Research article

Inhibitory effects of total saponin from Korean Red Ginseng on $[Ca^{2+}]_i$ mobilization through phosphorylation of cyclic adenosine monophosphate-dependent protein kinase catalytic subunit and inositol 1,4,5-trisphosphate receptor type I in human platelets

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A R T I C L E   I N F O

A B S T R A C T

Background: Intracellular Ca^{2+}($[Ca^{2+}]_i$) is a platelet aggregation-inducing molecule. Therefore, understanding the inhibitory mechanism of $[Ca^{2+}]_i$ mobilization is very important to evaluate the antiplatelet effect of a substance. This study was carried out to understand the Ca^{2+}-antagonistic effect of total saponin from Korean Red Ginseng (KRG-TS).
Methods: We investigated the Ca^{2+}-antagonistic effect of KRG-TS on cyclic nucleotides-associated phosphorylation of inositol 1,4,5-trisphosphate receptor type I (IP$_3$RI) and cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) in thrombin (0.05 U/mL)-stimulated human platelet aggregation.
Results: The inhibition of $[Ca^{2+}]_i$ mobilization by KRG-TS was increased by a PKA inhibitor (Rp-8-Br-cAMPS), which was more stronger than the inhibition by a cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG) inhibitor (Rp-8-Br-cGMPS). In addition, Rp-8-Br-cAMPS inhibited phosphorylation of PKA catalytic subunit (PKAc) (Thr^{197}) by KRG-TS. The phosphorylation of IP$_3$RI (Ser^{1756}) by KRG-TS was very strongly inhibited by Rp-8-Br-cAMPS compared with that by Rp-8-Br-cGMPS. These results suggest that the inhibitory effect of $[Ca^{2+}]_i$ mobilization by KRG-TS is more strongly dependent on a cAMP/PKA pathway than a cGMP/PKG pathway. KRG-TS also inhibited the release of adenosine triphosphate and serotonin. In addition, only G-Rg3 of protopanaxadiol in KRG-TS inhibited thrombin-induced platelet aggregation.
Conclusion: These results strongly indicate that KRG-TS is a potent beneficial compound that inhibits $[Ca^{2+}]_i$ mobilization in thrombin–platelet interactions, which may result in the prevention of platelet aggregation-mediated thrombotic disease.

1. Introduction

Platelet aggregation is absolutely essential for the formation of a hemostatic plug when normal blood vessels are injured. However, the interactions between platelets and thrombin can also cause circulatory disorders, such as thrombosis, atherosclerosis, and myocardial infarction [1]. Accordingly, inhibition of the platelet–thrombin interaction might be a promising approach for the prevention of thrombosis. It is well-known that thrombin, a platelet agonist, stimulates platelet aggregation by binding to the Gq-

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coupled proteinase-activated receptor, which is involved in activating phospholipase C-β (PLC-β). The activated PLC-β hydrolyzes phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG) [2–5]. Moreover, IP_3 mobilizes cytosol-free Ca^{2+} ($[Ca^{2+}]_i$) from the dense tubular system by binding to the IP_3 receptor type I (IP_3RI). The increased $[Ca^{2+}]_i$ activates both Ca^{2+}/calmodulin-dependent phosphorylation of myosin light chain (20 kDa) and DG-dependent phosphorylation of pleckstrin (47 kDa) to induce granule secretion [adenosine triphosphate (ATP) secretion] and platelet aggregation [6,7]. The Ca^{2+}-antagonistic effects of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are mediated through cAMP- and cGMP-dependent protein kinases (PKA and PKG, respectively), which phosphorylate the substrate protein IP_3RI. The phosphorylation of IP_3RI involves in inhibition of $[Ca^{2+}]_i$ mobilization [8–10]. Therefore, understanding the mechanism of IP_3RI phosphorylation is very useful for evaluating the Ca^{2+}-antagonistic effect of various substances.

Ginseng, the root of *Panax ginseng* C.A. Meyer, has been used frequently in traditional Oriental medicine, and is known to possess various pharmacological effects such as anti-inflammatory, anti-oxidation, antitumor, antidiabetes, and antihepatotoxicity [11,12]. Recently, it has been reported that Korean Red Ginseng (KRG) also has an effect on cardiovascular disease, which is characterized by reduction in blood pressure and arterial stiffness by inhibition of Rho kinase activity [13], anticoagulation by prolonging prothrombin and activated partial thromboplastin times [14], endothelium relaxation by the nitric oxide–cGMP pathway [15], and inhibition of hypercholesterolemia-induced platelet aggregation [16]. In a previous report, it has been demonstrated that total saponin from KRG (KRG-TS) inhibits the production of thromboxane A_2 (TXA_2) in platelet-mediated thrombotic disease by suppressing the activities of cyclooxygenase-1 (COX-1) and thromboxane A_2 synthase (TXAS) [17]. In addition, KRG-TS was reported to be involved in increasing the cAMP level and subsequent reduction of $[Ca^{2+}]_i$ mobilization in thrombin-induced rat platelet aggregation [18]. With regard to the effects of ginsenosides on platelet aggregation, it is well-known that ginsenoside Rg3 (G-Rg3) and its chemical derivatives (dihydroxyginsenoside Rg3 and ginsenoside Rp1) exhibit antiplatelet effects by regulating the aggregation-inhibiting molecule (cAMP) and aggregation-stimulating molecules (extracellular kinase 2, tyrosine kinase-dependent phosphoproteins, TXA_2, intracellular Ca^{2+}, integrin αIIb/β₃, etc.) [19–21]. In this study, to understand the inhibition of $[Ca^{2+}]_i$ mobilization by KRG-TS [18], we investigated whether KRG-TS is involved in phosphorylation of IP_3RI. We also examined which cyclic nucleotide of cAMP and cGMP participates in IP_3RI phosphorylation to attenuate $[Ca^{2+}]_i$ mobilization in thrombin-induced human platelet aggregation. In addition, our results showed that only G-Rg3 of protopanaxadiol in KRG-TS inhibited thrombin-induced platelet aggregation.

2. Materials and methods

2.1. Materials

KRG-TS was obtained from Korea Ginseng Corporation R&D Headquarters (Daejeon, Korea). Thrombin was purchased from Chrono-log Corporation (Havertown, PA, USA). The ATP assay kit was purchased from Biomedical Research Service Center (Buffalo, NY, USA). Serotonin enzyme-linked immunosorbent assay (ELISA) kit was purchased from Labor Diagnostika Nord GmbH & Co. (Nordhorn, Germany). Fura 2-acetoxymethyl (Fura 2-AM) and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Anti-phospho-IP_3RI (Ser^{1756}), antiphospho-PKA catalytic subunit (PKAc; Thr^{197}), antirabbit immunoglobulin G (IgG)–horseradish peroxidase

conjugate (HRP), and lysis buffer were obtained from Cell Signaling (Beverly, MA, USA). Polyvinylidene difluoride (PVDF) membrane was obtained from GE Healthcare (Piscataway, NJ, USA). Enhanced chemiluminescence (ECL) solution was purchased from GE Health-care (Chalfont St, Giles, Buckinghamshire, UK). Protopanaxadiol saponins [PPDSs; G-Ra1, G-Rb1, G-Rb2, G-Rb3, G-Rc, G-Rd, G-Rg3 (20R), G-Rg3 (20S), and G-Rh2 (20R)] and protopanaxatriol saponins [PPTSs; G-Re, G-Rf, G-Rg1, G-Rg2 (20R), and G-Rh1 (20S)] were purchased from Ambo Institute (Daejeon, Korea).

2.2. Preparation of washed human platelets

Human-platelet-rich plasma (PRP) anticoagulated with acid–citrate–dextrose solution (0.8% citric acid, 2.2% sodium citrate, and 2.45% glucose) was obtained from Korean Red Cross Blood Center (Changwon, Korea). The PRP was centrifuged for 10 min at 125g to remove little red blood cells, and was centrifuged for another 10 min at 1,300g to obtain the platelets. The platelets were washed two times with washing buffer (138mM NaCl, 2.7mM KCl, 12mM NaHCO₃, 0.36mM NaH₂PO₄, 5.5mM glucose, and 1mM EDTA; pH 6.5). The washed platelets were then resuspended in suspension buffer (138mM NaCl, 2.7mM KCl, 12mM NaHCO₃, 0.36mM NaH₂PO₄, 0.49mM MgCl₂, 5.5mM glucose, and 0.25% gelatin; pH 6.9) to make up a final concentration of 5×10^8/mL. All of the aforementioned procedures were carried out at 25°C to avoid platelet aggregation at low temperatures. The Korea National Institute for Bioethics Policy Public Institutional Review Board (Seoul, Korea) approved these experiments (PIRB12-072).

2.3. Measurement of platelet aggregation

Washed platelet (10^8/mL) were preincubated for 3 min at 37°C in the presence of 2mM exogenous $CaCl_2$ with or without (KRG-TS, A-kinase/G-kinase inhibitor), and then stimulated with thrombin (0.05 U/mL) for 5 min. Aggregation was monitored using an aggregometer (Chrono-log Corporation) at a constant stirring speed of 1,000 rpm. Each aggregation rate was evaluated as an increase in light transmission. The suspension buffer was used as the reference (transmission 0). KRG-TS was dissolved in the platelet suspension buffer (pH 6.9).

2.4. Determination of cytosolic-free Ca^{2+}

PRP was incubated with 5μM Fura 2-AM at 37°C for 60 min. Because Fura 2-AM is light sensitive, the tube containing the PRP was covered with aluminum foil during loading. The Fura 2-loaded washed platelets were prepared using the aforementioned procedure and 10^8 platelets/mL were preincubated for 3 min at 37°C with or without KRG-TS in the presence of 2mM $CaCl_2$, and then stimulated with thrombin (0.05 U/mL) for 5 min to evaluate $[Ca^{2+}]_i$. Fura 2 fluorescence was measured using a spectrofluorometer (SFM 25; BioTek Instruments, Milan, Italy) with an excitation wavelength that was changed every 0.5 s from 340 nm to 380 nm. The emission wavelength was set at 510 nm. The $[Ca^{2+}]_i$ values were calculated using the method suggested by Schaeffer and Blaustein [22].

2.5. Determination of ATP and serotonin release

The washed platelets (10^8/mL) were preincubated for 3 min at 37°C with or without (KRG-TS, A-kinase/G-kinase inhibitor, A-kinase/G-kinase activator) in the presence of 2mM $CaCl_2$, and then stimulated with thrombin (0.05 U/mL). The reaction was terminated and the mixture was centrifused with 200xg at 4°C for 10 min. The supernatants were used for the ATP and serotonin release assays. ATP release was measured in a luminometer (GloMax 20/
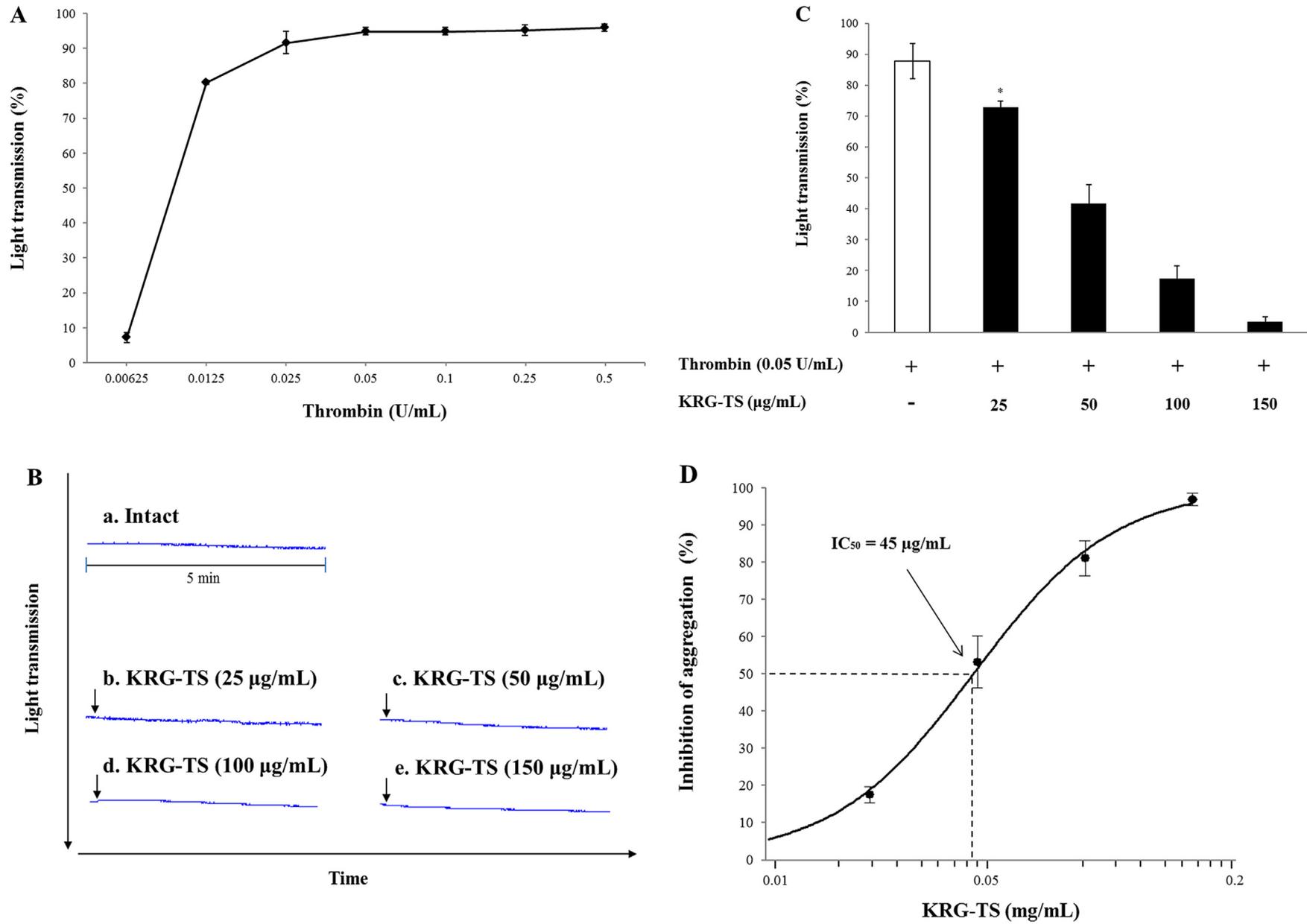
Fig. 1. Effects of total saponin from Korean Red Ginseng (KRG-TS) on thrombin-induced human platelet aggregation. (A) Concentration threshold of thrombin on human platelet aggregation. (B) Effects of KRG-TS on resting human platelets. (C) Effects of KRG-TS on thrombin-induced human platelet aggregation. (D) Half-maximal inhibitory concentration (IC_{50}) value of KRG-TS in thrombin-stimulated human platelet aggregation. Measurement of platelet aggregation was carried out as described in the "Materials and methods" section. The rate of inhibition by KRG-TS was recorded as the percentage of thrombin-induced aggregation rate. The IC_{50} value of KRG-TS was calculated according to the four-parameter log-fit method. Data are expressed as the mean ± standard deviation ($n = 4$). *$p < 0.05$ versus the thrombin-induced human platelet aggregation.

20; Promega, Madison, WI, USA) using an ATP assay kit. Serotonin release was measured with a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT, USA) using a serotonin ELISA kit.

2.6. Western blot analysis for evaluation of PKA catalytic subunit and IP$_3$RI phosphorylation

Washed platelets (10^8/mL) were preincubated with or without KRG-TS in the presence of 2mM CaCl$_2$ for 3 min and then stimulated with thrombin (0.05 U/mL) for 5 min at 37°C. The reactions were terminated by adding an equal volume (250 μL) of lysis buffer (20mM Tris−HCl, 150mM NaCl, 1mM Na$_2$EDTA, 1mM ethylene glycol tetraacetic acid, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1mM serine/threonine phosphatase inhibitor β-glycerophosphate, 1mM ATPase, alkaline and acid phosphatase, protein phosphotyrosine phosphatase inhibitor Na$_3$VO$_4$, 1 μg/mL serine and cysteine protease inhibitor leupeptin, and 1mM serine protease and acetylcholinesterase inhibitor phenylmethanesulfonyl fluoride; pH 7.5). Platelet lysates containing the same protein (15 μg) were used for analysis. Protein concentrations were measured using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Waltham, MA, USA). The effects of various substances on PKA catalytic subunit (PKAc) and IP$_3$RI phosphorylation were analyzed by Western blotting. A 6−8% sodium dodecyl sulfate polyacrylamide gel electrophoresis was used for electrophoresis and a PVDF membrane was used for protein transfer from the gel. The dilutions for anti-phospho-IP$_3$RI (Ser^{1756}), antiphospho-PKAc (Thr^{197}), and antirabbit IgG−HRP were 1:1,000, 1:1,000, and 1:10,000, respectively. The membranes were visualized using ECL. Blots were analyzed using the Quantity One version 4.5 software (BioRad, Hercules, CA, USA).

2.7. Statistical analyses

The experimental results were expressed as the mean ± standard deviation accompanied by the number of observations. Data were assessed by analysis of variance. If this analysis indicated significant differences among the group means, then each group was compared by the Newman−Keuls method. Statistical analysis was performed using SPSS 21.0.0 (SPSS Inc., Chicago, IL, USA). A p value < 0.05 was taken to be significant.

3. Results

3.1. Effects of KRG-TS on thrombin-induced human platelet aggregation

The concentration of thrombin-induced maximal human platelet aggregation was approximately 0.05 U/mL (Fig. 1A). Therefore, 0.05 U/mL thrombin was used as the human platelet agonist in this study. The light transmission in response to various concentrations of KRG-TS in intact platelets was 1.3 ± 0.6% (at 25 μg/mL of KRG-TS), 1.3 ± 0.6% (at 50 μg/mL of KRG-TS), 1.3 ± 0.6% (at 100 μg/mL of KRG-TS), and 1.3 ± 0.6% (at 150 μg/mL of KRG-TS), which was not significantly different from that observed in resting platelets [1.0 ± 0.0%; Fig. 1B(a−e)]. This result indicated that KRG-TS alone did not have an effect on platelet aggregation. When washed human platelets (10^8/mL) were activated with thrombin, the aggregation rate was increased up to 87.8 ± 5.7%. However, various concentrations of KRG-TS (25−150 μg/mL) significantly reduced thrombin-stimulated platelet aggregation in a dose-dependent manner (Fig. 1C), and its half-maximal inhibitory concentration (IC$_{50}$) was approximately 45 μg/mL (Fig. 1D). This IC$_{50}$ is low compared with that (81 μg/ml) by rat platelets [18]. In addition,
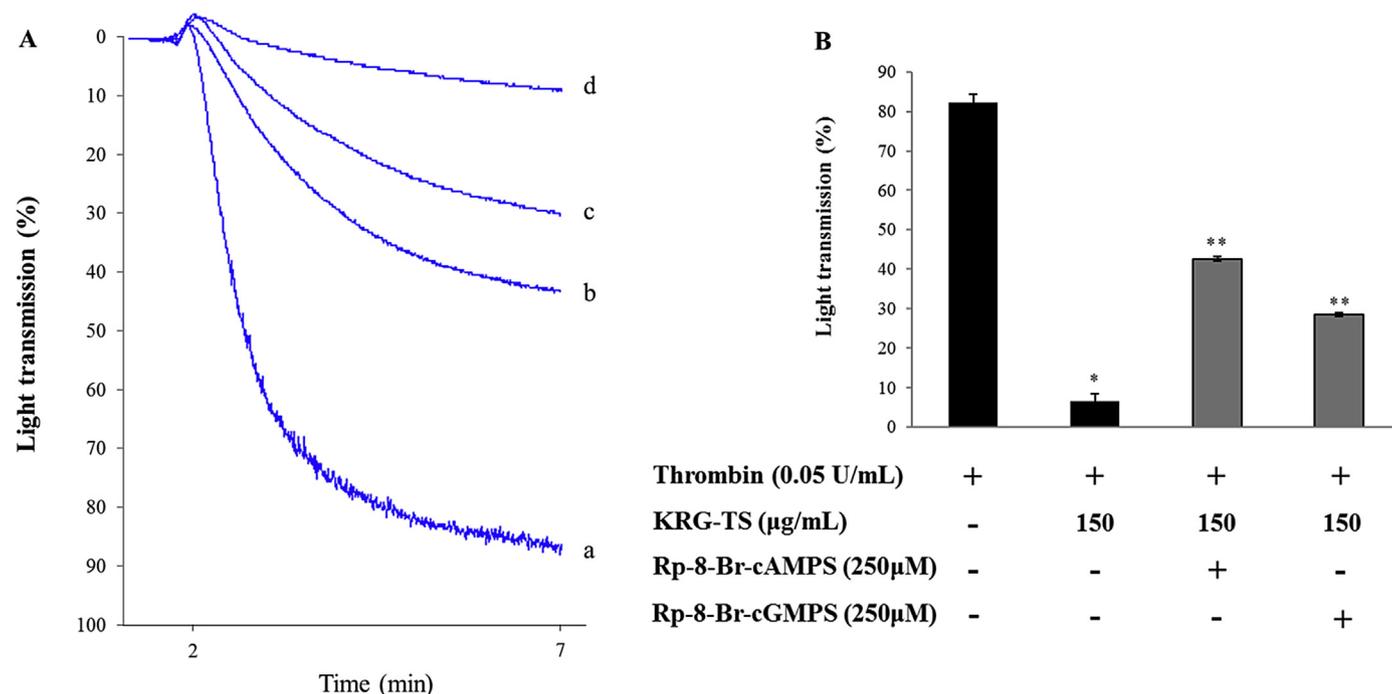

Fig. 2. Effects of total saponin from Korean Red Ginseng (KRG-TS) on thrombin-induced human platelet aggregation in the presence of cyclic adenosine monophosphate-dependent protein kinase (PKA) or cyclic guanosine monophosphate-dependent protein kinase (PKG) inhibitor. (A) Effects of KRG-TS, PKA inhibitor, or PKG inhibitor on thrombin-elevated light transmission: (a) thrombin (0.05 U/mL); (b) thrombin (0.05 U/mL) + KRG-TS (150 μg/mL) + Rp-8-Br-cAMPS (250μM); (c) thrombin (0.05 U/mL) + KRG-TS (150 μg/mL) + Rp-8-Br-cGMPS (250μM); and (d) thrombin (0.05 U/mL) + KRG-TS (150 μg/mL). (B) Effects of KRG-TS, PKA inhibitor, or PKG inhibitor on thrombin-induced human platelet aggregation. Washed human platelets (10^8/mL) were preincubated with KRG-TS (150 μg/mL) in the presence of PKA inhibitor (Rp-8-Br-cAMPS) or PKG inhibitor (Rp-8-Br-cGMPS) for 3 min at 37°C, and then thrombin (0.05 U/mL) was added. Data are expressed as the mean ± standard deviation ($n = 4$). *$p < 0.05$ versus the thrombin-stimulated human platelets. **$p < 0.05$ versus the thrombin-stimulated platelets in the presence of KRG-TS (150 μg/mL).

A

[Ca²⁺]ᵢ (nM)

	[Ca²⁺]ᵢ (nM)	Inhibition (%)
Thrombin (0.05 U/mL)	590.6 ± 18.3	0
KRG-TS (150 μg/mL) + Thrombin (0.05 U/mL)	110.9 ± 1.9	81.2

Thrombin (0.05 U/mL)	−	+	+	+	+	+
KRG-TS (μg/mL)	−	−	25	50	100	150

B

[Ca²⁺]ᵢ (nM)

Thrombin (0.05 U/mL)	−	+	+	+	+
KRG-TS (μg/mL)	−	150	150	150	150
Rp-8-Br-cAMPS (μM)	−	−	50	150	250

C

[Ca²⁺]ᵢ (nM)

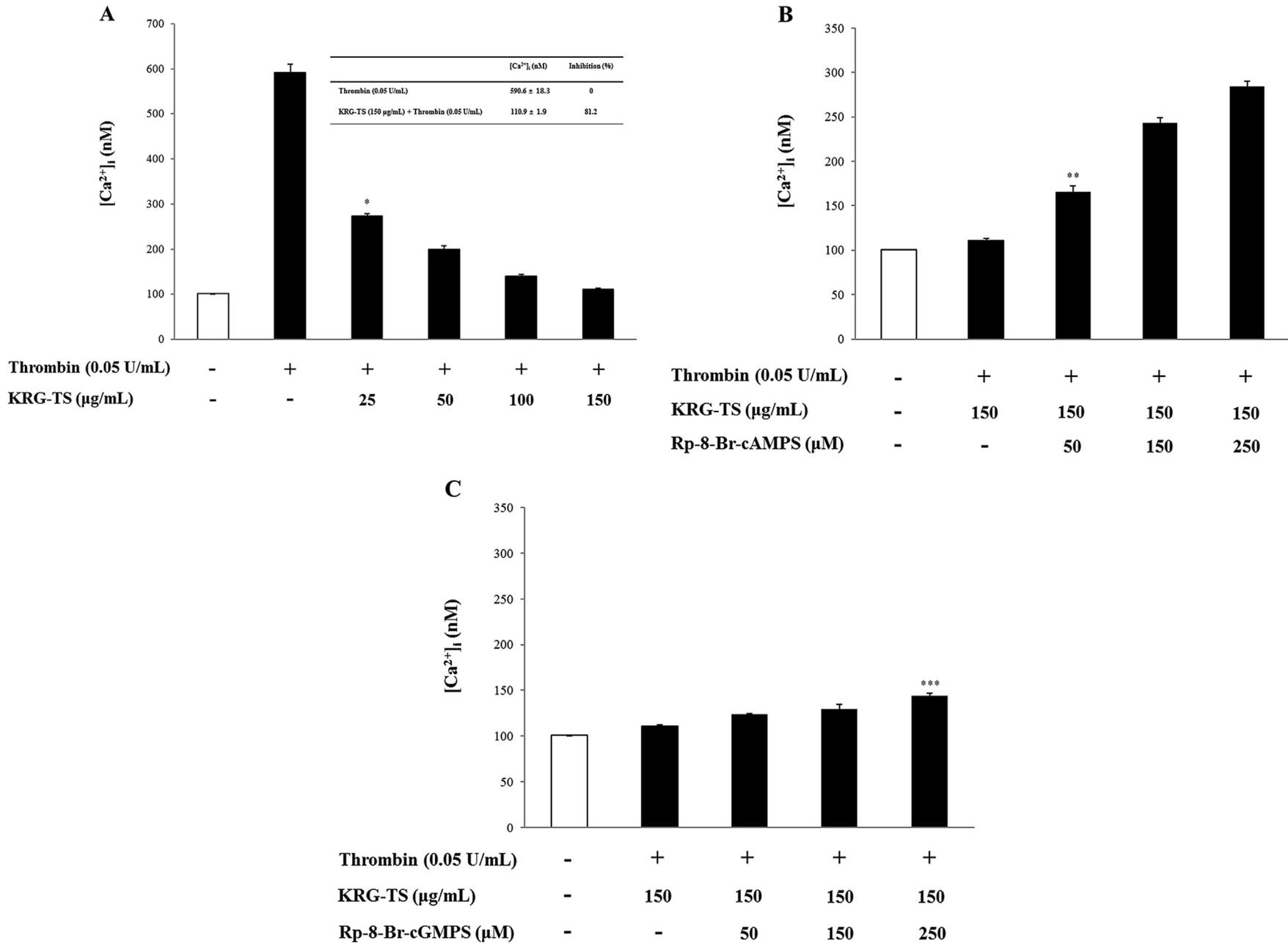| Thrombin (0.05 U/mL)	−	+	+	+	+
KRG-TS (μg/mL)	−	150	150	150	150
Rp-8-Br-cGMPS (μM)	−	−	50	150	250

Fig. 3. Effects of total saponin from Korean Red Ginseng (KRG-TS) on thrombin-induced [Ca²⁺]ᵢ mobilization. (A) Inhibitory effects of KRG-TS on thrombin-induced [Ca²⁺]ᵢ mobilization. (B) Effects of KRG-TS on [Ca²⁺]ᵢ mobilization in the presence of cyclic adenosine monophosphate-dependent protein kinase inhibitor (Rp-8-Br-cAMPS). (C) Effects of KRG-TS on [Ca²⁺]ᵢ mobilization in the presence of cyclic guanosine monophosphate-dependent protein kinase inhibitor (Rp-8-Br-cGMPS). [Ca²⁺]ᵢ was determined as described in the "Materials and methods" section. Data are expressed as the mean ± standard deviation ($n = 4$). *$p < 0.05$ versus the thrombin-stimulated human platelets. **$p < 0.05$ versus the thrombin-stimulated human platelets in the presence of KRG-TS (150 μg/mL). ***$p < 0.05$ versus the thrombin-stimulated human platelets in the presence of KRG-TS (150 μg/mL).

Table 1
Effects of Rp-8-Br-cAMPS and Rp-8-Br-cGMPS on [Ca^{2+}]$_i$ mobilization

	[Ca^{2+}]$_i$ (nM)	Increase (%)
KRG-TS (150 µg/mL) + thrombin (0.05 U/mL)	110.9 ± 1.9	–
KRG-TS (150 µg/mL) + Rp-8-Br-cAMPS (250µM) + thrombin (0.05 U/mL)	238.6 ± 5.9	155.1[1)]
KRG-TS (150 µg/mL) + Rp-8-Br-cGMPS (250µM) + thrombin (0.05 U/mL)	143.6 ± 2.8	29.4[2)]

Data are from Figs. 3B and 3C
KRG-TS, total saponin from Korean Red Ginseng
[1)] Increase (%) = [(KRG-TS + thrombin + Rp-8-Br-cAMPS) − (KRG-TS + thrombin)]/(KRG-TS + thrombin) × 100
[2)] Increase (%) = [(KRG-TS + thrombin + Rp-8-Br-cGMPS) − (KRG-TS + thrombin)]/(KRG-TS + thrombin) × 100

150 µg/mL of KRG-TS inhibited up to 95.9% of thrombin-induced human platelet aggregation (87.8 ± 5.7%).

3.2. Effects of KRG-TS on thrombin-induced human platelet aggregation in the presence of PKA or PKG inhibitor

Because [Ca^{2+}]$_i$ is essential for platelet aggregation, we investigated the effect of KRG-TS on thrombin-induced platelet aggregation in the presence of PKA or PKG inhibitor, both of which are intracellular Ca^{2+}-antagonistic compounds. As shown in Fig. 2A, thrombin increased light transmission [Fig. 2A(a)], whereas KRG-TS decreased thrombin-induced light transmission [Fig. 2A(d)]. The PKA inhibitor Rp-8-Br-cAMPS [Figs. 2A(b) and 2B] and the PKG inhibitor Rp-8-Br-cGMPS [Figs. 2A(c) and 2B] increased KRG-TS-attenuated light transmission in thrombin-activated platelets. The inhibitory degree [Figs. 2A(b) and 2B] achieved by the PKA inhibitor was more stronger than that achieved by the PKG inhibitor [Figs. 2A(c) and 2B]. These results suggest that the inhibition of thrombin-induced human platelet aggregation by KRG-TS is greatly dependent on cAMP-dependent Ca^{2+}-antagonistic condition than cGMP-dependent Ca^{2+}-antagonistic condition.

3.3. Effects of KRG-TS on [Ca^{2+}]$_i$ mobilization

We investigated the effect of KRG-TS on Ca^{2+}-antagonistic activity. As shown in Fig. 3A, thrombin increased the [Ca^{2+}]$_i$ level from 101.2 ± 0.5nM (basal level) to 590.6 ± 18.3nM (Fig. 3A, inset table). However, this was significantly decreased by various concentrations (25−150 µg/mL) of KRG-TS in a dose-dependent manner (Fig. 3A). If the inhibition of [Ca^{2+}]$_i$ mobilization by KRG-TS results from IP$_3$R phosphorylation through the cAMP/PKA or cGMP/PKG pathway, then the KRG-TS-reduced [Ca^{2+}]$_i$ level would be increased by the PKA or PKG inhibitor. Accordingly, we investigated the effects of PKA inhibitor (Rp-8-Br-cAMPS) and PKG inhibitor (Rp-8-Br-cGMPS) on KRG-TS-reduced [Ca^{2+}]$_i$ mobilization. As shown in Fig. 3B, the PKA inhibitor Rp-8-Br-cAMPS (50−250µM) dose dependently increased the [Ca^{2+}]$_i$ level compared with that (110.9 ± 1.9 nM) by KRG-TS (150 µg/mL) in the thrombin-induced platelet aggregation. By contrast, the PKG inhibitor Rp-8-Br-
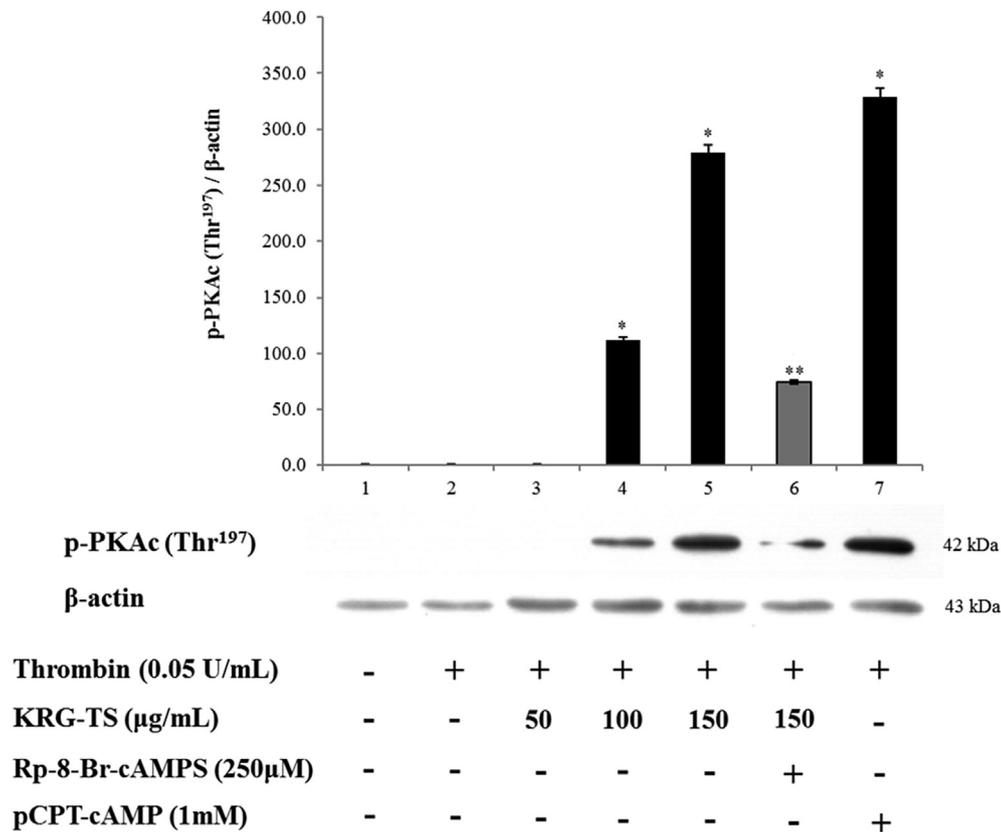

Fig. 4. Effect of total saponin from Korean Red Ginseng (KRG-TS) on cyclic adenosine monophosphate-dependent protein kinase catalytic subunit (PKAc) phosphorylation. Lane 1, intact platelets (base); Lane 2, thrombin (0.05 U/mL); Lane 3, thrombin (0.05 U/mL) + KRG-TS (50 µg/mL); Lane 4, thrombin (0.05 U/mL) + KRG-TS (100 µg/mL); Lane 5, thrombin (0.05 U/mL) + KRG-TS (150 µg/mL); Lane 6, thrombin (0.05 U/mL) + KRG-TS (150 µg/mL) + Rp-8-Br-cAMPS (250µM); and Lane 7, thrombin (0.05 U/mL) + 8-(4-chlorophenylthio)-cyclic adenosine monophosphate (pCPT-cAMP; 1mM). Western blotting was performed as described in the "Materials and methods" section. Data are expressed as the mean ± standard deviation ($n = 4$). *$p < 0.05$ versus the thrombin-stimulated human platelets. **$p < 0.05$ versus the thrombin-stimulated human platelets in the presence of KRG-TS (150 µg/mL).
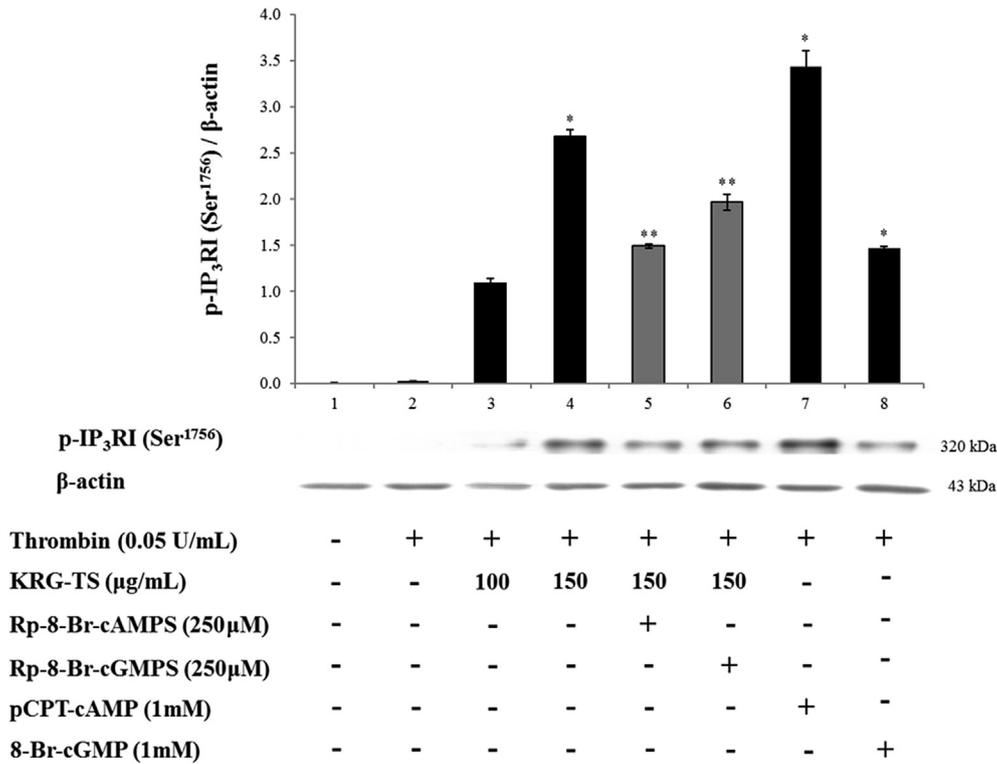
Fig. 5. Effect of total saponin from Korean Red Ginseng (KRG-TS) on inositol 1,4,5-trisphosphate receptor type I (IP$_3$RI) (Ser^{1756}) phosphorylation. Lane 1, intact platelets (base); Lane 2, thrombin (0.05 U/mL); Lane 3, thrombin (0.05 U/mL) + KRG-TS (100 μg/mL); Lane 4, thrombin (0.05 U/mL) + KRG-TS (150 μg/mL); Lane 5, thrombin (0.05 U/mL) + KRG-TS (150 μg/mL) + Rp-8-Br-cAMPS (250μM); Lane 6, thrombin (0.05 U/mL) + KRG-TS (150 μg/mL) + Rp-8-Br-cGMPS (250μM); Lane 7, thrombin (0.05 U/mL) + 8-(4-chlorophenylthio)-cyclic adenosine monophosphate (pCPT-cAMP; 1mM); and Lane 8, thrombin (0.05 U/mL) +8-Br-cGMP (1mM). Western blotting was performed as described in the "Materials and methods" section. Data are expressed as the mean ± standard deviation ($n = 4$). *$p < 0.05$ versus the thrombin-stimulated platelets, **$p < 0.05$ versus the thrombin-stimulated human platelets in the presence of KRG-TS (150 μg/mL).

cGMPS (50—250μM) very weakly increased the [Ca^{2+}]$_i$ level compared with that by KRG-TS (150 μg/mL; 110.9 ± 1.9nM) in thrombin-induced platelet aggregation (Fig. 3C). As shown in Table 1, Rp-8-Br-cAMPS (250μM) increased the [Ca^{2+}]$_i$ level to 155.1% and Rp-8-Br-cGMPS (250μM) increased the [Ca^{2+}]$_i$ level to 29.4%, indicating that the reduction of [Ca^{2+}]$_i$ mobilization (Fig. 3A) by KRG-TS may be more dependent on the pathway of cAMP/PKA than cGMP/PKG. We, therefore, investigated the effect of KRG-TS on phosphorylation of PKAc as an indicator of cAMP/PKA activation.

3.4. Effects of KRG-TS on PKAc (Thr^{197}) phosphorylation

It is well-known that the phosphorylation of PKAc (Thr^{197}) is necessary to produce biological functions through the cAMP/PKA pathway [23]. As shown in Fig. 4, KRG-TS dose dependently phosphorylated Thr^{197} in PKAc (42 kDa), and abruptly increased the ratio of phosphorylated PKAc (Thr^{197}) to β-actin in thrombin-induced platelet aggregation (Fig. 4, lanes 4 and 5). The PKA inhibitor Rp-8-Br-cAMPS strongly decreased KRG-TS-elevated phosphorylation of PKAc (Thr^{197}; Fig. 4, lane 6), meaning that the phosphorylation of PKAc (Thr^{197}) by KRG-TS depends on the cAMP/PKA pathway. This is also supported by the fact that the PKA activator 8-(4-chlorophenylthio)-cAMP (pCPT-cAMP) increased the phosphorylation of PKAc (Thr^{197}; Fig. 4, lane 7).

3.5. Effects of KRG-TS on IP$_3$RI (Ser^{1756}) phosphorylation in the presence of PKA and PKG inhibitors

We then investigated the IP$_3$RI phosphorylation-associated PKAc (Thr^{197}) phosphorylation. The phosphorylation of IP$_3$RI

(Ser^{1756}) and the ratio of phosphorylated IP$_3$RI (p-IP$_3$RI; Ser^{1756}) to β-actin were increased in the presence of the PKA activator pCPT-cAMP (Fig. 5, lane 7) in thrombin-induced human platelet aggregation, indicating that cAMP/PKA is involved in the phosphorylation of IP$_3$RI (Ser^{1756}). As shown in Fig. 5 (lanes 3 and 4), the phosphorylation of IP$_3$RI (Ser^{1756}) and the ratio of p-IP$_3$RI (Ser^{1756}) to β-actin were dose dependently increased in the presence of both thrombin and KRG-TS. To investigate whether cAMP/PKAc (Thr^{197}) phosphorylation by KRG-TS (Fig. 4, lane 5) is involved in IP$_3$RI (Ser^{1756}) phosphorylation (Fig. 5, lane 4), we used the PKA inhibitor Rp-8-Br-cAMPS (Fig. 4, lane 6) that inhibited KRG-TS-induced PKAc (Thr^{197}) phosphorylation. As shown in Fig. 5 (lane 5), the PKA

Table 2
Changes of p-IP$_3$R to β-actin ratio

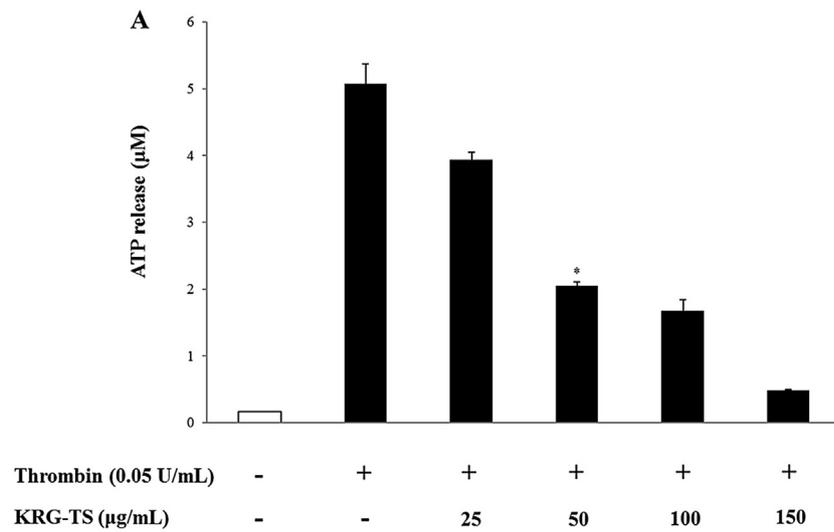|	Ratio of p-IP$_3$R to β-actin	Δ (%)
Thrombin (0.05 U/mL)	2.7 ± 0.1	
KRG-TS (150 μg/mL) + thrombin (0.05 U/mL)	267.6 ± 7.3	+9811.1[1]
KRG-TS (150 μg/mL) + thrombin (0.05 U/mL) + Rp-8-Br-cAMPS (250μM)	148.9 ± 2.1	−44.3[2]
KRG-TS (150 μg/mL) + thrombin (0.05 U/mL) + Rp-8-Br-cGMPS (250μM)	196.2 ± 8.5	−26.6[3]

Data are from Fig. 5
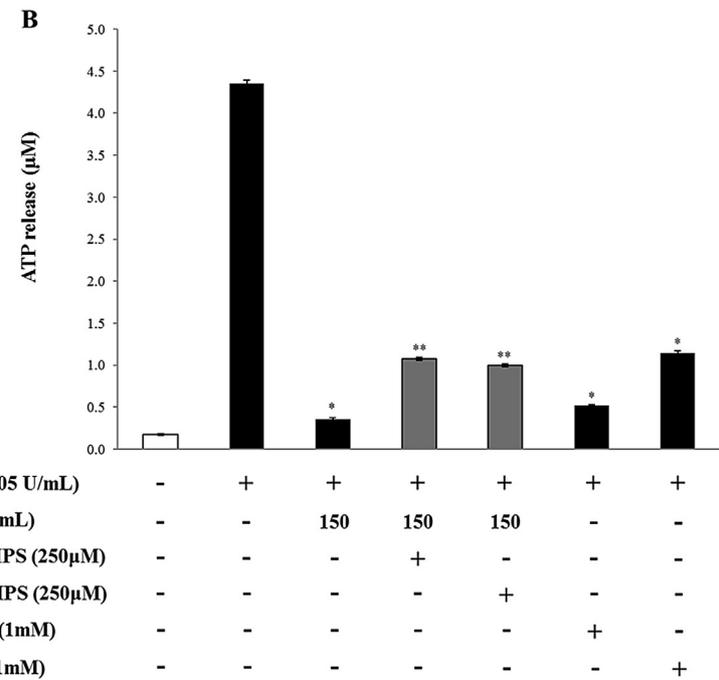IP$_3$R, inositol 1,4,5-trisphosphate receptor; KRG-TS, total saponin from Korean Red Ginseng
[1] Δ (%) = [(KRG-TS + thrombin) − thrombin]/thrombin × 100
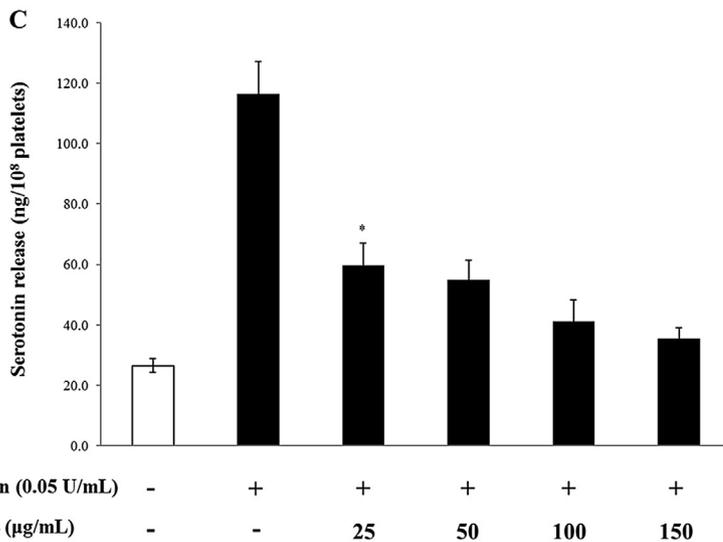[2] Δ (%) = [(KRG-TS + thrombin + Rp-8-Br-cAMPS) − (KRG-TS + thrombin)]/(KRG-TS + thrombin) × 100
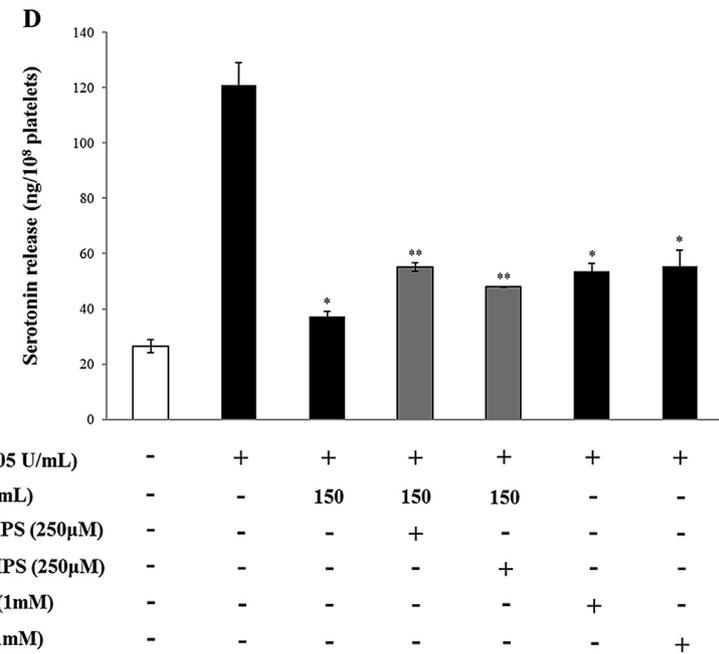[3] Δ (%) = [(KRG-TS + thrombin + Rp-8-Br-cGMPS) − (KRG-TS + thrombin)]/(KRG-TS + thrombin) × 100

A

ATP release (μM)

Thrombin (0.05 U/mL)	-	+	+	+	+	+
KRG-TS (μg/mL)	-	-	25	50	100	150

B

ATP release (μM)

Thrombin (0.05 U/mL)	-	+	+	+	+	+	+
KRG-TS (μg/mL)	-	-	150	150	150	-	-
Rp-8-Br-cAMPS (250μM)	-	-	-	+	-	-	-
Rp-8-Br-cGMPS (250μM)	-	-	-	-	+	-	-
pCPT-cAMP (1mM)	-	-	-	-	-	+	-
8-Br-cGMP (1mM)	-	-	-	-	-	-	+

C

Serotonin release (ng/10⁸ platelets)

Thrombin (0.05 U/mL)	-	+	+	+	+	+
KRG-TS (μg/mL)	-	-	25	50	100	150

D

Serotonin release (ng/10⁸ platelets)

Thrombin (0.05 U/mL)	-	+	+	+	+	+	+
KRG-TS (μg/mL)	-	-	150	150	150	-	-
Rp-8-Br-cAMPS (250μM)	-	-	-	+	-	-	-
Rp-8-Br-cGMPS (250μM)	-	-	-	-	+	-	-
pCPT-cAMP (1mM)	-	-	-	-	-	+	-
8-Br-cGMP (1mM)	-	-	-	-	-	-	+

Fig. 6. Effects of total saponin from Korean Red Ginseng (KRG-TS) on adenosine triphosphate (ATP) and serotonin release. (A) Effects of KRG-TS on ATP release in thrombin-activated platelets. (B) Effects of KRG-TS on ATP release in the presence of cyclic adenosine monophosphate-dependent protein kinase (PKA) or cyclic guanosine monophosphate-dependent protein kinase (PKG) inhibitor. (C) Effects of KRG-TS on serotonin release. (D) Effects of KRG-TS on serotonin release in the presence of PKA or PKG inhibitor. Data are expressed as mean ± standard deviation ($n = 4$). *$p < 0.05$ versus the thrombin-stimulated human platelets. **$p < 0.05$ versus the thrombin-stimulated human platelets in the presence of KRG-TS (150 μg/mL).

inhibitor Rp-8-Br-cAMPS potently decreased p-IP$_3$RI (Ser^{1756}) and the ratio of p-IP$_3$RI (Ser^{1756}) to β-actin achieved by both thrombin and KRG-TS. These results indicate that the increase of p-IP$_3$RI (Ser^{1756}) by KRG-TS is dependent on cAMP/PKAc (Thr^{197}). The PKG inhibitor Rp-8-Br-cGMPS also decreased KRG-TS-induced p-IP$_3$RI (Ser^{1756}; Fig. 5, lane 6). However, its inhibitory degree (22.6%) was lower compared with that of the PKA inhibitor Rp-8-Br-cAMPS (44.3%; Table 2), indicating that the increase of p-IP$_3$RI (Ser^{1756}) by KRG-TS is effected through the cAMP/PKA pathway.

3.6. Effects of KRG-TS on ATP and serotonin release

Intracellular [Ca^{2+}]$_i$ mobilization is known to be involved in the release of ATP and serotonin from a dense body of platelets [24]. Because KRG-TS inhibited thrombin-elevated [Ca^{2+}]$_i$ mobilization (Fig. 3A), we investigated whether KRG-TS is involved in the inhibition of ATP and serotonin release. As shown in Fig. 6, KRG-TS dose dependently inhibited thrombin-induced ATP (Fig. 6A) and serotonin release (Fig. 6C). Because the inhibition of [Ca^{2+}]$_i$ mobilization by KRG-TS was dependent on cAMP/PKA and cGMP/PKG pathways, we investigated whether the inhibition of ATP and serotonin release by KRG-TS resulted from cAMP/PKA and cGMP/PKG pathways. The PKA inhibitor Rp-8-Br-cAMPS (Fig. 6B) and the PKG inhibitor Rp-8-Br-cGMPS (Fig. 6D) increased KRG-TS-decreased ATP and serotonin release.

These results indicate that the inhibition of ATP and serotonin release by KRG-TS (Figs. 6A and 6C) is dependent on the cAMP/PKA and cGMP/PKG-pathways. The results are supported by the fact that the PKA activator pCPT-cAMP (Fig. 6B) and the PKG activator 8-Br-cGMP (Fig. 6D) inhibit thrombin-induced ATP and serotonin release.

3.7. Effects of ginsenosides in KRG-TS on thrombin-induced platelet aggregation

In a previous report [18], five ginsenosides (G-Rb1, G-Rb2, G-Rc, G-Rd, and G-Rg3) were reported as 20(S)-PPDS and three ginsenosides (G-Re, G-Rg1, and G-Rg2) as 20(S)-PPTS. Besides these ginsenosides, it is known that G-Ra1, G-Rb3, and G-Rh2 as PPDS and G-Rf and G-Rh1 as PPTS are also present in *P. ginseng* [25]. Therefore, to investigate which ginsenoside of KRG-TS was involved in the inhibition of thrombin-induced human platelet aggregation, we investigated the effect of ginsenosides in KRG-TS and other ginsenosides on thrombin-induced human platelet aggregation. In this study, G-Ra1, G-Rb1, G-Rb2, G-Rb3, G-Rc, G-Rd, and G-Rh2(20R) of PPDS did not inhibit thrombin-induced human platelet aggregation, but only G-Rg3 (20R, 20S) dose dependently inhibited thrombin-induced human platelet aggregation (Table 3). In addition, none of the PPTSs [G-Re, G-Rf, G-Rg1, G-Rg2 (20R), and G-Rh1 (20S)] inhibited thrombin-induced human platelet aggregation (Table 4).

4. Discussion

Although KRG-TS elevated cAMP only in thrombin-induced human platelets [17], to confirm whether the cAMP/PKA or cGMP/PKG pathway contributed to the inhibition of platelet aggregation by KRG-TS, we investigated the effect of PKA inhibitor and PKG inhibitor on thrombin-induced human platelet aggregation in the presence of KRG-TS. The results showed that the PKA inhibitor Rp-8-Br-cAMPS and the PKG inhibitor Rp-8-Br-cGMPS increased KRG-TS-decreased light transmission in thrombin-induced human platelet aggregation. However, the elevation of light transmission by the PKA inhibitor was stronger than that by the PKG inhibitor in KRG-TS-inhibited thrombin-induced human

Table 3
Effects of protopanaxadiol on thrombin-induced human platelet aggregation

Treatment (µM)	Aggregation (%)	Treatment (µM)	Aggregation (%)
Thrombin (0.05 U/mL)	86.7 ± 1.5	–	–
Ginsenoside Ra1		Ginsenoside Rd	
50	83.3 ± 1.2	50	85.0 ± 3.0
10	83.0 ± 1.7	100	84.0 ± 1.7
200	83.0 ± 1.0	200	86.3 ± 1.5
300	86.7 ± 1.5	300	84.3 ± 4.0
Ginsenoside Rb1		Ginsenoside Rg3 (20R)	
50	83.0 ± 1.7	50	86.7 ± 1.5
100	84.3 ± 0.6	100	86.0 ± 2.0
200	85.3 ± 3.1	200	75.7 ± 3.2
300	83.0 ± 3.5	300	62.0 ± 2.0
Ginsenoside Rb2		Ginsenoside Rg3 (20S)	
50	86.7 ± 1.5	50	79.7 ± 2.1 *
100	84.7 ± 0.6	100	39.7 ± 2.1 **
200	86.3 ± 2.1	200	9.7 ± 2.1 **
300	84.7 ± 0.6	300	3.7 ± 1.5 **
Ginsenoside Rb3		Ginsenoside Rh2 (20R)	
50	82.3 ± 3.5	50	83.3 ± 1.2
100	85.7 ± 1.2	100	84.7 ± 0.6
200	83.7 ± 1.5	200	85.3 ± 3.1
300	84.0 ± 3.6	300	81.0 ± 1.7
Ginsenoside Rc			
50	84.7 ± 3.2	–	–
100	84.7 ± 0.6	–	–
200	86.3 ± 2.1	–	–
300	84.0 ± 2.6	–	–

Results are expressed as percentage of aggregation induced by thrombin. The data are expressed as the mean ± standard deviation ($n = 4$)
*$p < 0.05$, **$p < 0.01$ versus the thrombin-stimulated human platelets

platelet aggregation. These results show that the cAMP/PKA pathway mainly contributed to the inhibition of thrombin-induced platelet aggregation and [Ca^{2+}]$_i$ mobilization by KRG-TS. Several aggregation-inducing molecules (Ca^{2+}, TXA$_2$, etc.) are commonly generated by agonists such as thrombin, collagen, and adenosine diphosphate (ADP). The IP$_3$ mobilizes [Ca^{2+}]$_i$, and subsequently activates Ca^{2+}-dependent PLC or phospholipase-A$_2$ to separate the TXA$_2$ precursor arachidonic acid (20:4) from glycerophospholipids, and TXA$_2$ is produced by activation of COX-1/TXAS. TXA$_2$ produces IP$_3$ to mobilize [Ca^{2+}]$_i$ through the G-protein-coupled receptor/PLC-β pathway, and constricts the blood vessel tract [5,26–28], which enforces thrombus formation. Therefore, the inhibition of [Ca^{2+}]$_i$ mobilization by IP$_3$ and TXA$_2$ production by COX-1/TXAS are very important to evaluate the antiplatelet effect of a substance. A previous report [17] confirmed that KRG-TS inhibits TXA$_2$

Table 4
Effects of protopanaxatriol on thrombin-induced human platelet aggregation

Treatment (µM)	Aggregation (%)	Treatment (µM)	Aggregation (%)
Thrombin (0.05 U/mL)	86.7 ± 1.5	–	–
Ginsenoside Re		Ginsenoside Rg2 (20R)	
50	82.7 ± 1.2	50	84.0 ± 2.6
100	82.3 ± 1.5	100	87.0 ± 1.0
200	85.3 ± 2.5	200	85.3 ± 2.9
300	84.7 ± 0.6	300	86.0 ± 2.6
Ginsenoside Rf		Ginsenoside Rh1 (20S)	
50	82.7 ± 2.1	50	84.3 ± 1.5
100	86.7 ± 1.5	100	83.3 ± 2.1
200	84.7 ± 0.6	200	82.7 ± 1.2
300	83.0 ± 1.0	300	85.3 ± 2.5
Ginsenoside Rg1			
50	84.3 ± 2.1	–	–
100	83.0 ± 1.7	–	–
200	84.7 ± 4.0	–	–
300	85.0 ± 1.0	–	–

Results are expressed as percentage of aggregation induced by thrombin. The data are expressed as the mean ± standard deviation ($n = 4$)

production by attenuating COX-1 and TXAS activities. Although KRG-TS inhibited thrombin-induced $[Ca^{2+}]_i$ mobilization, its inhibitory mechanism is unknown. The Ca^{2+}-antagonistic reaction by cAMP and cGMP is mediated by both PKA/IP$_3$RI and PKG/IP$_3$RI phosphorylation pathways. Because KRG-TS elevated the level of cAMP [18], if KRG-TS stimulates IP$_3$RI (Ser^{1756}) phosphorylation in thrombin-activated human platelets, it is clear evidence that KRG-TS inhibits $[Ca^{2+}]_i$ mobilization through the cAMP/PKA/IP$_3$RI (Ser^{1756}) phosphorylation pathway. In this report, we confirmed that KRG-TS inhibited $[Ca^{2+}]_i$ mobilization through IP$_3$RI (Ser^{1756}) phosphorylation by cAMP/PKAc, which is supported by the fact that the cAMP inhibitor Rp-8-Br-cAMPS inhibited KRG-TS-elevated phosphorylation of both IP$_3$RI (Ser^{1756}) and PKAc (Thr^{197}) in thrombin-induced human platelet aggregation, otherwise the cAMP inhibitor Rp-8-Br-cAMPS would not increase KRG-TS-decreased $[Ca^{2+}]_i$ mobilization in thrombin-induced human platelet aggregation. It is known that IP$_3$ induces serotonin release from platelet-dense bodies, meaning that IP$_3$ is involved in serotonin release by elevating $[Ca^{2+}]_i$ through IP$_3$RI [29]. This reflects the fact that KRG-TS may be involved in the inhibition of serotonin release by phosphorylating IP$_3$RI (Ser^{1756}).

A lot of agonists such as collagen, thrombin, and ADP mobilize $[Ca^{2+}]_i$ to phosphorylate Ca^{2+}/calmodulin-dependent myosin light chain (20 kDa), which plays a role in secretion of granules such as serotonin and ATP [6,7], and platelet aggregation. It is thought that the inhibition of ATP and serotonin secretion by KRG-TS results from the elevation of Ca^{2+}-antagonistic molecule cAMP and subsequent inhibition of $[Ca^{2+}]_i$ mobilization, which is also supported by the fact that KRG-TS stimulated the phosphorylation of both PKAc (Thr^{197}) and IP$_3$RI (Ser^{1756}).

Platelet aggregation is generated at the site of vascular wall injury, and is involved in the formation of thrombus. During the formation of thrombus, platelets release cell growth proteins [e.g., platelet-derived growth factor (PDGF)] and vascular endothelial growth factor (VEGF) in α-granules [30,31]. It is well-established that PDGF and VEGF induce the proliferation of fibroblast, vascular smooth cells, and epithelial cells, and subsequently enhance the rate of atherosclerosis lesion progression [32−36]. The progression of atherosclerosis is strongly induced by inflammatory cells such as monocytes/macrophages and neutrophils [37]. Although KRG-TS shows antiplatelet effects, if KRG-TS does not inhibit inflammation by leukocytes, progression of atherosclerosis lesion occurs at the site of vascular wall injury, which raises questions about the antiplatelet effects of KRG-TS. Byeon et al [38] reported that saponin fraction inhibits lipopolysaccharide (LPS)-induced inflammation, and it is well-known that ginsenosides exhibit anti-inflammatory effects by inhibiting the production of various proinflammatory mediators such as prostaglandin E$_2$ and NO [39]. Recently, it was reported that saponin fractions of KRG downregulate LPS-induced proinflammatory mediators (i.e., NO and interleukin-1β) [40]. Considering these three previous reports [38−40], it is thought that KRG-TS may exhibit antithrombotic and antiatherosclerotic effects without causing inflammation and progression of atherosclerotic lesion at the site of vascular wall injury. Therefore, KRG-TS is highlighted as a "nontoxic antiplatelet compound," and could be clinically applied to the prevention of platelet-mediated thrombosis. This result is supported by a previous study suggesting the protective effects of KRG on carotid artery thrombosis *in vivo* in rats [41]. In addition, both ginseng and ginsenosides are very useful for prevention of cardiovascular disease [42].

With regard to the antiplatelet effects of ginsenosides in KRG-TS, only G-Rg3 (20R, 20S) inhibited thrombin-induced platelet aggregation. This is in accordance with the reports that G-Rg3 (20R, 20S) inhibited arachidonic acid- or U46619-induced platelet aggregation

[43], and its analog (G-Rp1, dihydroxy G-Rg3) inhibited collagen- or thrombin-induced platelet aggregation [19,20]. Other reports also suggested that G-Rg1 [molecular weight (MW) = 800.94] and G-Rg2 (MW = 781.01) inhibit various agonists (i.e., thrombin, collagen, ADP)-induced platelet aggregation [44−46], but the inhibitory concentrations were 1 mg/mL (1.2mM) to 4 mg/mL (5mM) for G-Rg1 [44,45] and 1 mg/mL (1.3mM) for G-Rg2 [45,46]. These inhibitory concentrations (1.2−5mM) of G-Rg1 or G-Rg2 (1.3mM) [44−46] are very high compared with those of G-Rg3 and its analogues (G-Rp1, dihydroxy G-Rg3), which exhibited antiplatelet effect at concentrations in the range of micromoles [19,20,43]. Therefore, it is thought that the antiplatelet effects exhibited by G-Rg1 and G-Rg2 should be re-evaluated. Because only G-Rg3 in KRG-TS inhibited thrombin-induced human platelet aggregation, it is thought that G-Rg3 in KRG-TS might have contributed to the inhibition of platelet aggregation and $[Ca^{2+}]_i$ mobilization, which also supports the report that G-Rg3 elevated Ca^{2+}-antagonistic cAMP levels [20].

Thrombosis mainly results from the irreversible aggregation, which is closely related to the serotonin released from platelets activated by agonists (i.e., collagen and thrombin) [47−49]. In addition, the released serotonin is involved in causing migraine [50−52].

In conclusion, the most important result of this study is that KRG-TS significantly phosphorylates IP$_3$RI (Ser^{1756}) to inhibit thrombin-induced $[Ca^{2+}]_i$ mobilization, which contributed to attenuating the release of ATP and serotonin. Therefore, our results suggest that KRG-TS may be a physiologically effective negative regulator in platelet aggregation, a cause of thrombosis, atherosclerosis, and myocardial infarction. Because KRG-TS also inhibits serotonin release, it should also be evaluated as an antimigraine substance.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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