradford method were about 9.4, 24.1, 20.5, 35.8, 37.1, and 39.6% for gintonin E1, E2, E3, L1, L2, and L3, respectively. Thus, protein
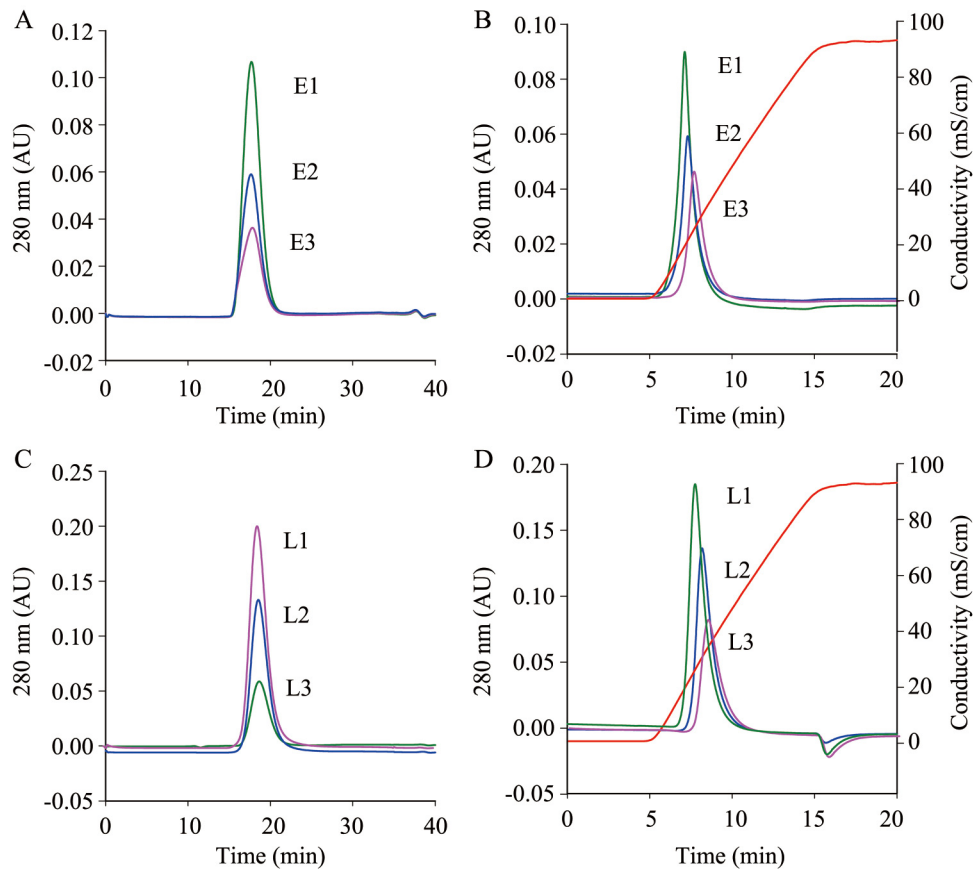
Fig. 2. Elution patterns of individual gintonins by gel chromatography or continuous anion exchange chromatography. (A,C) Overlay of gel filtration chromatograms on Superdex 75 column with phosphate buffer saline (pH 7.2) of purified gintonin E1, E2, E3, L1, L2, and L3. (B,D) Overlay of anion exchange chromatograms of purified gintonin E1, E2, E3, L1, L2, and L3. Analysis through two chromatograms shows that the individual gintonins are isolated as a single peak, reflecting that the molecular weights of six individual gintonins are almost same (A,C) but that the charges that they carry might differ between them (B,D).
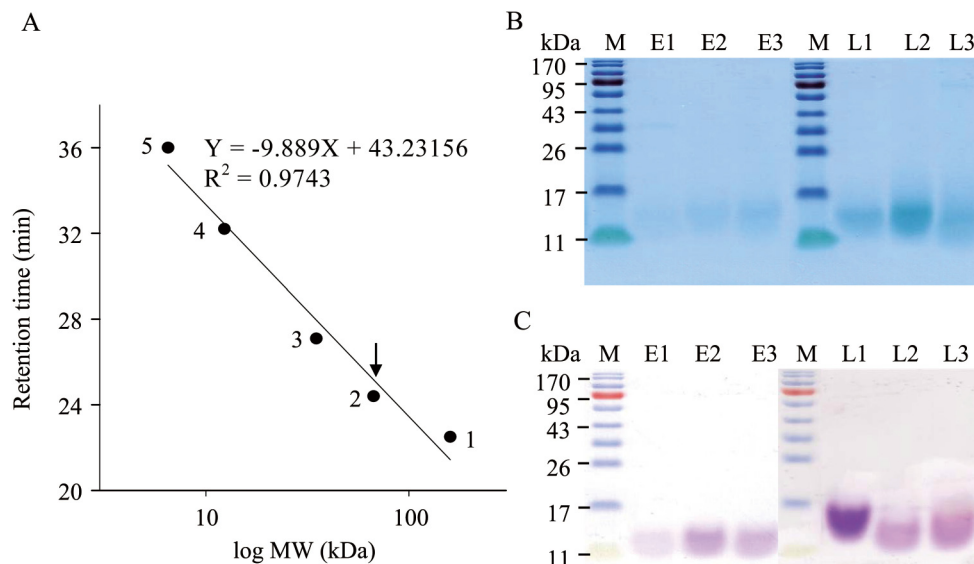
Fig. 3. Determination of molecular weight of gintonin and gel staining of carbohydrate components of individual gintonins. (A) Determination of molecular weight of gintonin through gel filtration chromatography. Gel filtration chromatogram of gintonin L2 in Superdex 75 column with phosphate buffer saline (pH 7.2). The numbers represent standard proteins used: 1) IgG (160 kDa), 2) bovine serum albumin (BSA) (67 kDa), 3) β-lactoglobulin (35 kDa), 4) cytochrome C (12 kDa), 5) aprotinin (6.5 kDa). The arrow indicates gintonin L2 that is almost coincided with BSA standard. (B) Determination of molecular weight of gintonin through SDS-PAGE. M represents molecular markers. Comassie Brilliant blue staining was used to stain protein moieties of gintonins. Six gintonins in SDS-PAGE showed that apparent molecular weight of gintonins is about 13 kDa. (C) Periodic acid-schiff staining demonstrates that individual gintonins contain carbohydrate moiety and shows that the amount of carbohydrate moiety of each gintonin differs between them.

contents of gintonin L1-L3 were about three times higher than those of gintonin E1. Amino acid composition of individual gintonins was summarized in Table 1.

Carbohydrate and lipid composition of individual gintonins

As shown in Fig. 3B, since the bands of individual gintonins from SDS-PAGE are single but broad and are not strongly stained with Comassie brilliant blue staining, there is a possibility that gintonin might contain carbohydrate moieties. Therefore, we used PAS technique to know whether gintonin contains carbohydrates. Individual gintonins were strongly stained by PAS as shown in Fig. 3C. Next, we examined carbohydrate composition of gintonins using HPAEC-PAD system. Table 2 showed that carbohydrate compositions of six gintonins were almost similar to each other. Glucose was major component, while rhamnose, mannose, and xylose were minor. Interestingly, galactose and mannose in gintonin L1-L3 was not detected (Table 2). Finally, the content of total carbohydrate in gintonins were about 34.3, 45.3, 38.1, 36.0, 29.8, and 45.2% for gintonin E1, E2, E3, L1, L2, and L3, respectively. The above results again confirmed that gintonins are glycoproteins.

Since gintonins were co-fractionated with ginsenosides by butanol extraction, we examined the possibility that

Table 1. Amino acid composition of gintonins

Amino acid	E1	E2	E3	L1	L2	L3
CYA	2.90	3.87	1.29	0.97	1.14	0.94
ASX	8.27	8.60	6.11	1.89	3.71	3.98
GLX	6.67	7.22	4.57	2.43	4.89	4.25
Ser	4.57	4.29	3.29	2.29	5.00	3.59
Gly	12.40	13.67	11.55	12.88	11.57	10.56
His	0.00	0.00	0.00	0.00	0.00	0.00
Arg	1.68	1.85	1.19	0.63	1.69	1.10
Thr	3.11	3.29	2.20	1.37	1.46	1.79
Ala	5.62	6.98	3.59	4.52	4.07	4.67
Pro	4.52	4.90	3.30	3.18	3.09	4.86
Tyr	0.00	0.00	0.00	0.00	0.00	0.00
Val	3.13	3.89	3.74	3.99	4.68	6.00
Met	0.00	0.00	0.00	0.00	0.00	0.00
Ile	6.03	5.16	7.54	10.36	8.83	7.76
Leu	3.96	5.09	3.43	4.26	6.83	10.09
Phe	26.40	18.66	35.10	28.89	21.37	22.47
Trp	3.59	7.10	3.62	4.88	8.13	6.10
Lys	7.14	5.42	9.78	17.48	13.56	11.85

Data are presented as percentage.
The detail methods for amino acid compositions of each gintonin are described in Materials and Methods.
CYA, the sum of cysteine and cystine; ASX, the sum of asparagine and aspartic acid, GLX, the sum of glutamine and glutamic acid.

Table 2. Carbohydrate composition of gintonins

Carbohydrate	E1	E2	E3	L1	L2	L3
Rhamnose	0.86	1.02	0.91	1.11	0.93	1.09
Arabinose	5.66	5.10	5.66	9.11	8.32	7.36
Glucose	84.84	84.41	83.97	77.16	74.64	71.59
Manose				1.76	2.30	3.37
Xylose	1.12	0.94	0.95	2.73	2.55	2.50
Glucosamine	7.52	8.53	8.51	8.13	11.26	14.09

Data are presented as percentage.
The detail methods for carbohydrate compositions of each gintonin are described in Materials and Methods.

gintonin also contained lipid moieties. As shown in Table 3, individual gintonins contained fatty acids such as palmitic, stearic, oleic and linoleic acids. The portion of polyunsaturated fatty acid, linoleic acid in gintonin E1 and L1 were higher than saturated fatty acids such as palmitic and stearic acid or oleic acid. Palmitic acid was predominant in gintonin E2, E3, L2, and L3. Next, we estimated total contents of lipid moiety in gintonins by GC. The contents of total lipids in gintonins were 20.7, 4.4, 2.4, 11.0, 6.2 and 7.0% for gintonin E1, E2, E3, L1, L2 and L3, respectively. Thus, gintonin E1 has the highest lipid contents among them. These results indicate that gintonins are novel glycolipoproteins.

Effects of crude and individual gintonins on endogenous CaCC in *Xenopus* oocytes

We examined whether gintonin but not ginsenosides induce CaCC activation. As shown in Fig. 4, treatment of crude gintonin from Korean red ginseng and American ginseng induced inward Cl⁻ currents in a concentration-dependent manner at –70 mV holding potential (Fig. 4A). The ED_{50} was 4.0±0.2 and 4.4±0.5 µg/mL for Korean red ginseng and American ginseng (Fig. 4C), respectively. Individual ginsenosides tested had no effects on CaCC activity at low and high concentrations (Fig. 4C). Next, we examined the effects of individual gintonin E1, E2 and E3 or gintonin L1, L2 and L3 on CaCC activation in *Xenopus* oocytes. As shown in Fig. 4D, we found that gintonin E1, E2, and E3 showed almost the

same degree of effects on CaCC activation. The ED_{50} values were 1.5±0.1, 2.3±0.1, 1.5±0.1 µg/mL for E1, E2 and E3, respectively. Interestingly, although gintonin L1 showed almost the same degree of effects on CaCC activation with E1 to E3, gintonin L2 and L3 showed weaker CaCC activation than other individual gintonins. The ED_{50} values were 1.0±0.1, 3.2±0.2, and 4.3±0.2 µg/mL for L1, L2 and L3, respectively. Thus, these results indicate that although individual gintonins are similar to each other in molecular weights and physico-chemical properties, there are slight differences in activating CaCC in *Xenopus* oocytes among individual gintonins.

Effect of gintonin or ginsenosides on $[Ca^{2+}]_i$ in mouse Ehrlich ascite tumor cells

Treatment of GTP-binding protein coupled receptor agonists in fura 2-AM-loaded mouse EAT cells induces transient increases in $[Ca^{2+}]_i$. In the present study, we examined whether gintonin or individual ginsenosides such as ginsenoside Ra, Rb₁, Rb₂, Rc and Rg₁ exhibit their effects on transient increases in $[Ca^{2+}]_i$ in fura 2-AM-loaded EAT cells. Because of limitation of available individual gintonins, we used crude gintonin for the study on elevation of $[Ca^{2+}]_i$ in mouse EAT cells. When EAT cells in suspension are first treated with ginsenoside alone (each 100 µM), none of the ginsenosides tested induced any responses in EAT cells (Fig. 5A). In contrast to ginsenosides, crude gintonin treatment (100 ng/mL) caused a transient elevation of $[Ca^{2+}]_i$, as illustrated by the data in Fig. 5A. Fig. 5B also shows that the elevation of crude gintonin-induced $[Ca^{2+}]_i$ was in a concentration-dependent manner. Interestingly, crude gintonin-induced transient elevation of $[Ca^{2+}]_i$ was greatly reduced by removal of external Ca^{2+} (Ca^{2+}-free medium in the presence of 0.2 mM EGTA), indicating that extracellular Ca^{2+} is also required (Fig. 5B). However, treatment of crude gintonin still increased $[Ca^{2+}]_i$ in a concentration-dependent manner, indicating that crude gintonin-induced elevation of $[Ca^{2+}]_i$ is likely due to Ca^{2+} release from intracellular Ca^{2+} stores and Ca^{2+} entry from extracellular side. The EC_{50} values were 33.6±8.2

**Table 3.** Lipids composition of gintonins

| Lipids	E1	E2	E3	L1	L2	L3
Palmitic acid (16:0), esterform	29.82	57.39	56.59	36.48	41.78	39.5
Stearic acid (18:0), esterform	4.22	8.73	8.20	4.74	5.49	5.44
Oleic acid (18:1) or oleic acid ester form	7.08	26.06	27.52	14.32	30.04	26.64
Linoleic acid (18:2) or linoleic acid ester form	58.88	7.82	7.69	44.46	22.69	28.42

Data are presented as percentage.
The detail methods for lipid compositions of each gintonin are described in Materials and Methods.
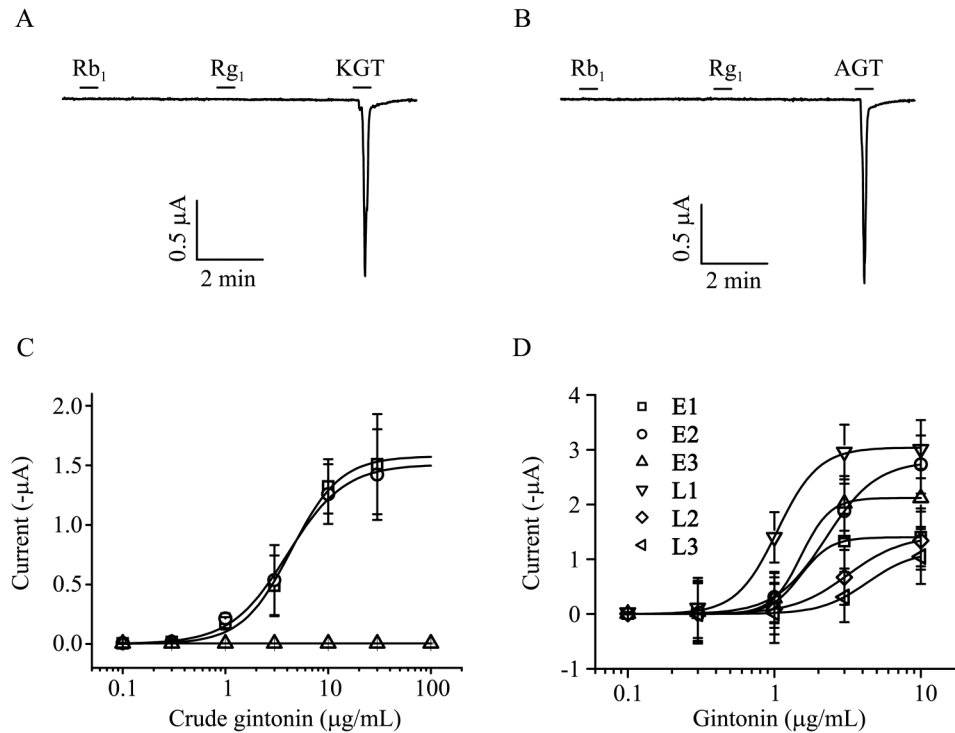
Fig. 4. Crude gintonin and individual gintonins but not ginsenosides activate Ca^{2+}-activated Cl^- channel (CaCC) with concentration-dependent manner. (A,B) Treatment of crude gintonin (10 µg/mL) from Korean red ginseng (KGT) (A) or from American ginseng (AGT) (B) induced inward Cl^-currents in a concentration-dependent manner, whereas treatment of ginsenoside Rb_1 and Rg_1 (100 µM each) did not. (C) Crude gintonin from KGT (□), AGT (○), or ginsenoside Rb_1 (△) and symbols of other ginsenosides including Rc, Rd, Re, Rf, and Rg_1 were omitted for clarification. (D) Individual gintonins increased inward CaCC currents in a concentration-dependent manner. Crude gintonin fraction of AGT (*Panax quinquefolis*) roots was prepared as described in Materials and Methods. Data represent means±SEM (*n*=7-8).
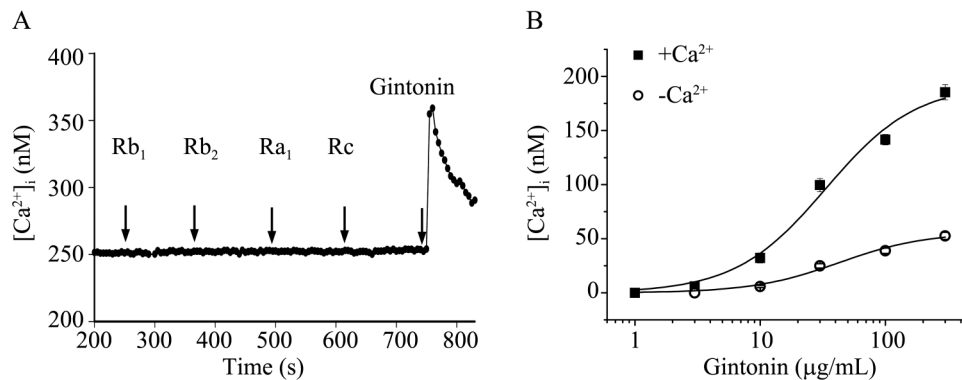
Fig. 5. Gintonin but not ginsenosides elevates $[Ca^{2+}]_i$ in mouse Ehrlich ascites tumor (EAT) cells. (A) Fura 2 loaded cells (2-$4×10^6$/mL) were incubated in Ca^{2+} buffer (1.5 mM Ca^{2+}) for 10 min. Sequential addition of 100 µM ginsenosides such as ginsenoside Ra, Rb_1, Rb_2, Rc and Rg_1 did not induce any responses to EAT cells. However, addition of 100 ng/mL crude gintonin exhibited transient increases of $[Ca^{2+}]_i$ in fura 2-AM loaded EAT cells. (B) Fura 2 cell loaded cells (2-$4×10^6$/mL) were incubated in either Ca^{2+} buffer (1.5 mM Ca^{2+}) or Ca^{2+} free buffer (0.2 mM EGTA) for 10 min. Gintonin transiently increased $[Ca^{2+}]_i$ in EAT cells in a concentration-dependent manner. Data are represented as means±SEM (*n*=5-6/group).

and 43.6±14.2 ng/mL in the presence and absence of extracellular Ca^{2+}, respectively. These results indicate that gintonin but not ginsenosides are responsible for elevation of $[Ca^{2+}]_i$ in mammalian cells.

Effects of phospholipase C inhibitor, IP_3 receptor antagonist, or Ca^{2+} chelator on crude gintonin-mediated CaCC activation and $[Ca^{2+}]_i$ elevation

Next, to explain the possible role of PLC on gintonin-mediated signaling pathway of increases in cytoplasmic

$[Ca^{2+}]_i$ and CaCC activations, we first investigated the effect of active PLC inhibitor, U-73122 and inactive analog, U-73343 using *Xenopus* oocytes and mouse EAT cells. In *Xenopus* oocytes, crude gintonin-induced CaCC currents in U-73122 were 3.9±0.3% of the control, whereas the current in U-73343 was 97.2±0.2% of the control (Fig. 6A). These observations indicate that crude gintonin-mediated CaCC activations are mediated through PLC activation. As a next step, we examined whether or not intracellular Ca^{2+} release through IP_3 receptor was responsible for gintonin-induced CaCC activations. For this, we tested the effects of IP_3 receptor antagonist, membrane permeable 2-aminoethxydiphenyl borate (2-APB) [22], on gintonin-induced CaCC activation. As shown in Fig. 6B, addition of 2-APB almost completely abolished inward CaCC currents induced by gintonin (control currents from 1.3±0.2 to 0.03±0.01 μA). In addition, buffering intracellular free Ca^{2+} by

treatment of membrane permeable 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester (BAPTA-AM) (10 μM final) also almost abolished gintonin-mediated CaCC activation (Fig. 6C). In mouse EAT cells, Fig. 6D shows the increase in $[Ca^{2+}]_i$ in suspensions of mouse EAT cells in Ca^{2+}-containing or Ca^{2+}-free buffer after addition of gintonin in the presence of the active U-73122 or inactive analog U-73343 (1 μM). Thus, application of U-73122 but not U-73343 almost abolished the action of crude gintonin (Fig. 6D). These results again signify that gintonin is the main active components of CGSF for the activation of CaCC via the phospholipase C-IP_3-Ca^{2+} pathway in *Xenopus* oocytes and mouse EAT cells.

In the present study, we identified novel glycolipoproteins from ginseng root; that we named and refer to as gintonin, which was co-fractionated with ginsenosides during CGSF preparation of ginseng. Gintonin treatment
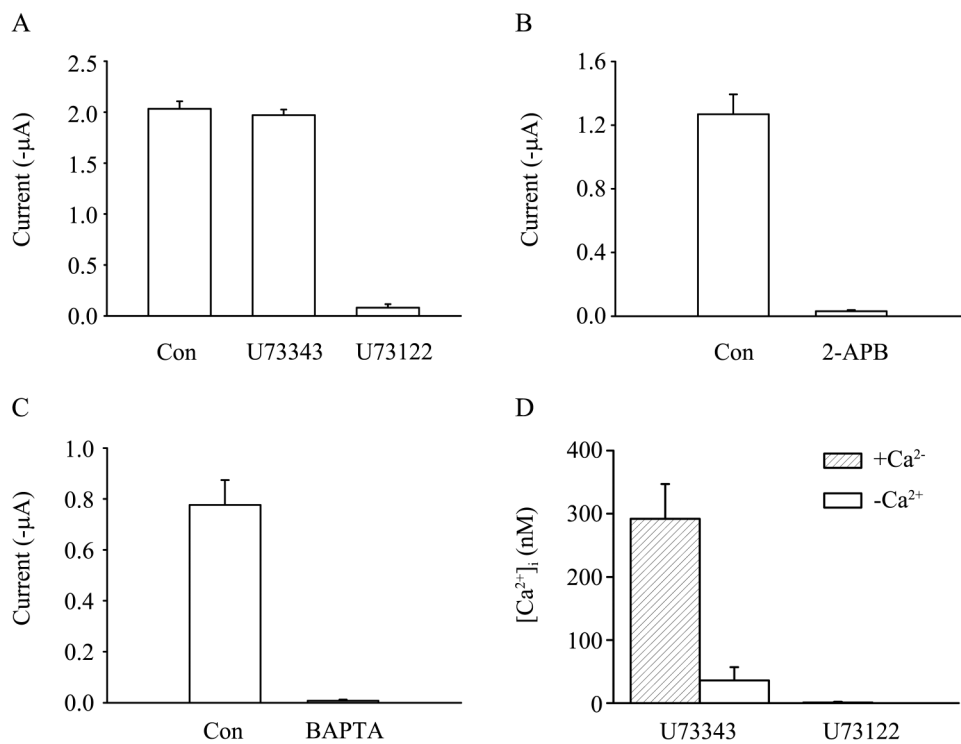
Fig. 6. The activation of Ca^{2+}-activated Cl⁻ channel by gintonin depends on phospholipase C (PLC) and inositol 1,4,5-trisphosphate receptor activation and intraoocyte Ca^{2+} in *Xenopus* oocytes and in Ehrlich ascites tumor (EAT) cells. **(A)** Histograms of current amplitudes were first measured in the presence of crude gintonin (10 μg/mL) at −80 mV holding potential and oocytes were perfused for 5 min with 1 μM of either the inactive (U73343) or the active (U73122) inhibitor of PLC. (B) Histograms of current amplitudes measured from oocytes treated with 1 μM of either H_2O (Con) or 2-aminoethxydiphenyl borate (2-APB) prior to recording and incubated for 5 min. (C) Histograms of current amplitudes measured from oocytes treated with H_2O (Con) or 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester (BAPTA-AM) (10 μM final) prior to recording. After H_2O or BAPTA-AM treated oocytes were further incubated for 2 h in ND96 medium. Current amplitudes are represented as means±SEM (*n*=6). (D) Effects of active or inactive PLC inhibitor on gintonin-mediated increase in cytoplasmic $[Ca^{2+}]_i$ in EAT cells in Ca^{2+} buffer (1.5 mM Ca^{2+}) and in Ca^{2+} free buffer (0.2 mM EGTA). Pretreatment of active PLC inhibitor (U73122, 10 μM) but not inactive PLC inhibitor (U73343, 10 μM) blocked gintonin (100 ng/mL)-mediated elevation of $[Ca^{2+}]_i$ in Ca^{2+} and Ca^{2+} free buffer (0.2 mM EGTA). Data are represented as means±SEM (*n*=4-5/group).

induced a transient elevation of $[Ca^{2+}]_i$ in mouse EAT cells and caused activation of CaCC in *Xenopus* oocytes, whereas individual ginsenosides did not. However, in water-soluble fractions of ginseng, which might also contain proteins and polysaccharides, and in petroleum ether extracts of ginseng that might contain main lipid soluble components, we could not observe any stimulating effects on CaCC activations in *Xenopus* oocytes and elevation of $[Ca^{2+}]_i$ in mouse EAT cells (data not shown). Thus, these results indicate that gintonin is a novel bioactive ligand derived from ginseng.

It is noteworthy that the amino acid composition of individual gintonins revealed the highest contents of phenylalanine, although we do not currently know the functional role of phenylalanine in gintonin (Table 1). In addition, individual gintonins contain the high abundance of hydrophobic amino acids such as alanine, isoleucine, leucine, phenylalanine, tryptophan, and valine. They might be involved in structural roles in gintonins' chemical and physical properties by forming α-helix. Since glucosamine is usually involved in N- or O-linkages of carbohydrate and proteins of glycoproteins, it shows a possibility that glucosamine might couple to asparagine, serine, or threonine of gintonins (Table 1) [23]. In the present study we also found that gintonin contained lipid components. The lipid analysis revealed that individual gintonins also contain palmitic, oleic, linoleic, and stearic acids. Interestingly, gintonin E1 contains linoleic acid more than seven times compared to E2 and E3. Gintonin L1 also contains linoleic acid near twice compared to L2 and L3 (Table 3). However, we could not exclude the possibility that gintonin content or composition is variable from batch-to-batch and from different sources of ginseng. Further studies will be needed to establish standardization of gintonin.

In present study we further revealed that gintonin from ginseng induced CaCC activation in *Xenopus* oocytes and transiently elevate $[Ca^{2+}]_i$ in mouse EAT cells. However, treatment of active PLC inhibitor U73122 but not inactive PLC inhibitor, U73343, IP_3 receptor antagonist, 2-APB or buffering of $[Ca^{2+}]_i$ with membrane permeable BAPTA-AM greatly diminished or abolished gintonin-mediated CaCC activations in *Xenopus* oocytes. Thus, gintonin but not ginsenosides are main agents that are responsible for induction of CaCC activation from CGSF through sequential $PLC-IP_3-Ca^{2+}$ pathway. Although CGSF activates CaCC through $G\alpha_{q/11}$-phospholipase $C-IP_3-Ca^{2+}$ pathway in *Xenopus* oocytes [5,6] and CGSF treatment also causes transient elevation of $[Ca^{2+}]_i$ in EAT cells [24], ginsenosides devoid of gin-

tonin failed to activate CaCC and to elevate $[Ca^{2+}]_i$ (Figs. 4 and 5). Thus, in regarding to previous reports on the regulation of Ca^{2+}-related membrane signaling pathways by CGSF [5,11,12,25], the newly identified gintonin is the main active ingredient of CGSF. Interestingly, gintonin isolated from Korean red ginseng or American ginseng did not show much difference in CaCC activations in *Xenopus* oocytes (Fig. 4). Taken together, these results further showed the possibility that gintonin rather than ginsenosides might be main contributors of ginseng's multiple pharmacological effects through unidentified membrane component(s). In addition, since ginseng is usually taken through oral route, it might be questioned whether oral administration of gintonin could show the same biological effects as it did *in vitro* experiments. Thus, in future study, it will be necessary to study gintonin bioavailability for the explanation of gintonin-mediated *in vivo* biological actions through $[Ca^{2+}]_i$ regulations.

We for the first time identified that ginseng contains novel glycolipoproteins, which we refer to as gintonin, that cause the mobilization of $[Ca^{2+}]_i$ in EAT cells and activation of CaCCs in *Xenopus* oocytes through well-known membrane signaling pathways. Since intracellular Ca^{2+} release is a convergence point for many hormones- and neurotransmitters-induced cell signals, the present findings also show the possibility that gintonin could be a novel first messenger and could utilize Ca^{2+} as a mediator of intracellular signaling pathways of a variety of physiological and pharmacological actions of ginseng.

ACKNOWLEDGEMENTS

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