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RESEARCH PAPER

Ginsenoside-Rp1 inhibits platelet activation and thrombus formation via impaired glycoprotein VI signalling pathway, tyrosine phosphorylation and MAPK activation

M Endale[1], WM Lee[1], SM Kamruzzaman[1], SD Kim[1], JY Park[1], MH Park[2], TY Park[2], HJ Park[3], JY Cho[4] and MH Rhee[1]

[1]*Laboratory of Physiology & Cell Signaling, College of Veterinary Medicine, Kyungpook National University, Daegu, Korea,* [2]*Ambo Institute, Seoul, Korea,* [3]*College of Biomedical Science and Engineering, and Regional Research Center, Inje University, Gimhae, Korea, and* [4]*Department of Genetic Engineering, Sungkyunkwan University, Suwon, Korea*

Correspondence
Man-Hee Rhee, Laboratory of Veterinary Physiology and Cell Signaling, College of Veterinary Medicine, Kyungpook National University, Daegu 702-701, Korea. E-mail: rheemh@knu.ac.kr

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BACKGROUND AND PURPOSE

Ginsenosides are the main constituents for the pharmacological effects of *Panax ginseng*. Such effects of ginsenosides including cardioprotective and anti-platelet activities have shown stability and bioavailability limitations. However, information on the anti-platelet activity of ginsenoside-Rp1 (G-Rp1), a stable derivative of ginsenoside-Rg3, is scarce. We examined the ability of G-Rp1 to modulate agonist-induced platelet activation.

EXPERIMENTAL APPROACH

G-Rp1 *in vitro* and *ex vivo* effects on agonist-induced platelet-aggregation, granule-secretion, $[Ca^{2+}]_i$ mobilization, integrin-$\alpha_{IIb}\beta_3$ activation were examined. Vasodilator-stimulated phosphoprotein (VASP) and MAPK expressions and levels of tyrosine phosphorylation of the glycoprotein VI (GPVI) signalling pathway components were also studied. G-Rp1 effects on arteriovenous shunt thrombus formation in rats or tail bleeding time and *ex vivo* coagulation time in mice were determined.

KEY RESULT

G-Rp1 markedly inhibited platelet aggregation induced by collagen, thrombin or ADP. While G-Rp1 elevated cAMP levels, it dose-dependently suppressed collagen-induced ATP-release, thromboxane secretion, p-selectin expression, $[Ca^{2+}]_i$ mobilization and $\alpha_{IIb}\beta_3$ activation and attenuated $p38^{MAPK}$ and ERK2 activation. Furthermore, G-Rp1 inhibited tyrosine phosphorylation of multiple components (Fyn, Lyn, Syk, LAT, PI3K and PLCγ2) of the GPVI signalling pathway. G-Rp1 inhibited *in vivo* thrombus formation and *ex vivo* platelet aggregation and ATP secretion without affecting tail bleeding time and coagulation time, respectively.

CONCLUSION AND IMPLICATIONS

G-Rp1 inhibits collagen-induced platelet activation and thrombus formation through modulation of early GPVI signalling events, and this effect involves VASP stimulation, and ERK2 and $p38^{MAPK}$ inhibition. These data suggest that G-Rp1 may have therapeutic potential for the treatment of cardiovascular diseases involving aberrant platelet activation.

Abbreviations

GPVI, glycoprotein VI; G-Rp 1, ginsenoside-Rp1; LAT, linker for activation of T-cells; PI3K, phosphoinositide 3-kinase; Syk, spleen tyrosine kinase; VASP, vasodilator-stimulated phosphoprotein; $\alpha_{IIb}\beta_3$, glycoprotein IIb/IIIa or integrin-$\alpha_{IIb}\beta_3$

Introduction

Following blood vessel injury, platelets play a key role in haemostasis by forming thrombi that seal damaged tissue. But aberrant platelet activation triggered by pathophysiological factors can lead to the development and progression of cardiovascular disorders such as thrombosis (Furie and Furie, 2008), atherosclerosis (Huo *et al.*, 2003), hypertension (Perros *et al.*, 2008) and inflammation (Gawaz *et al.*, 2005; Zarbock *et al.*, 2007).

Collagen binding to the glycoprotein VI (GPVI) receptor leads to the Src family kinase mediated Fc receptor γ-chain tyrosine phosphorylation (Gibbins, 2004) followed by Syk and LAT tyrosine phosphorylations (Poole *et al.*, 1997). Then activated LAT (Pasquet *et al.*, 1999a) and phosphoinositide 3-kinase (PI3K) (Gibbins *et al.*, 1998) mediate the recruitment and tyrosine phosphorylation of PLCγ2, which results in 1,2-diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3) release, PKC activation and Ca^{2+} mobilization. Such platelet activations and granule secretions lead to the release of fibrinogen and p-selectin as well as prothrombotic factors, including thrombin, ADP and thromboxane A_2 (TXA_2), which act in an autocrine/paracrine fashion and activate other platelets (Ruggeri, 2002). All factors indicated above contribute to inside-out activation of integrin-$\alpha_{IIb}\beta_3$ for stable adhesion and aggregation (Andrews and Berndt, 2004). MAPKs, including ERK-2, $p38^{MAPK}$ and JNK-1, are also activated by agonist-stimulated platelets (Adam *et al.*, 2008). In general, platelet activation is multifaceted and responsive to different stressors, indicating the possibility of specific pharmacological targeting aimed at a selective inhibition of the pathways more relevant to the atherothrombosis than to haemostasis.

Current anti-platelet therapy can be improved with the development of agents against new platelet surface targets such as proteinase-activated receptor 1 (PAR_1) and 4, GPVI, integrin-α2β1 and PGE receptors (Barrett *et al.*, 2008), with the goal of better separating reduced thrombotic events from increased bleeding events (Michelson, 2010). However, the question that remains is how to improve treatment and prevention of thrombosis without increased haemorrhagic side effects? In our attempt to screen such agents, we have screened ginseng-derived compounds with anti-platelet activity and relative safety.

Ginseng (*Panax ginseng* C.A. Meyer) has been used as a traditional oriental medicine to treat illness and promote health. Recent reviews have discussed a multitude of *in vivo* and *in vitro* studies that have shown ginseng's beneficial effects in a wide range of pathological conditions such as nervous, cardiovascular, immune diseases and cancer (Jia *et al.*, 2009; Lu *et al.*, 2009; Kim and Park, 2012). Antioxidant, anti-inflammatory and anti- or pro-apoptotic activities are reported to be the possible ginseng-mediated protective mechanisms (Lu *et al.*, 2009; Edzard, 2010). Ginseng extracts and some ginsenosides are also known to have anti-platelet activities (Hwang *et al.*, 2008; Lee *et al.*, 2008). However, they are relatively unstable and show low bioavailability (Tawab *et al.*, 2003). Because of the stability limitations of ginseng ginsenosides, we aimed to synthesize novel ginsenoside-originated compounds with improved chemical stability, bioavailability and mass production rate (Park *et al.*, 2008). Ginsenoside (G)-Rp1 was prepared on a large scale from ginsenosides (e.g. Rg3, 2H-Rg3, G-Rg5 and Rk1) by means of a reduction with hydrogenation and was verified to be chemically stable (Park and Park, 2005). In addition, G-Rp1 displayed 10-fold more potent anti-cancer activity compared with G-Rg3 and G-Rg5 (Park *et al.*, 2008; Kumar *et al.*, 2009).

Previously, we showed that ginsenoside-2H-Rg3 had more potent anti-platelet activity than its parent compound, ginsenoside-Rg3 (Lee *et al.*, 2008). We also showed that G-Rp1, another derivative of Rg3 or 2H-Rg3, inhibits CD29-mediated cell adhesion (Kim and Cho, 2009), LPS-induced IL-1β production (Kim *et al.*, 2009a) and metastsis (Park *et al.*, 2008). However, information on the effect of G-Rp1 on platelet activation is limited.

In this study, the anti-platelet properties of G-Rp1 were investigated using agonist-induced platelet aggregation and thrombus formation together with its mechanism of action on collagen-activated platelets. The data reveal that G-Rp1 displays potent anti-platelet activity mediated through the suppression of the GPVI signalling molecules, tyrosine phosphorylation followed by inhibition of ERK2 and $p38^{MAPK}$ activation associated with an enhanced PKA-dependent vasodilator-stimulated phosphoprotein (VASP) phosphorylation. In addition, G-Rp1 suppressed *ex vivo* platelet aggregation and ATP secretion, and inhibited *in vivo* thrombus formation without affecting the coagulation time and bleeding time, respectively.

Methods

Animals

Male Sprague–Dawley rats weighing from 240 to 250 g and male C57BL/6J mice (16 weeks age and 20–23 g) were obtained from Orient Co. (Seoul, Korea) and maintained in a standard laboratory animal facility with free access to feed, water and were acclimatized to these conditions for at least two weeks before use. All studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). The experiments were carried out in accordance to internationally accepted guidelines on the use of laboratory animals, and the protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Kyungpook National University.

Platelet preparation and aggregation

Rats were anaesthetized with ethyl ether before blood (8 mL) was obtained using a venipuncture (by inserting a 23-gauge needle into the abdominal aorta) and transferred to a 15 mL test tube containing 1 mL citrate phosphate dextrose solution (CPD in mM: 90 $Na_3C_6H_5O_7{\cdot}2H_2O$, 14 $C_6H_8O_7{\cdot}H_2O$, 128.7 $NaH_2PO_4{\cdot}H_2O$ and 2.55 g·100 mL^{-1} dextrose). Platelet-rich plasma (PRP) was obtained following blood sample centrifugation at 170× *g* for 7 min. The PRP samples were again centrifuged at 120× *g* for 7 min to remove residual erythrocytes. In order to remove the CPD solution and isolate the platelets, PRP was centrifuged twice at 350× *g* for 10 min. The platelets of the precipitate were adjusted to the proper number (10^8 mL^{-1}) for the aggregation assay in Tyrode buffer (in mM: 137 NaCl, 12 $NaHCO_3$, 5.5 glucose, 2 KCl, 1 $MgCl_2$, 0.3 $NaHPO_4$, pH 7.4). All platelet preparations were conducted at room temperature.

Platelet aggregation was performed as previously described (Lee *et al.*, 2010). Aggregation was monitored by measuring light transmission via an aggregometer (Chronolog Corp., Havertown, PA, USA). The washed platelets were pre-incubated at 37°C for 3 min with either G-Rp1 or vehicle. The reaction mixture was further incubated for 8 min with stirring at 250× *g*. The concentration of the vehicle was held at less than 0.05%.

ATP release assay

Washed platelets were pre-incubated for 3 min at 37°C with various concentrations of G-Rp1 and then stimulated with collagen. The reaction was terminated, samples were centrifuged, and supernatants were used for the assay. ATP release was measured in a luminometer (GloMax 20/20, Promega, Madison, WI, USA) using ATP assay kit (Biomedical Research Service Center, Buffalo, NY, USA).

Measurement of TXA_2 generation

Washed platelets were pre-incubated with G-Rp1 or vehicle for 3 min in the presence of 1 mM $CaCl_2$ and stimulated with collagen for 5 min before the termination of the reactions by the addition of ice-cold 5 mM EDTA and 0.2 mM indomethacin. Samples were centrifuged, and the TXB_2 levels (the stable metabolite of TXA_2) in the supernatants were measured using the TXB_2 EIA Kit (Enzo Life Sciences, Ann Arbor, MI, USA), by following the manufacturer's instructions.

Flow cytometry

P-selectin secretion. Washed platelets, treated with either G-Rp1 or vehicle were stimulated with collagen and incubated for 5 min at 37°C. The reaction was terminated, and cells were centrifuged followed by resuspension in ice-cold PBS, containing 10% FCS and 1% sodium azide. Cells were then incubated with CD62P primary antibody in 3% BSA/PBS for 30 min at 4 °C in the dark. Next, platelets were fixed and washed three times by centrifugation at 400× *g* for 5 min and resuspended in ice-cold PBS followed by FITC-conjugated secondary antibody incubation in 3% BSA/PBS for 30 min at 4 °C in the dark. Platelets were again washed three times by centrifugation at 400× *g* for 5 min and resuspended in ice-cold PBS, 3% BSA and 1% sodium azide. Flow cytometry analysis was performed on a FACSCalibur™ flow cytometer® using CellQuest software (BD Biosciences, San Jose, CA, USA).

Fibrinogen binding to platelets. Washed platelets were initially treated with G-Rp1 or vehicle and incubated for 5 min at room temperature. Then 2.5 μg mL^{-1} collagen was added together with 200 μg mL^{-1} Alexa Fluor 488-human fibrinogen, and the sample was incubated at 37°C for 15 min. Non-specific binding of fibrinogen was estimated by measuring fibrinogen binding in the presence of a specific integrin inhibitor, RGDS peptide (1 mM). Alexa Fluor 488-fibrinogen binding to platelets was determined by flow cytometry using FACSCalibur flow cytometer and data were analysed using CellQuest software (BD Immunocytometry Systems, San Jose, CA, USA).

Determining the $[Ca^{2+}]_i$

The $[Ca^{2+}]_i$ was determined with fura-2/AM as described previously (Kamruzzaman *et al.*, 2010). Briefly, the platelet-rich plasma was incubated with 5μM of fura-2/AM for 60 min at 37°C. The fura-2-loaded washed platelets (10^8 mL^{-1}) were then pre-incubated with G-Rp1 for 3 min at 37°C in the presence of 1 mM $CaCl_2$. Next, the platelets were stimulated with thrombin for 5 min. Fura-2 fluorescence was measured in a spectrofluorometer (F-2500, Hitachi, Tokyo, Japan) with an excitation wavelength that ranges from 340 to 380 nm, changing every 0.5 s, and with an emission wavelength of 510 nm. The $[Ca^{2+}]_i$ was calculated by the method of Schaeffer and Blaustein (1989): $[Ca^{2+}]_i$ in cytosol = 224 nM × $(F - F_{min})$ / $(F_{max} - F)$, where 224 nM is the dissociation constant of the fura-2-Ca^{2+} complex, and F_{min} and F_{max} represent the fluorescence intensity levels at very low and very high Ca^{2+} concentrations, respectively. In our experiment, F_{max} is the fluorescence intensity of the fura-2-Ca^{2+} complex at 510 nm after the platelet suspension containing 1 mM $CaCl_2$ had been solubilized by Triton X-100 (0.1%). F_{min} is the fluorescence intensity of the fura-2-Ca^{2+} complex at 510 nm, after the platelet suspension containing 20 mM Tris/3 mM of EGTA had been solubilized by Triton X-100 (0.1%). *F* represents the fluorescence intensity of the fura-2-complex at 510 nm after the platelet suspension was stimulated by thrombin, with G-Rp1 or vehicle, in the presence of 1 mM $CaCl_2$.

Measurement of cAMP and cGMP

Washed platelets were pre-incubated for 3 min at 37°C with various concentrations of G-Rp1 in the presence or absence of IBMX, a non-selective PDE inhibitor, or vehicle, and then stimulated with collagen for 5 min in the presence of 1 mM $CaCl_2$ in a platelet aggregometer. The reaction was terminated by addition of equal volumes of 80% ice-cold ethanol. Samples were then centrifuged at 2000× *g* for 10 min at 4°C, and the supernatant cAMP and cGMP levels were determined with a cAMP and cGMP EIA Kit (Ann Arbor, MI, USA).

Immunoprecipitation studies

For protein precipitation assays, platelets were suspended at 8 × 10^8 cells·mL^{-1} in modified Tyrode's-HEPES buffer containing 1 mM EGTA to prevent aggregation. Platelets were incubated with G-Rp1 dissolved in DMSO or with DMSO alone (0.1% v v^{-1}) for 5 min (after 10 s stirring) followed by stimulation with collagen for 3 min at 37°C in an optical platelet aggregometer (Chronolog Corp.) with continuous stirring (1200 r.p.m.). Platelet stimulation was terminated by the addition of an equal volume of ice-cold NP40 lysis buffer [2% (v v^{-1}) Nonidet P40, 50 mM Tris, 20 mM NaF, 25 mM β-glycerolphosphate, 120 mM NaCl, 10 mM EDTA, 1 mM PMSF, 2 mM Na_3VO_4, 5 μg mL^{-1} leupeptin, 5 μg mL^{-1} aprotonin, 1 μg mL^{-1} pepstatin A, 1 mM benzamide; pH 7.5]. Detergent-insoluble debris was removed and the lysates were pre-cleared by mixing with protein A-Sepharose (PAS) for 1 h at 4°C. PAS was removed, and lysates were incubated in rotation with antibody (3 μg mL^{-1}) overnight at 4°C. Protein/antibody complexes were isolated with PAS for 4 h at 4°C. After 5 washes with lysis buffer, the beads were boiled in Laemmli sample buffer (2% SDS, 1% β-mercaptoethanol, 0.008% bromophenol blue, 80 mM Tris pH 6.8, 1 mM EDTA) and the proteins were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked with 5% milk, probed with respective antibodies, visualized by enhanced chemiluminescence and quantified using a Bio-Rad GS710 densitometer

(Bio-Rad Laboratories, Hercules, CA, USA) with Quantity One analysis software.

Immunoblotting

Platelets treated with G-Rp1 or vehicle were stimulated with collagen and incubated for 5 min in an aggregometer before the reaction was terminated, and cells were centrifuged. Lysates were then prepared by solubilizing and centrifuging platelets in sample buffer (125 mM Tris–HCl at pH 6.8, 2% SDS, 2% β-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue in the presence of protease inhibitors, μg mL^{-1}:1 PMSF, 2 aprotinin, 1 leupeptin and 1 pepstatin A). Protein concentration was determined using BCA Assay (PROMEASURE, iNtRON Biotechnology, Seongnam, Korea). Equal volumes of platelet proteins were resolved in (8 to 12%) SDS-PAGE and transferred to PVDF membrane in a transfer buffer (25 mM Tris, pH 8.5, 0.2 M glycerin and 20% methanol). Immunoblots were blocked with TBS-T containing 5% nonfat dry milk and incubated with primary antibody diluted in a blocking solution. The immunoblots were again incubated with HRP secondary antibody, and the membranes were visualized using enhanced chemiluminescence, ECL (iNtRON Biotechnology).

PI3-kinase homogenous time-resolved fluorescent (HTRF) assay

The PI3-Kinase HTRF Assay, mouse KinaseProfiler™ kit from Millipore (Dudee, UK) was used. The assay was performed as previously described (Kong and Yamori, 2007) with some modifications. The kinase reaction was carried out in a reaction mixture of 20 μL. Each class I PI3Kα, β and δ isoform protein was incubated in the assay buffer containing 10 μM PIP2 and ATP (200 μM) in the presence of G-Rp1 (10–20 μM) in a 384-well plate at room temperature. The reaction was initiated by the addition of ATP and stopped by adding 5 μL stop solution containing EDTA and biotin-PIP3 after 20 min. Then 5 μL detection buffer was added, which contained the Europium-labelled anti-glutathione S-transferase (GST) antibody, GST-tagged GRP1 PH domain and streptavidin–allophycocyanin (APC). After incubation at room temperature for 14 h, the plate was read using the EnVision 2103 Multilabel Reader in time-resolved fluorescence mode, and the HTRF signal was determined.

Effect of gingenoside-Rp1on thrombus formation in extracorporeal shunts

The *in vivo* antithrombotic activity of G-Rp1 was evaluated in a rat extracorporeal shunt model by the method of Umetsu and Sanai (1978), with a slight modification. Briefly, 1 h after administration of G-Rp1 (15–50 mg kg^{-1}, p.o.) or vehicle, non-fasted male rats were anaesthetized with urethane (1.75 g kg^{-1}, i.p.), and an incision was made over the trachea. The right jugular vein and left carotid artery were exposed, and the two ends of the extracorporeal shunt were inserted into them. The shunt consisted of two 12 cm lengths of polyethylene tubing (0.81 mm and 0.58 mm external and internal diameter, respectively) connected by 5 mm silicone rubber plugs to a 6 cm length of polyvinyl tubing (3 mm internal diameter). A 6 cm length of cotton thread was secured between the two plugs so that it remained longitudinally orientated in the blood flow through the cannula. Before cannulation the tubing was filled with 0.9% w v^{-1} sodium chloride solution (saline). The shunt was left in place for 15 min after the extracorporeal circulation was started. The flow was then stopped, the thread was removed and the thrombus formed was separated from the thread and unclotted blood and then weighed.

In vivo *bleeding assay*

Male mice were divided into three treatment groups (eight mice per group). The treatments were administered i.p. 60 min before bleeding; these included 0.1% vehicle control, aspirin (10 mg kg^{-1}), G-Rp1 (50 mg kg^{-1}). They were anaesthetized i.p. with a mixture of ketamine (75 mg kg^{-1}) and xylazine (10 mg kg^{-1}). The tail bleeding model was based on previous methods with minor modifications (Beviglia *et al.*, 1993; Lau *et al.*, 2009). Briefly, the tail was pre-warmed for 3 min in a 0.9% saline solution at 37°C. The bleeding was induced by precise transection of the mouse tail at 5 mm from the tip. The distal portion of the tail (3 cm) was immersed vertically into the 0.9% saline solution at 37°C. The time between the start of transection to bleeding cessation was recorded as the bleeding time. Bleeding cessation was considered to be the time when the flow of blood stops.

Ex vivo *platelet aggregation and ATP secretion*

G-Rp1 effect on *ex vivo* aggregation and ATP secretion was examined as described above. Forty male C57BL/6J mice (20–23 g) were allocated to four groups of 10 mice each and administered, p.o., vehicle (group 1), 50 mg kg^{-1} G-Rp1 (group 2), 100 mg kg^{-1} G-Rp1 (group 3) or 50 mg kg^{-1} aspirin (group 4) suspended in 0.5% carboxymethylcellulose solution for 1 week. Blood was collected 60 min after administration of the last dose, and platelets were prepared as described above. Platelet aggregation and ATP secretion were induced by 2.5 μg mL^{-1} collagen or 10 μM ADP. G-Rp1 effects on aggregation and ATP secretion were tested.

Ex vivo *coagulation assay*

Anti-coagulation activity of G-Rp1 was evaluated by measuring plasma clotting time of PPP. The activated partial thromboplastin time (APTT) and prothrombin time (PT) were measured according to Hara *et al.* (1994), with a slight modification using an Automated Coagulation Laboratory 100 Instrument (Instrumentation Laboratory Co., Milan, Italy). Briefly, the PPP from mice treated with G-Rp1, aspirin or vehicle was incubated at 37°C for 7 min. One hundred microlitres of the incubated PPP was mixed with 50 mL of cephalin in the process plate, and coagulation was started with the addition of 1 mM $CaCl_2$ and 100 mL thromboplastin to the PPP for the APTT and PT assays, respectively.

Statistical analysis

Data were analysed by a one-way ANOVA, followed by a *post hoc* Dunnett's test in order to determine the statistical significance of the differences by Statistical Analysis Software, version 9.1 (SAS Institute Inc., Cary, NC, USA). All data are presented as means ± SEM, and *P*-values of 0.05 or less were considered to be statistically significant.

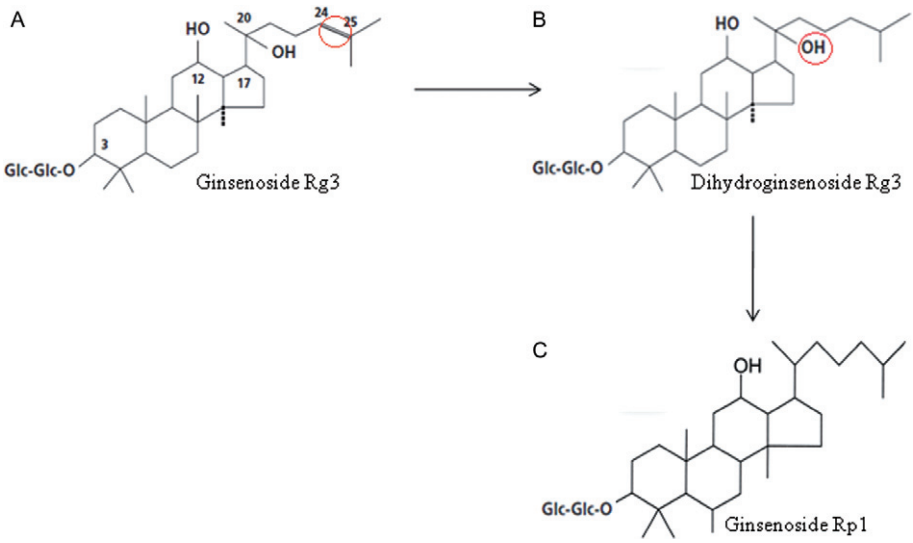

Figure 1

Chemical structure of ginsenoside Rp1 (3-O-β-D-glucopyranosyl (1→2)-β-D-glucopyranosyl dammarane-3β,12β-diol) (C) and its parent compounds ginsenoside Rg3 (A) and dihydroginsenoside-Rg3 (B).

Materials

Ginsenoside-Rp1 with 100% purity (Figure 1) was obtained from Ambo Institute (Seoul, Korea). Thrombin, ADP, forskolin and Fura-2/AM were obtained from Sigma (St. Louis, MO, USA). Collagen was procured from Chronolog. cAMP EIA kit was from Cayman Chemicals (Ann Arbor, MI, USA), and ATP assay kit was from Biomedical Research Service Center (Buffalo, NY, USA). Fibrinogen Alexa Fluor® 488 conjugate was obtained from Invitrogen Molecular Probes (Eugene, OR, USA). Antibodies against total-ERK, phospho-ERK, total JNK, phospho-JNK, total-$p38^{MAPK}$, phospho-$p38^{MAPK}$, $VASP^{ser157}$, phospho-$VASP^{ser157}$, PLCγ_2, phoshpo-PLCγ_2, PI3K (p85), phospho-PI3K (phoshpo-p85), PKAαβγ, phosphor-tyrosine, Syk and β-Actin were from Cell Signaling (Beverly, MA, USA). Antibodies against Fyn, Lyn and LAT were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibody to phospho-$VASP^{ser239}$ was from ALEXIS Biochemicals (Bingham, UK). The PI3-Kinase HTRF Assay kit was from (Millipore, Watford, UK). All other chemicals were of reagent grade.

Results

Effects of ginsenoside-Rp1 on platelet aggregation

In our previous report, we compared anti-platelet activities of ginsenoside-Rg3 and its derivative dihydroginsenoside-Rg3, both of which are parent compounds of G-Rp1. We have shown that 2H-Rg3 was about twofold more potent than that of ginsenoside-Rg3 (Lee *et al.*, 2008). Here, we demonstrated, for the first time, that another semi-synthetic ginsenoside, derived from either ginsenoside-Rg3 or 2H-Rg3, has strong anti-platelet activity, fivefold more potent than that of 2H-Rg3 in collagen-stimulated platelets (Figure 2B). In our model, we previously determined that collagen (2.5 μg mL^{-1}), thrombin (0.1 U mL^{-1}) and ADP (10 μM) induced complete platelet aggregation and, in the present study, these agonist concentrations were used to induce platelet activation. As shown in Figure 2A and B, G-Rp1 potently inhibited collagen-induced platelet aggregation in a concentration-dependent manner. The IC_{50} of G-Rp1 against collagen-activated platelets was 10.1 ± 0.1 μM. We further examined the compound against thrombin- and ADP-induced platelet aggregation. G-Rp1 suppressed thrombin- and ADP-activated platelet aggregations in a concentration-dependent fashion. It exhibited a similar pattern of inhibition for both agonists with an IC_{50} of 6.8 ± 0.9 μM for thrombin (Figure 2C and D) and 6.1 ± 0.3 μM for ADP (Figure 2E).

Effects of G-Rp1 on collagen-induced ATP release, p-selectin secretion, thromboxane A_2 production and calcium mobilization

Since granule secretions are critical markers of platelet activation prior to aggregation (Zarbock *et al.*, 2007; Barrett *et al.*, 2008; Mackman, 2008), we determined whether G-Rp1 pre-incubation affects collagen-induced-platelet granule secretions. As such, agonist-induced ATP release and p-selectin secretion were significantly and dose-dependently inhibited in G-Rp1-treated platelets (Figure 3A–C). At the doses of 10 and 20 μM, the compound completely inhibited the release of ATP and the expression of p-selectin, respectively (Figure 3A–C), suggesting that platelet-dense and α-granule secretory activities were impaired by the G-Rp1 pretreament. In addition, since TXA_2 by acting as a positive-feedback mediator amplifies the initial signals to ensure the rapid activation and recruitment of platelets into a growing thrombus (Offermanns, 2006); we examined the effect of G-Rp1 on its secretion. As shown in Figure 3D, G-Rp1 inhibited collagen-induced TXA_2 generation in a concentration-dependent manner.

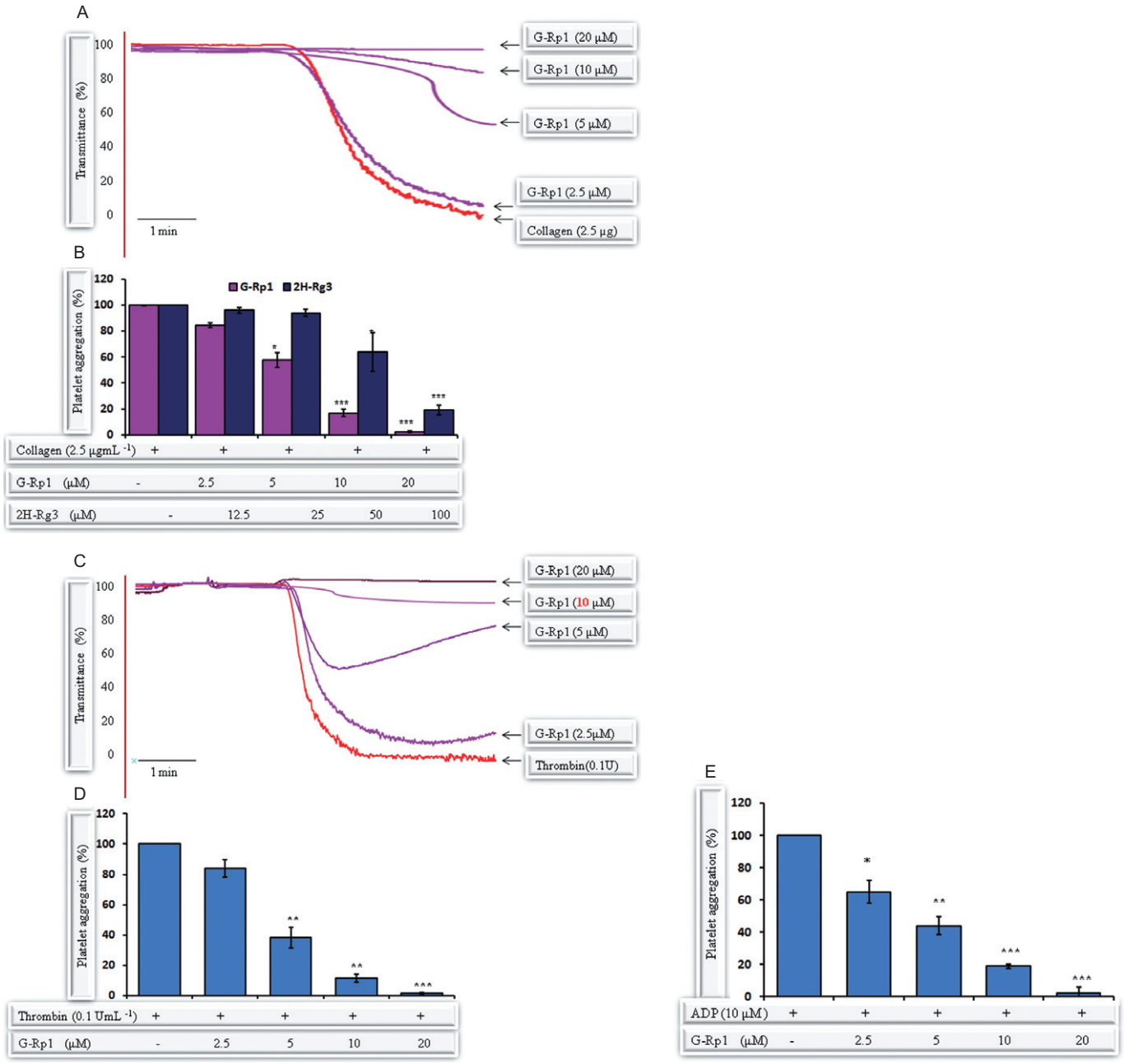

Figure 2

The inhibitory effect of G-Rp1 on thrombin-, collagen- and ADP-induced platelet aggregation. Platelets ($3 \times 10^8 mL^{-1}$) were pre-incubated with or without the G-Rp1 in the presence of 1 mM $CaCl_2$ for 2 min at 37°C. The platelets were stimulated with collagen (A and B), thrombin (C and D) and ADP (E). The aggregation reaction was terminated at 5 min, and the % aggregation rate was determined. Tracings (A and C) are representative of platelet aggregation at the indicated concentrations, and the bar graphs (B, D and E) are a summary of 6 to 9 independent experiments. Each column shows the mean ± SEM of at least six independent experiments performed. *: $P < 0.05$, **: $P < 0.01$ or ***: $P < 0.001$ versus agonist-activated control.

Intracellular calcium concentration plays a crucial role in platelet aggregation, activation and thrombus formation (Jackson *et al.*, 2003). Thus, we investigated the effects of G-Rp1 on thrombin-induced $[Ca^{2+}]_i$ mobilization. Pretreatment of rat platelets with the test compound significantly inhibited the rise in thrombin-induced $[Ca^{2+}]_i$ (Figure 3E).

G-Rp1 inhibits collagen-induced fibrinogen binding to integrin $\alpha_{IIb}\beta_3$

The principal outcome of adhesion and activation in platelets is a change in the ligand-binding function of integrin-$\alpha_{IIb}\beta_3$ (Calderwood, 2004), which leads to aggregation mediated by

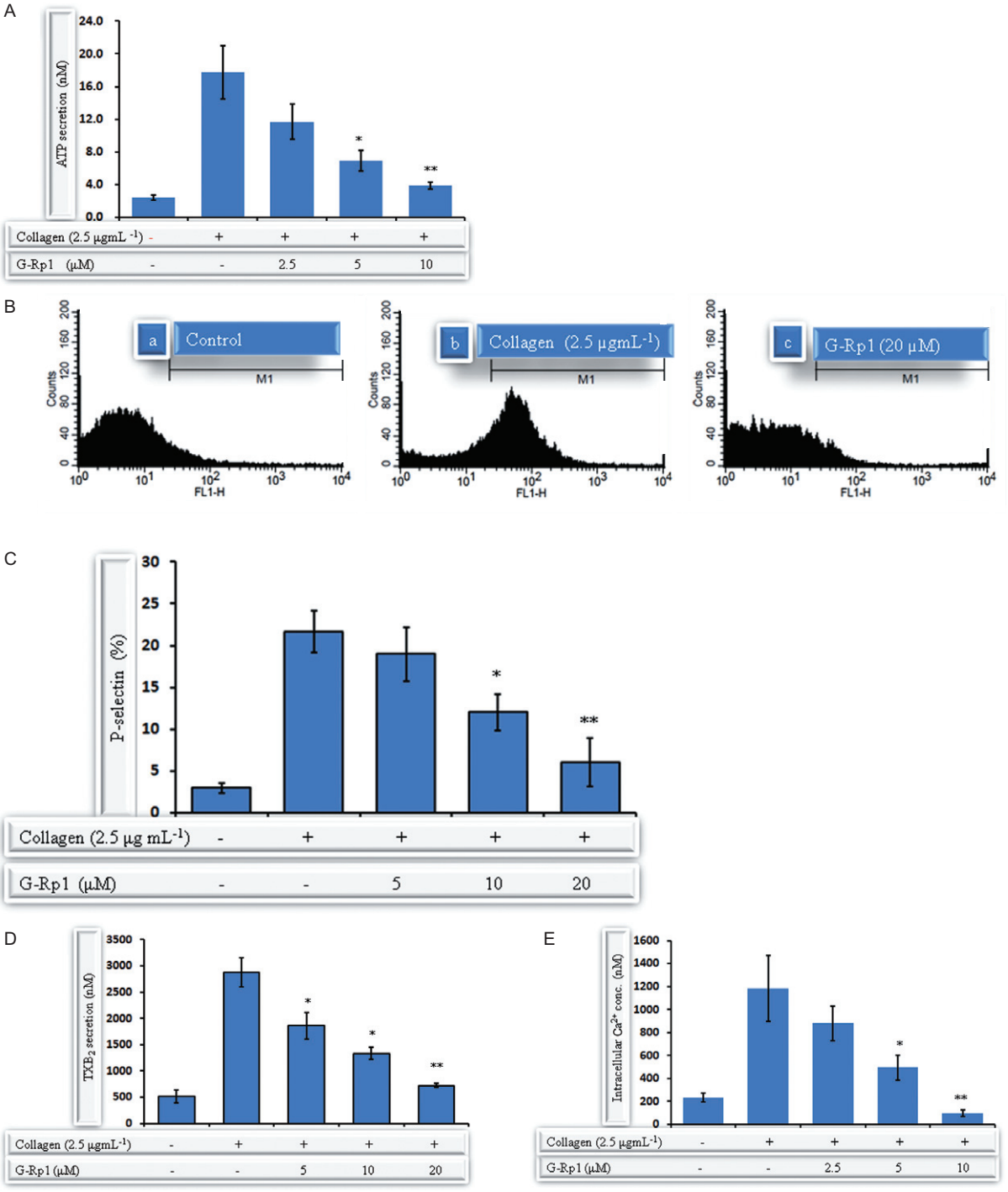

Figure 3

Effects of G-Rp1 on collagen-activated platelet ATP release, p-selectin expression TXA_2 production and $[Ca^{2+}]_i$ concentration. Washed platelets were pre-incubated with G-Rp1 (at the concentrations indicated) and stirred in an aggregometer for 2 min prior to collagen or thrombin stimulation for 5 min, and then the reactions were terminated followed by granule secretion assay. (A) ATP release in response to agonist stimulation was performed and G-Rp1, dose-dependently, suppressed collagen stimulated platelet ATP release. (B and C) Collagen-induced surface p-selectin expression was analysed. G-Rp1 significantly attenuated collagen-activated platelet surface p-selectin expression. (B), a and b represent untreated basal and collagen-stimulated, and c represents G-Rp1 (20 μM). (C) The bar graph shows a summary of four independent experiments. (D) G-Rp1 was pre-incubated with platelets at 37°C for 3 min followed by the addition of collagen (2.5 μg mL^{-1}). The TXB_2 formation was terminated by addition of 2 mM EDTA and 200 μM indomethacin 5 min after the addition of collagen. (E) Platelets were loaded with Fura-2/AM. The platelets (3×10^8 mL^{-1}) were pre-incubated with or without a G-Rp1 in the presence of 1 mM $CaCl_2$ for 2 min at 37°C. The platelets were stimulated with thrombin (0.1 U mL^{-1}) for 3 min at 37°C. $[Ca^{2+}]_i$ levels were determined. Bar graphs show mean ± SEM of at least three independent experiments performed. *: $P < 0.05$ or **: $P < 0.01$ versus agonist-activated control.

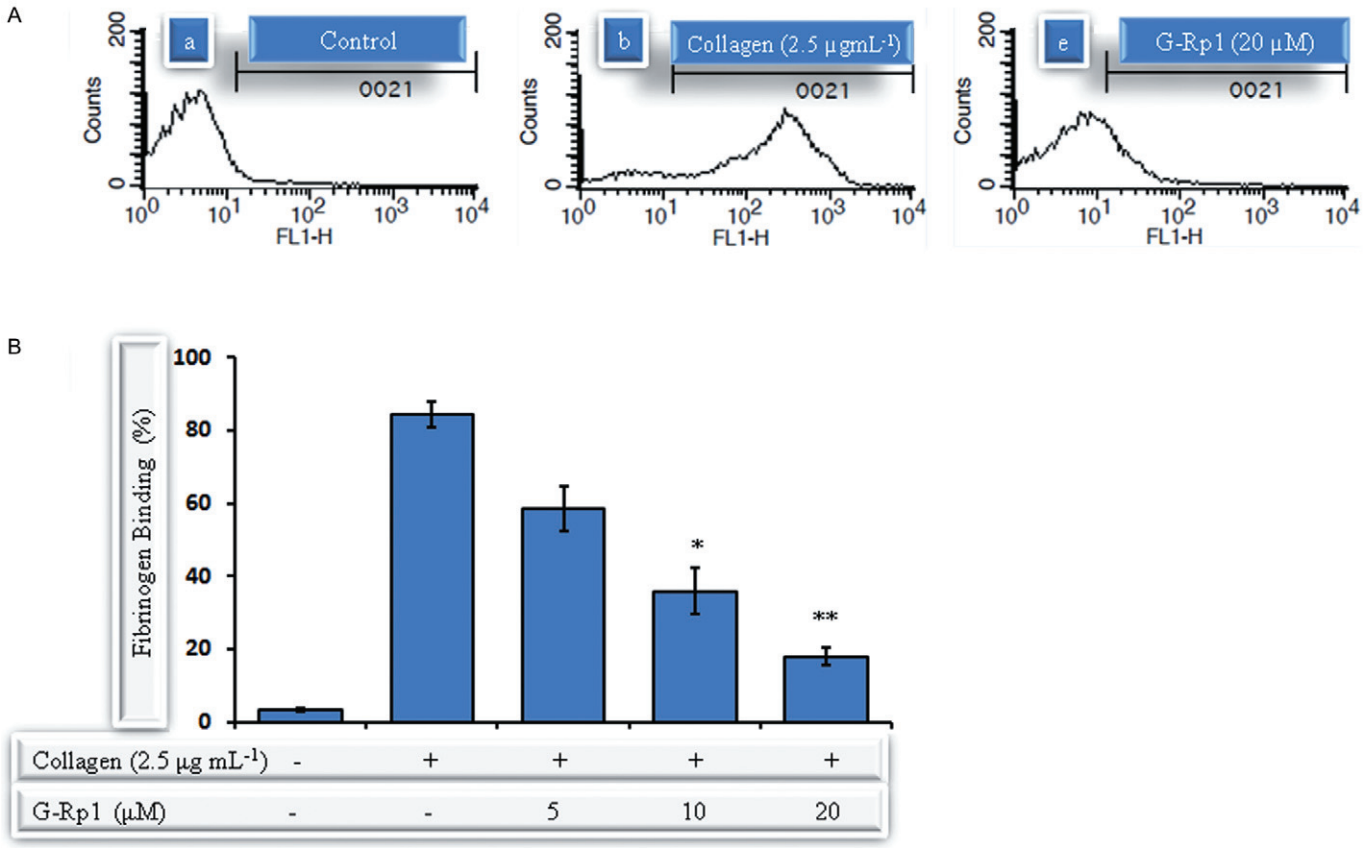

Figure 4

G-Rp1 inhibits collagen-induced fibrinogen binding to integrin $\alpha_{IIb}\beta_3$. The inhibitory effect of G-Rp1 on fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ in collagen-stimulated platelets was examined by flow cytometric analysis. Washed platelets were pretreated with G-Rp1, and then collagen (2.5 μg mL^{-1}) was added together with Alexa Fluor 488-human fibrinogen (200 μg mL^{-1}), and the sample was incubated at 37°C for 15 min. Bar graph represents summary of inhibitory effect of G-Rp1 on fibrinogen binding. *: $P < 0.05$, **: $P < 0.01$ versus control.

adhesive substrates bound to the membranes of activated platelets (Ruggeri, 2002). Thus, we examined the effect of the compound on the functional response induced by integrin $\alpha_{IIb}\beta_3$ activation. Figure 4A and B shows that collagen-activated soluble fibrinogen bindings were significantly decreased in G-Rp1-treated platelets, as compared with the control group.

Effects of G-Rp1 on cAMP/cGMP activity in platelets

Since platelet activation has been known to inhibit cAMP generation and cyclic nucleotide-dependent protein kinase activity (Weber *et al.*, 1999; Li *et al.*, 2003; Sudo *et al.*, 2003), we next investigated whether G-Rp1 influences basal levels of cAMP in the compound-treated platelets. Platelets were incubated with various concentrations of G-Rp1 alone or in combination with IBMX (50 μM), and the generation of intracellular cAMP or cGMP was assessed in resting platelets. G-Rp1 increased the intracellular cAMP levels in a concentration-dependent manner (Figure 5A and B), whereas it had no significant effect on the cGMP level (data not shown). In addition, though slightly enhanced, co-incubation of G-Rp1 with IBMX did not significantly change the increase in cAMP levels (Figure 5B).

G-Rp1 enhanced VASP phosphorylation

VASP is a substrate of cyclic nucleotide (cAMP/cGMP)-dependent protein kinases (PKA/PKG) and its stimulation by the increased activity of these kinases inhibits platelet activation (Aszodi *et al.* 1999; Pula *et al.*, 2006). As shown in Figure 6A lanes 4 and 5, G-Rp1 treatment increased phospho-$VASP^{ser157}$ activation in a concentration-dependent manner. At a higher dose (20 μM), the compound also exerted a modest increase in phospho-$VASP^{ser239}$ (Figure 6A, lane 5). Figures 6A, upper panel and 6B, middle panel show that G-Rp1 exerted both activation of phospho-$VASP^{ser157}$ and translocation of total $VASP^{157}$ from 46 to 50 kDa protein. G-Rp1-mediated VASP phosphorylation was compared with its ability to activate forskolin. As shown in Figure 6B, lane 2, forskolin stimulation exhibited a strong p-$VASP^{ser157}$ and total $VASP^{ser157}$ activation, which was inhibited by H-89, a potent PKA inhibitor (de Rooij *et al.*, 1998). Similarly, G-Rp1 evoked VASP activation in Figure 6B, lane 3, was totally reversed by H-89 (Figure 6B, lane 5). VASP is one of the major PKA substrates in platelets and has been used to monitor the activa-

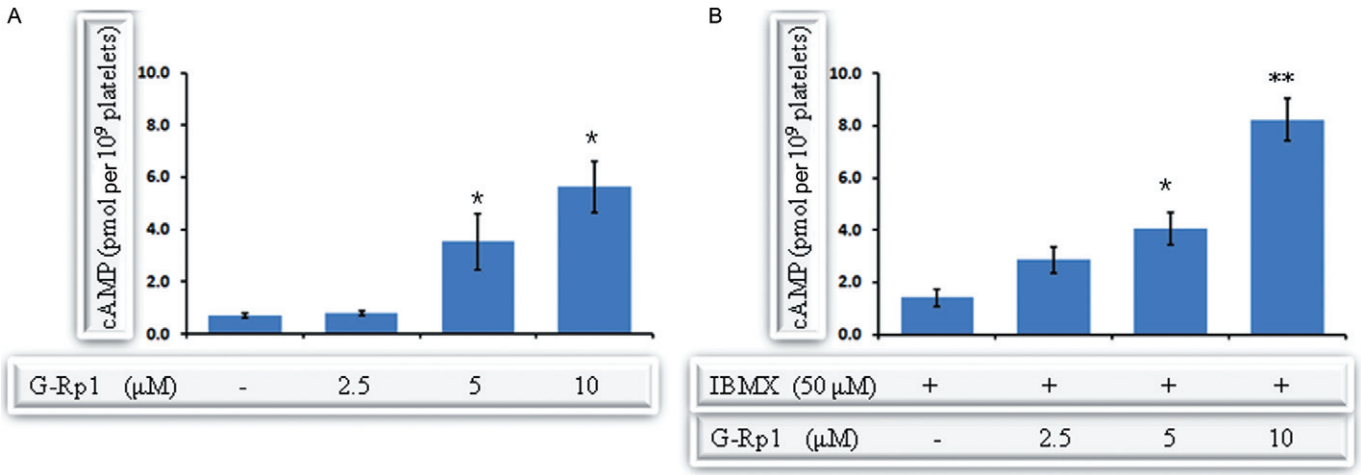

Figure 5

G-Rp1 elevates basal levels of cAMP activity. Rat washed platelets were stirred either in the presence of vehicle or G-Rp1 (at the concentrations indicated) in an aggregometer; the reaction was terminated and then cAMP enzyme immunoassays were performed. (A) G-Rp1 significantly increased cAMP accumulation in resting platelets in a dose-dependent manner. (B) IBMX pretreatment slightly potentiated G-Rp1-induced cAMP elevation. Results are a summary of at least three independent experiments performed and results in bar graphs are presented as mean ± SEM *: $P < 0.05$ or **: $P < 0.01$ versus control.

tion state of the kinase (Walter *et al.*, 1993). Treatment of platelets with forskolin or G-Rp1 resulted in phosphorylation of VASP at Ser^{157}, the site preferred by PKA (Figure 6C). To investigate whether G-Rp1-induced VASP phosphorylation at Ser^{157} was mediated by PKA, we examined catalytic PKAαβγ activation and used the PKA inhibitor, H-89 (inhibits ATP binding to PKAc). H-89 strongly decreased PKA activation and VASP phosphorylation caused by forskolin and G-Rp1 (Figure 6B and C, upper and middle lanes). A similar pattern of inhibition was also observed with the PI3K inhibitor, LY297002, suggesting that PKA activation is downstream of PI3K signalling (Figure 6C).

G-Rp1 attenuated collagen-activated platelet p38 and ERK2 phosphorylations

Since it is a well-established fact that ERK2, p38 and JNK1 are present in platelets and activated by various agonists (Adam *et al.*, 2008), we determined whether modulation of the signalling pathway that includes collagen-induced platelet JNK1, ERK2 and p38 phosphorylation is involved in the anti-platelet activity of G-Rp1. G-Rp1 significantly and dose-dependently suppressed collagen-induced p38 and ERK2 phosphorylations (Figure 7A and B) but not JNK activation (data not shown). Collagen binding to GPVI initiates a signalling cascade, leading to the activation of PI3K (Pasquet *et al.*, 1999b) and PLCγ2 (Pasquet *et al.*, 1999a). To determine whether G-Rp1-mediated inhibition of collagen-induced ERK2 and $p38^{MAPK}$ expressions is associated with its effect on early events of collagen receptor signalling pathways, we employed pharmacological inhibitors of PI3K and PLC activation and examined the levels of MAPK expression. Figure 7B shows that, in addition to G-Rp1 and their inhibitors (PD98059 and SB203580), collagen-induced ERK2 and $p38^{MAPK}$ activations were also inhibited by LY297002 (PI3K) and U73122 (PLC) inhibitors (Figure 7B), suggesting that collagen-induced activation of the indicated MAPKs are downstream of PI3K and PLCγ2 signalling pathways.

G-Rp1 inhibits the tyrosine phosphorylation of proteins involved in GPVI signalling pathway

Since PI3K plays an important role in signalling by the platelet collagen receptor, GPVI (Gibbins *et al.*, 1998) and the activation of PLCγ2 is critical for the majority of functional responses to collagen, including aggregation and dense-granule secretion (Watson and Gibbins, 1998), we examined the effect of G-Rp1 on the expression levels of the indicated proteins. G-Rp1 suppressed collagen-induced PLCγ2 and PI3K (p85) activations in a concentration-dependent manner (Figure 8A).

In collagen signalling studies, where non-aggregation conditions are necessary, collagen concentrations required to observe signalling were increased to fourfold in order to observe tyrosine phosphorylation of components of the GPVI pathway as described previously (Hubbard *et al.*, 2003; Tucker *et al.*, 2008; Moraes *et al.*, 2010). The G-Rp1 concentrations used were therefore also increased accordingly. To begin to explore the mechanism through which G-Rp1 inhibits collagen receptor-mediated signalling, the effect of the compound on the tyrosine phosphorylation of a number of receptor components of the GPVI signalling pathway was examined. Since activation of the GPVI collagen receptor results in tyrosine phosphorylations of Fyn and Lyn (Gibbins *et al.*, 1996; Quek *et al.*, 2000), the effect of G-Rp1 on the level of tyrosine phosphorylations of these proteins following stimulation with collagen was therefore examined. G-Rp1 diminished the level of collagen-stimulated tyrosine phosphorylation of the Fyn and Lyn in a concentration-dependent manner (Figure 8B). This indicates that G-Rp1 is able to inhibit very early signalling events in the collagen signalling pathway. To

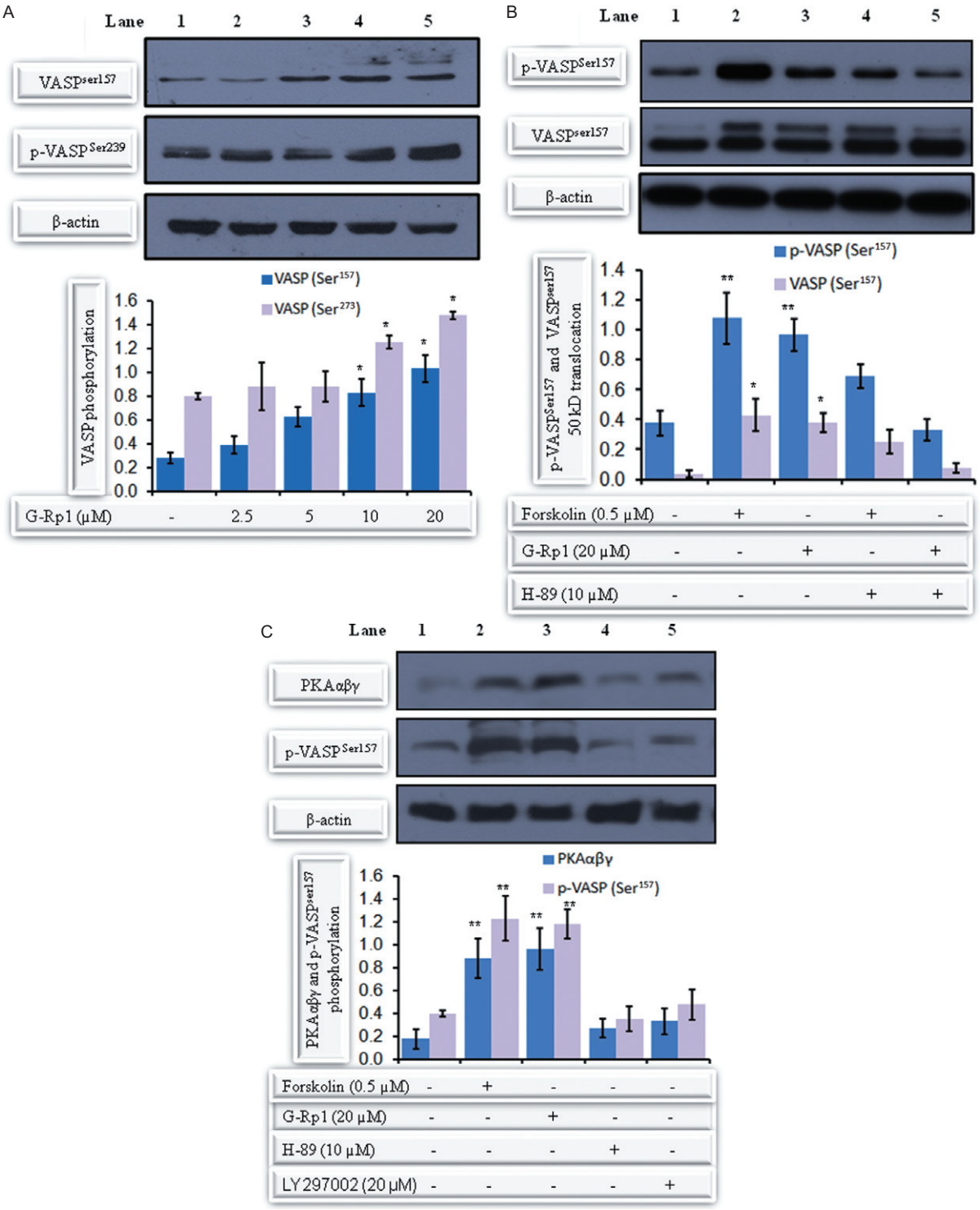

Figure 6

Effect of G-Rp1 on VASP and PKAαβγ phosphorylation. (A–C) Washed rat platelets (3×10^8 mL^{-1}) were incubated with vehicle, forskolin, G-Rp1, H-89 or LY297002 (at the concentrations indicated) and were stirred in an aggregometer for 10 min before termination. Then, proteins were extracted, separated, transferred to nitrocellulose and immunoblotted using anti-phospho-VASP^{Ser239}, VASP^{Ser157}, PKAαβγ and β-actin antibodies. (A) Concentration-dependent effect of G-Rp1 in VASP$^{Ser-157}$ (upper lane) and VASP$^{Ser-239}$ (middle lane) activation. (B) Effects of forskolin, G-Rp1 or H-89 pretreatment on phospho-VASP$^{Ser-157}$ (upper lane) and total-VASP$^{Ser-157}$ (middle lane) phosphorylations. (C) Forskolin and G-Rp1 increased, and H-89 and LY297002 suppressed, the expressions of catalytic PKAαβγ (upper lane) and its substrate phospho-VASP$^{Ser-157}$ (middle lane), respectively. Images are representative of three independent experiments. Values in bar graphs are means ± SEM of at least four independent experiments performed in triplicate. *: $P < 0.05$ or **: $P < 0.01$ versus control.

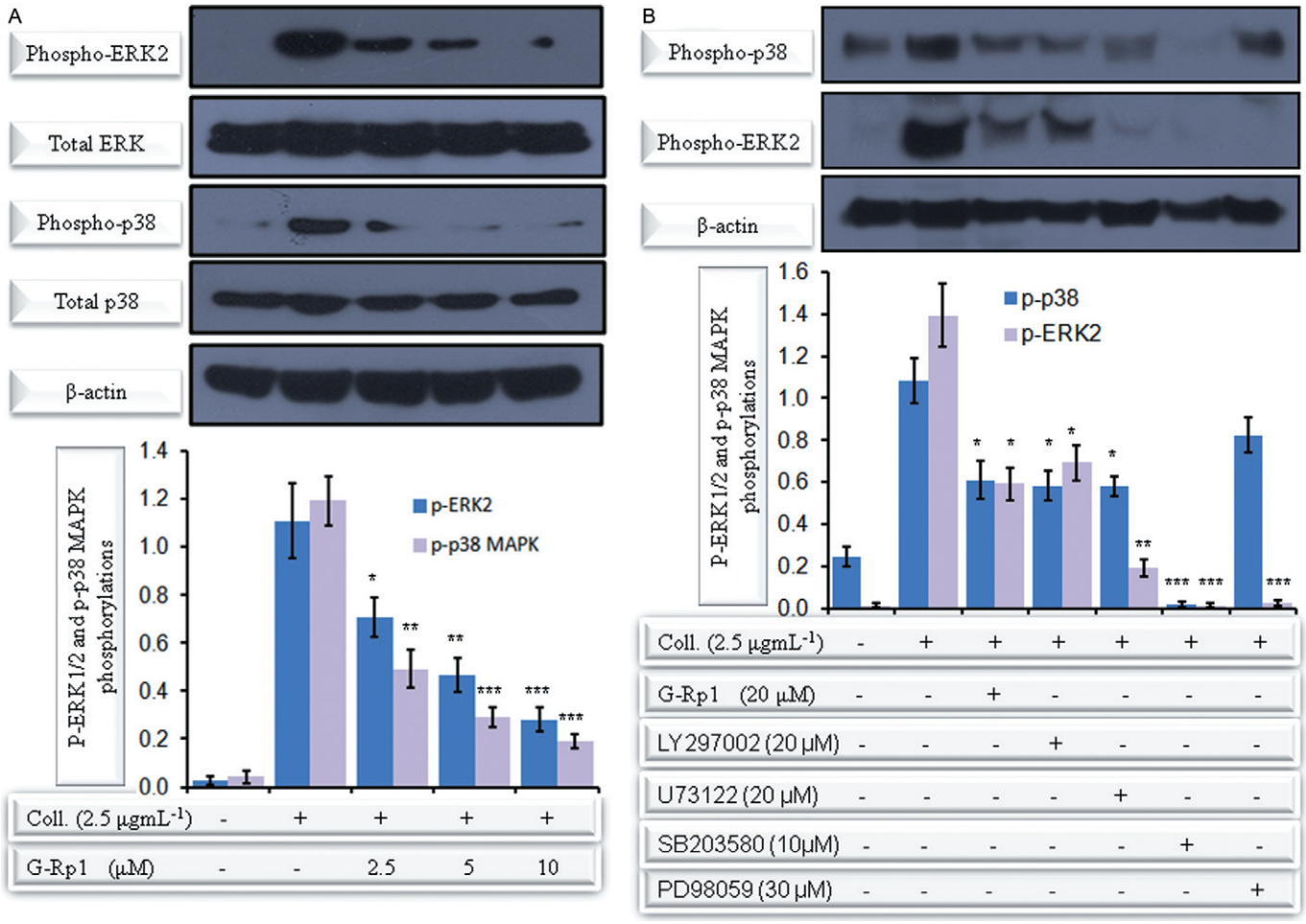

Figure 7

G-Rp1 attenuated collagen-activated platelet p38 and ERK2 phosphorylations. (A and B) Washed platelets (3×10^8 mL^{-1}) were stirred in an aggregometer with vehicle, G-Rp1, LY297002, U73122, SB203580 or PD98059 at the concentrations indicated for 3 min prior to stimulation with collagen for 5 min before termination of the reactions. Proteins were extracted, separated by SDS-PAGE transferred to nitrocellulose and immunoblotted with antibodies against ERK and p38 MAPKs. Blots were visualized by ECL, and all immunoblots are representative of three to four similar experiments. (A) G-Rp1 dose-dependently attenuated p38 and ERK2 phosphorylations. (B) Effects of pre-incubation of platelets with G-Rp1 or the above indicated inhibitors on collagen-induced ERK2 and p38 phosphorylations at the doses shown in the figure. Images are representative of three independent experiments. Values in bar graphs are means ± SEM of at least four independent experiments performed in triplicate. *: $P < 0.05$, **: $P < 0.01$ or ***: $P < 0.001$ versus control.

confirm this, molecules that had been shown to lie downstream of the Fyn and Lyn were also examined. Interestingly, collagen-stimulated tyrosine phosphorylation of the non-receptor tyrosine kinase Syk was also inhibited by G-Rp1 in a concentration-dependent manner (Figure 8B) with a similar profile to Fyn and Lyn. Activation of Syk following its association with the activated receptor complex results in tyrosine phosphorylation of the transmembrane adapter protein LAT, which in turn leads to the recruitment of PLCγ2 (Pasquet *et al.*, 1999a). We, therefore, investigated the effect of G-Rp1 on collagen-stimulated tyrosine phosphorylation of LAT and PLCγ2. As shown in Figure 8, G-Rp1 dose-dependently exhibited a significant decrease in the levels of tyrosine phosphorylations of LAT and PLCγ2 (Figure 8C), and similar result was observed with this GPVI specific agonist in convulxin-stimulated platelets (data not shown). Collagen-induced tyrosine phosphorylations of LAT has also been reported to recruit PI3K that influences the recruitment and activation of PLCγ2, which in turn liberates DAG and IP3 for the subsequent mobilization of $[Ca^{2+}]_i$ and activation of PKC leading to secretion and aggregation (Blake *et al.*, 1994; Gibbins *et al.*, 1998). Next, PI3K and PLCγ2 from platelets treated with G-Rp1 for 5 min prior to collagen stimulation were immunoprecipitated. G-Rp1 treatment markedly inhibited the collagen-induced interactions of these proteins (Figure 8D). Moreover, we used HTRF kinase assay to examine whether G-Rp1 inhibits PI3K activity. The result revealed that G-Rp1 inhibited all three isoforms of class IA PI3K tested in an ATP-competitive manner. Amongst all of the PI3K isoforms, PI3Kβ and δ were inhibited most potently by G-Rp1, and PI3Kα was inhibited moderately at the two doses used (Figure 8E).

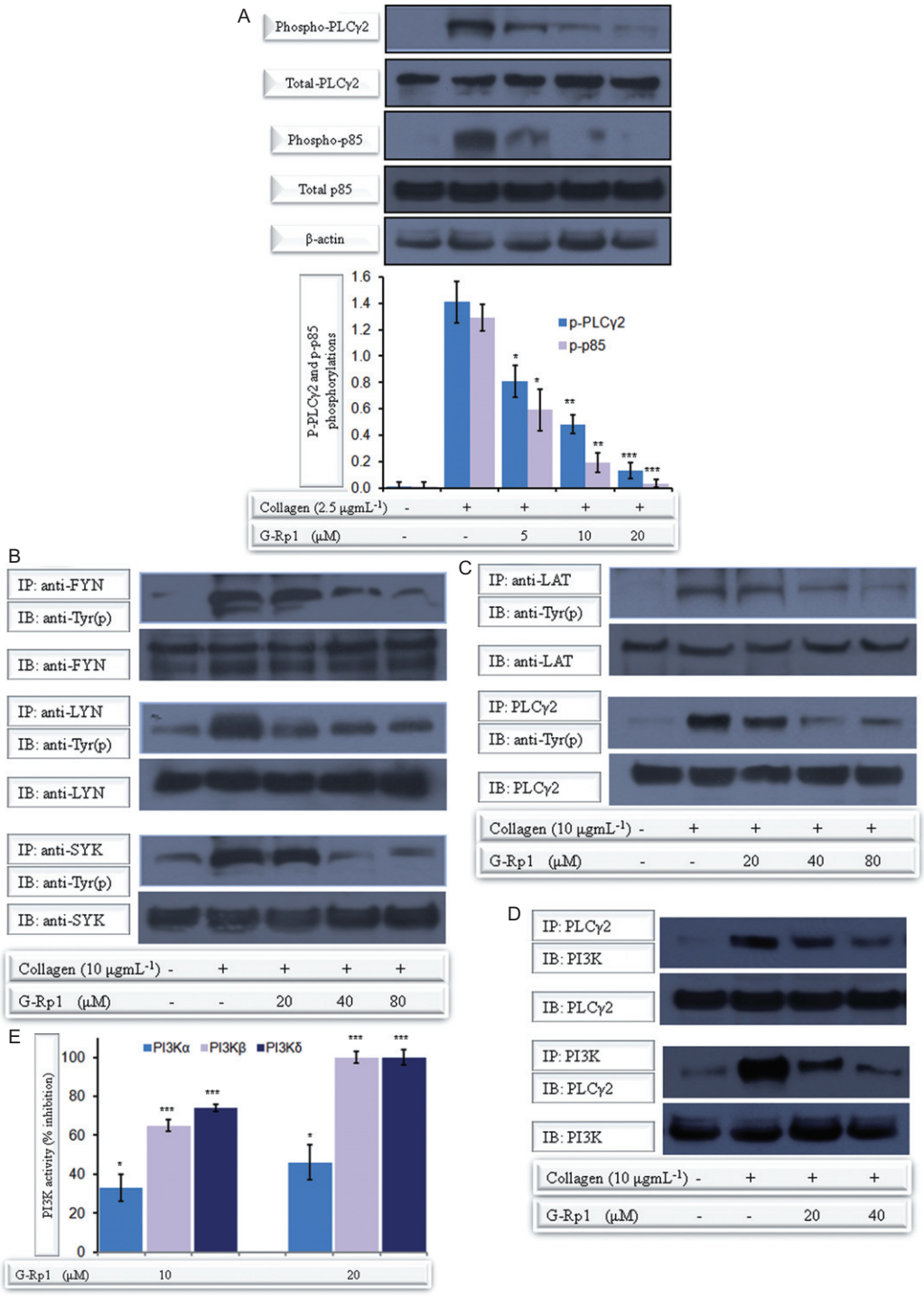

Figure 8

G-Rp1 suppresses collagen-induced PLCγ2 and p85 activations and inhibits the tyrosine phosphorylation of multiple components of the GPVI signalling pathway. Washed platelets in the presence of EGTA (1 mM) were incubated with G-Rp1 or vehicle for 5 min and then stimulated with collagen for 3 min. (A) Lysates from G-Rp1 (5–20 μM) treated platelets were immunoblotted to detect the expression levels of PLCγ2 and PI3K (p85) proteins. (B-D) Platelet lysates were immunoprecipitated by incubating overnight with anti-Fyn, anti-Lyn or anti-Syk (B), anti-LAT or anti-PLCγ2 (C), and anti-PI3K or anti-PLCγ2 (D), respectively, and then incubated with protein A-Sepharose (PAS) for 4 h at 4°C. Precipitated proteins were separated by SDS-PAGE and immunoblotted to detect phosphotyrosine residues. (E) PI3K HTRF assay was performed. Each class I PI3Kα, β and δ isoform protein was incubated in the assay buffer containing 10 μM PIP2 and ATP (200 μM) in the presence of G-Rp1 (10–20 μM) and HTRF signal was determined. Equivalent protein loading was verified by reprobing for Fyn, Lyn, Syk, LAT, PLCγ2 or PI3K. Data are representative of three separate experiments. Images are representative of three independent experiments. Values in bar graphs are means ± SEM of at least four independent experiments performed in triplicate. *: $P < 0.05$, **: $P < 0.01$ or ***: $P < 0.001$ versus control.

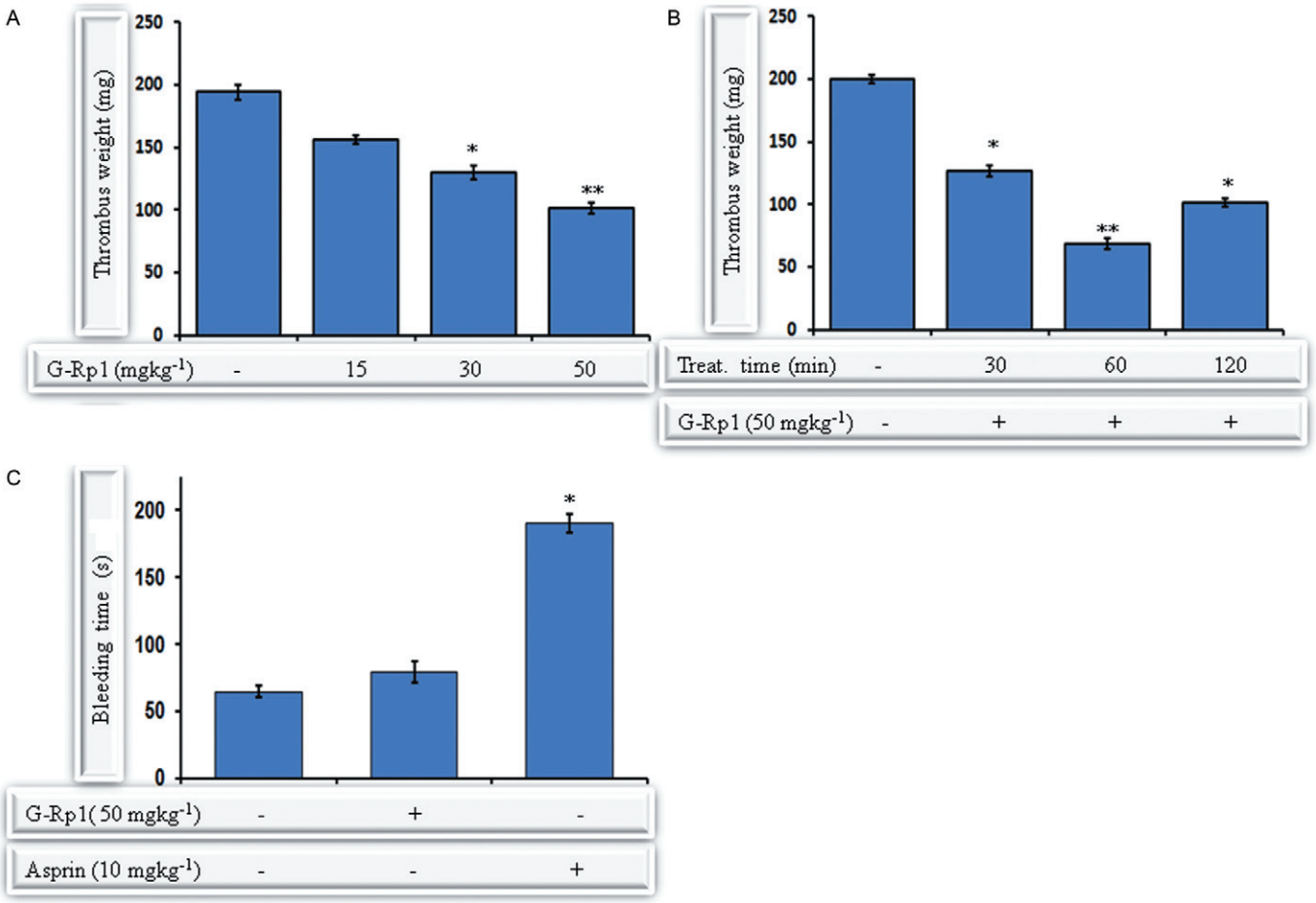

Figure 9

The effect of G-Rp1 on thrombus formation in rats and bleeding time in mice. The arteriovenous shunt model was used, and the blood circulation in the cannula shunt was carried out for 15 min, and the thrombus weight was immediately determined. (A) The G-Rp1 (15, 30 or 50 mg kg^{-1}) or vehicle was given orally 1 h before thrombus induction. (B) The G-Rp1 at the dose of (50 mg kg^{-1}) time-dependently inhibited thrombus formation with the highest inhibition after 30 min of the compound treatment. (C) Mice were administered G-Rp1 (50 mg kg^{-1}), aspirin (a positive control, 10 mg kg^{-1}) or vehicle. The mouse tail was transected to induce bleeding, and tail was immersed vertically into the 0.9% saline solution at 37°C, and then the time between the start of transection to bleeding cessation was recorded as the bleeding time. Bar graphs show mean ± SEM of at least six independent experiments performed. *: $P < 0.05$; **: $P < 0.01$ versus control.

Effects of G-Rp1 on thrombus formation and bleeding time in vivo

In rats, Umetsu and Sanai (1978) observed that the thrombus that developed in an arteriovenous shunt was comprised primarily of platelets, and its formation was inhibited by pretreatment with anti-platelet agents. We therefore, examined the compound effect on extracorporeal shunts model thrombus formation. As shown in Figure 9A and B, G-Rp1 inhibited arteriovenous shunt thrombus formation in a dose- and time-dependent fashion. At the doses of 15, 30 and 50 mg kg^{-1}, the compound exhibited 20%, 33% and 47%, respectively, inhibitory effect on thrombus formation compared with the vehicle control (Figure 9A). G-Rp1, at 100 mg kg^{-1}, inhibited thrombus formation by 37%, 76%, and 50% after 30, 60 and 120 min, respectively, of its administration where the compound's maximum effect was observed at 60 min of its treatment (Figure 9B).

Since anti-thrombic activity could be accompanied by adverse effects such as prolonged bleeding time and impaired blood coagulation (Schulman and Spencer, 2010), we investigated the effect of G-Rp1 on bleeding time *in vivo* using C57/BL6 mice. While aspirin (10 mg kg^{-1}), a positive control, significantly increased bleeding time when compared with vehicle control group, a single treatment of G-Rp1 (50 mg kg^{-1}, i.p.) had no effect on it (Figure 9C).

Ex vivo *effects of G-Rp1 on platelet aggregation, ATP secretion and blood coagulation*

The inhibitions of platelet aggregation and ATP secretion by G-Rp1 after 1 week oral administration into mice are shown in Figure 10. G-Rp1 significantly and dose-dependently inhibited the collagen- and ADP-induced platelet aggregation (Figure 10A) and ATP secretion (Figure 10B) *ex vivo*. The

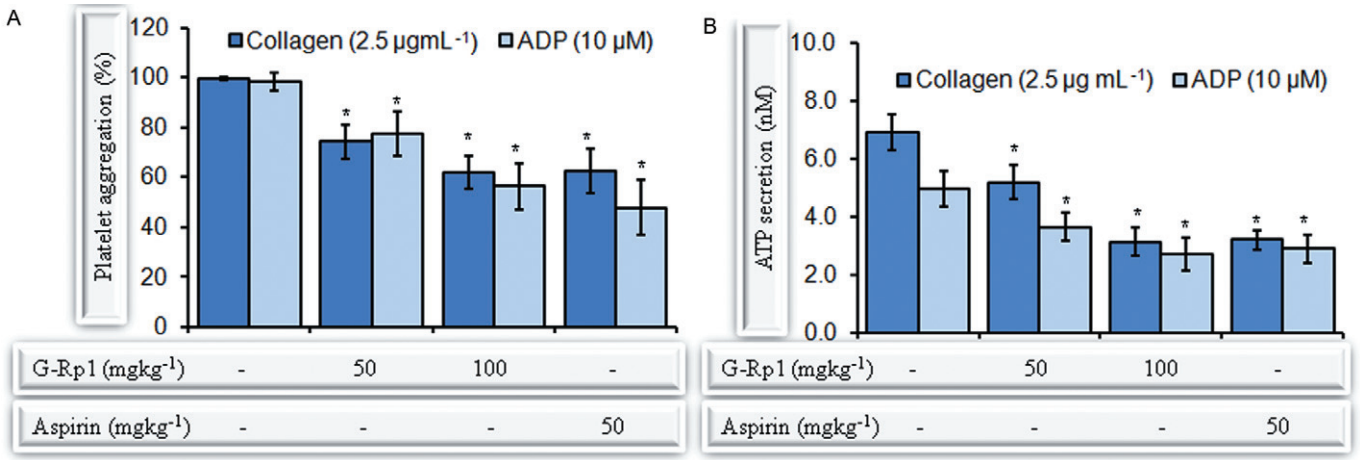

Figure 10

Ex vivo effect of G-Rp1 administered orally on the collagen- or ADP-induced platelet aggregation and ATP secretion in mice. G-Rp1 (50 or 100 mg kg^{-1}), aspirin (50 mg kg^{-1}) or vehicle was orally administered for 1 week to mice with the last dose 60 min before blood collection. Platelet aggregation (A) and ATP secretion (B) were induced by collagen or ADP at the indicated concentrations. Results are expressed as mean ± SEM (n = 10). *: $P < 0.05$ versus control.

Table 1

Effect of ginsenoside-Rp1 (G-Rp1) on mice APTT and PT *ex vivo*

Compounds	APPTT (s)	PT (s)
Vehicle control	38.6 ± 1.4[a]	13.5 ± 0.32
G-Rp1 (50 mg kg^{-1})	40.1 ± 1.6	13.6 ± 0.24
G-Rp1 (100 mg kg^{-1})	41.6 ± 1.7	14.1 ± 0.21
Aspirin (50 mg kg^{-1})	46.2 ± 2.0	15.2 ± 0.28

[a]Results are expressed as mean ± SEM (n = 10).

effects of G-Rp1 on coagulation time were also evaluated by APTT and PT assay using mouse PPP. APTT and PT were not affected by the G-Rp1 treatment at concentrations up to 100 mg kg^{-1} (Table 1).

Discussion

Ginseng is one of the commonly used herbal medicines reported to have a wide range of therapeutic and pharmacological applications. Ginsenosides, the major pharmacologically active ingredients of ginseng, appear to be responsible for most of the activities of ginseng including anti-oxidation, anti-inflammation and anti-cancer. Extensive reviews report that saponin fractions and the various gensenoside components of Korean red ginseng exhibit cardiovascular protective effects (Attele *et al.*, 1999; Radad *et al.*, 2006; Jia *et al.*, 2009). In addition, ginseng extracts and components have been shown to have anti-platelet and anti-atherosclerotic actions (Kimura *et al.*, 1988; Park *et al.*, 1996; Jung *et al.*, 1998; Jin *et al.*, 2007). However, the mechanisms by which ginsenosides induce anti-platelet activity still remain unknown. In addition, the various ginsenosides produce effects that differ from one another, a single ginsenoside initiates multiple actions in the same tissue and the overall pharmacology of ginseng is complex. Further, most importantly, due to the instability and low bioavailability of ginsenosides and their metabolites, most of these compounds do not reach the intended biological system when administered orally (Tawab *et al.*, 2003; Leung and Wong, 2010). To this effect, Leung and Wong (2010) concluded that the findings of ginsenoside studies would be more physiologically relevant only when (1) the pure compounds of the ginsenosides are made available in large quantities; and (2) the ginsenosides are biochemically stabilized to avoid degradation and enhance absorption in the gastrointestinal tract.

To overcome the above limitations, a new approach was initiated to develop novel ginsenoside-derived compounds with improved pharmacological activity, potency and stability. Recently, our group developed a novel semi-synthetic derivative known as ginsenoside-Rp1 (3-O-β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl dammarane-3β, 12β-diol) by means of a reduction with hydrogenation. We previously reported that ginsenoside-Rp1, derived from gingenoside-Rg3 or its derivatives (2H-Rg3, Rg5 and Rk1), exhibited improved stability and potency with strong anti-metastatic and anti-inflammatory activities (Kumar *et al.*, 2006; 2009; Park *et al.*, 2008; Kim and Cho, 2009); however, the anti-platelet effect of G-Rp1 was not then investigated.

In a previous study using rat platelets, we compared the anti-platelet activities of G-Rg3 and 2H-Rg3 (G-Rp1 parent compounds). We observed that the anti-platelet activity of 2H-Rg3 was more potent than that of ginsenoside-Rg3, and its mechanism of action was mediated via cAMP activation and ERK2 inhibition (Lee *et al.*, 2008). In the present study, we further provide evidence for the potent inhibitory effects of G-Rp1 on platelet activation and arteriovenous shunt thrombus formation. The anti-aggregating capacity of G-Rp1

in agonist-activated platelets was shown to be superior to that of its parent compounds, G-Rg3 and 2H-Rg3. It displayed broad-spectrum inhibitory activity on platelet activation, independent of ligands such as collagen, thrombin or ADP, each of which acts on different platelet receptors. In this study, the inhibitory potential of G-Rp1 in collagen-induced platelet activation was further confirmed by a marked suppression of granule secretion, TXA_2 production, $[Ca^{2+}]_i$ mobilization and integrin $\alpha_{IIb}\beta_3$ activation (common converging step of platelet activation). These findings suggest that G-Rp1 pretreatment may impair the $\alpha_{IIb}\beta_3$ conformational changes induced on exposure to its high-affinity fibrinogen binding site (inside-out signalling), which occurs as a result of previous platelet agonist interactions. Conversely, the results also suggest that the next step (i.e. fibrinogen binding followed by post-ligand occupancy events; outside-in signalling) that lead to platelet shape change and spreading may be attenuated by G-Rp1.

A large body of evidence has been obtained indicating that platelets are continually exposed to a variety of activating factors, including collagen, fibrinogen, ADP, vWF, thrombin and thromboxane (Davi and Patrono, 2007; Ruggeri and Mendolicchio, 2007; Varga-Szabo *et al*., 2008), and inhibitory factors such as endothelial-derived NO, prostacyclin (PGI_2) and ADPase (Ruggeri and Mendolicchio, 2007; Varga-Szabo *et al*., 2008). An impairment of this equilibrium will promote either thrombotic or bleeding disorders. Thus, a strong equilibrium between the two opposing processes of platelet stimulation and inhibition is thought to be essential for normal platelet and vascular function. Therefore, G-Rp1 pretreatment of activated platelets, in the present study, may contribute to the maintenance of this balance for normal homeostasis.

We found that treatment with G-Rp1 elevated cAMP levels in resting platelets and the addition of IBMX slightly increased these levels further. Moreover, elevated cAMP levels result in PKA-dependent VASP phosphorylations, providing a good rationale to consider G-Rp1 for potential therapeutic use either alone or in combination with other agents. These findings further support those from our previous study in U-937 cells, which demonstrated that G-Rp1 downregulates CD29 activation, blocks the rearrangement of actin and promotes phosphorylation of VASP at Ser^{157} in these cells, suggesting that the actin cytoskeleton may be affected by G-Rp1 (Kim and Cho, 2009).

It is a widely accepted that VASP is a major substrate for cAMP-dependent PKA and cGMP-dependent PKG and is an actin- and profilin-binding protein that is expressed in platelets. It plays a major role in promoting filopodia formation and actin polymerization that negatively regulates the secretory and adhesive properties of platelets (Pula *et al*., 2006; Wentworth *et al*., 2006), and phosphorylation of VASP on Ser^{157} and/or Ser^{239}, correlates with reduced activation of integrin $\alpha_{IIb}\beta_3$ and inhibition of platelet aggregation (Horstrup *et al*., 1994). In our experiments, G-Rp1-induced PKA$\alpha\beta\gamma$ and $VASP^{Ser157}$ phosphorylations were partially blocked by LY297002 (Figure 6C), suggesting that PI3K may be an upstream negative regulator of PKA activation and VASP phosphorylations (Gambaryan *et al*., 2010).

The observations in the present study indicate that G-Rp1 markedly inhibits collagen-induced ERK2 and $p38^{MAPK}$ but not JNK activation, indicating that the modulation of the former two may be involved in G-Rp1's anti-platelet activity. A similar pattern of inhibition of ERK2 and $p38^{MAPK}$ activation was observed on treatment of the platelets with LY297002 (PI3K inhibitor) or U73122 (PLC inhibitor), suggesting that collagen-induced activation of PI3K and PLC may be upstream events mediating ERK2 and $p38^{MAPK}$ phosphorylation. It has been established that of the MAPKs, ERK2, p38 and JNK1 are present in platelets and activated by various agonists (Adam *et al*., 2008; 2010). In accordance with our findings, Jackson and McNicol (2010) reported that adenylate cyclase activation inhibits collagen-induced MAPK phosphorylation. The involvement of collagen-induced ERK2 activation in platelet aggregation is dependent on TXA_2 formation and ADP release (Roger *et al*., 2004). The requirement for an ERK cascade in platelet activation was further confirmed from data showing its involvement in the activation of store-mediated Ca^{2+} entry and PLA2 phosphorylation (Rosado and Sage, 2001), and ERK2 amplifies collagen-induced platelet secretion (Toth-Zsamboki *et al*., 2003). P38 has been shown to be involved in collagen-induced platelet adhesion and spreading (Mazharian *et al*., 2005) as its inhibitors suppress platelet activation (Kuliopulos *et al*., 2004). Boilard *et al*. (2010) recently demonstrated that platelets and the microparticles induced by their activation have a role in inflammatory joint diseases. Their findings indicated that platelets amplify inflammation in arthritis via collagen-dependent microparticle production, which is mediated by the collagen receptor GPVI. Thus, because mice and humans lacking GPVI remain healthy (Nieswandt and Watson, 2003), antagonism of this receptor represents a novel therapeutic approach.

In the present study we established that the potent inhibitory action of G-Rp1 on platelet function is linked to its ability to inhibit multiple components of collagen-induced signalling pathways in platelets. The present findings confirm the inhibitory effect of G-Rp1 on Fyn and Lyn tyrosine phosphorylations upon collagen binding to GPVI. In addition, G-Rp1 suppressed Syk recruitment and activation, and LAT tyrosine phosphorylation. Moreover, it inhibited the interaction and activation of downstream signalling proteins, including PI3K and PLCγ2, with subsequent inhibition of PLCγ2-mediated activation. Our data thus suggest that G-Rp1 functions by inhibiting collagen-stimulated platelet activation via modulation of GPVI signalling. Activation of the GPVI collagen receptor results in tyrosine phosphorylations of Fyn and Lyn (Gibbins *et al*., 1996; Quek *et al*., 2000) followed by Syk activation and LAT tyrosine phosphorylation, which in turn leads to the recruitment of PI3K (Gibbins *et al*., 1998) and PLCγ2 (Pasquet *et al*., 1999a). PLCγ2 then liberates DAG and IP3 for subsequent PKC activation and $[Ca^{2+}]_i$ mobilization followed by secretion, aggregation and thrombus formation (Blake *et al*., 1994; Gibbins *et al*., 1998). Therefore, modulation of the indicated signalling pathway by anti-platelet agents including G-Rp1 can be a promising strategy for the treatment of cardiovascular diseases involving aberrant platelet activation. Above all, we showed that G-Rp1 strongly inhibits the activity of all of the three class IA PI3K isoforms tested in an ATP-competitive manner with potent inhibitory effects observed on PI3Kβ and δ. These results indicate that G-Rp1 may be an ATP-competitive pan-class I PI3K inhibitor.

PI3Kα, β and δ isoforms are reported to regulate platelet function (Jackson *et al.*, 2004), and while Gilio *et al.* (2009) noted a non-redundant roles of PI3Kα and β in GPVI-induced platelet signalling and thrombus formation, Senis *et al.* (2005) indicated a minor role for PI3Kδ in this receptor signalling. Amongst all the isoforms, however, PI3Kβ has been extensively studied in platelets where it appears to play an important role downstream of the GPVI receptor (Canobbio *et al.*, 2009; Kim *et al.*, 2009b; Martin *et al.*, 2010), Gi-coupled receptor (Kim *et al.*, 2009b; Garcia *et al.*, 2010) and integrin-$\alpha_{IIb}\beta_3$ receptor (Canobbio *et al.*, 2009; Martin *et al.*, 2010; Schoenwaelder *et al.*, 2010). In this study, we did not rule out a direct effect of G-Rp1 on PI3K activity downstream of the GPCR and the integrin-$\alpha_{IIb}\beta_3$ receptor. However, the capacity of G-Rp1 to inhibit class IA PI3K isoforms in general and its strong inhibition of the p110β isoform in particular supports previous reports showing that isoform-selective PI3K p110β inhibitors prevent formation of stable integrin αIIbβ3 adhesion, leading to defective platelet thrombus formation without prolonged bleeding time both *in vitro* and *in vivo* (Jackson *et al.*, 2005; Schoenwaelder *et al.*, 2010).

The ability of G-Rp1 to inhibit agonist-induced aggregation, granule secretion, TXA_2 production, $[Ca^{2+}]_i$ mobilization, $\alpha_{IIb}\beta_3$ activation, MAPK and tyrosine phosphorylations *in vitro* and thrombus formation *in vivo*, as well as aggregation and ATP secretion *ex vivo* without affecting the coagulation and bleeding time, reflects the potential use of the compound as a candidate antithrombotic agent. This conclusion is supported by previous findings that indicated initial platelet activation by adhesion molecules, such as vWF, collagen and p-selectin via their integrin receptors, initiates contact of the platelets to the vessel wall, which then leads to thrombus formation (Gibbins, 2004; Ruggeri and Mendolicchio, 2007; Varga-Szabo *et al.*, 2008). The generation and release of agonists stored in platelet granules that act on GPCRs is the main mechanism of further platelet recruitment and occlusion of the blood vessel during later thrombus formation (Davi and Patrono, 2007; Sachs and Nieswandt, 2007). Platelet aggregation and thrombus formation are mediated by platelet–platelet cross-linking of fibrinogen bound to activated-$\alpha_{IIb}\beta_3$ (Steinhubl and Moliterno, 2005), and by MAPK activation (Adam *et al.*, 2008). Therefore, anti-platelet therapy using compounds such as G-Rp1 may be considered for the treatment of thrombosis.

Conclusion

Our result show that G-Rp1 is a potent inhibitor of agonist-induced platelet aggregation. In addition, G-Rp1 significantly inhibited *in vivo* thrombus formation, *ex vivo* platelet aggregation and ATP secretion; while it had no effect on coagulation and bleeding times. Our findings indicate that G-Rp1 inhibits collagen-stimulated platelet function through modulation of signalling downstream of the collagen receptor GPVI.

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Conflict of interest

The authors declare no conflict of interest.

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