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Inhibitory mechanisms of dihydroginsenoside Rg3 in platelet aggregation: Critical roles of ERK2 and cAMP

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Abstract

Ginsenoside Rg3, a single ginseng saponin, is known to be a major anti-platelet component of protopanaxadiol that is isolated from Korean red ginseng. In this study, we investigated whether dihydroginsenoside Rg3, a stable chemical derivative of ginsenoside Rg3, also demonstrated anti-platelet activity. Dihydroginsenoside Rg3 inhibited thrombin-induced platelet aggregation in a concentration-dependent manner with an IC50 (concentration producing 50% inhibition) of 18.8 ± 0.4 μM. Ginsenoside Rg3 inhibited platelet aggregation which was induced by thrombin (0.1 U mL^{-1}) with an IC50 of 40.2 ± 0.9 μM. We next determined whether dihydroginsenoside Rg3 affected different types of ligand-induced platelet aggregation. We found that dihydroginsenoside Rg3 inhibited collagen-induced platelet aggregation with an IC50 of 20.0 ± 0.9 μM. To elucidate the inhibitory mechanism of dihydroginsenoside Rg3 on aggregation, we analysed its downstream signalling pathway. It was interesting to note that dihydroginsenoside Rg3 elevated cyclic AMP production in resting platelets, but did not affect cyclic GMP production. In addition, we found that dihydroginsenoside Rg3 potently suppressed phosphorylation of extracellular signal-regulated kinase 2 (ERK2), which was stimulated by collagen (2.5 μg mL^{-1}), but not of p38 mitogen-activated protein kinase. Taken together, our results indicate that dihydroginsenoside Rg3 potently inhibited platelet aggregation via the modulation of downstream signalling components such as cAMP and ERK2.

Introduction

Korean red ginseng has been used for more than 2000 years as a major component of Far East medicine to treat many ailments. Ginseng contains many active components such as ginsenosides, polysaccharides, peptides, fatty acids and mineral oils (Gillis 1997). Among these components, ginsenosides are believed to be most responsible for the pharmacological and immunological activity (Gillis 1997; Attele et al 1999). Dammarane types of ginsenosides are classified into 20(S)-protopanaxadiol and 20(S)-protopanaxatriol. 20(S)-protopanaxadiol, with two sugars, comprises the most abundant ginsenosides in ginseng, including Rb1, Rb2, Rc, Rd, Rg3 and Rh2. 20(S)-protopanaxatriol includes ginsenosides Re, Rg1, Rg2 and Rh1 (Li & Liu 2007). In addition, ginsenoside Rg3 is known to have anti-platelet activity (Lee et al 1997). However, it is relatively unstable under acidic and high-temperature conditions, like in the stomach. To overcome this instability, we obtained the chemical derivative of ginsenoside Rg3, dihydroginsenoside Rg3, which displayed a relatively stable form.

Platelets play an important role in the pathological thrombotic process (e.g. plaque formation) (Lee et al 2006). Once vascular injury occurs, platelets will be activated by endogenous agonists, such as ADP and collagen, and adhere to the site of injury (Corti et al 2002; Ruggeri & Mendolicchio 2007). Upon binding of agonists by their receptor, platelets are activated and the contents of the granules are released. The process of platelet activation is regulated, in part, by levels of the second messengers adenosine 3′:5′-cyclic monophosphate (cAMP) and guanosine 3′:5′-cyclic monophosphate (cGMP) (el-Daher et al 1996; Jang et al 2002). Increased intracellular cAMP or cGMP levels lead to inhibition of agonist-induced platelet activation, aggregation, adhesion and release of granule contents (Radomski et al 1987). Moreover, adenylate or guanylate cyclase activators and phosphodiesterase inhibitors synergistically elevate intracellular cAMP or cGMP levels,

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providing a good rationale for the therapeutic use of these agents alone or in combination with other agents (Liao et al 1998; Manns et al 2002; Yao et al 2007). Cytosolic Ca^{2+} also plays a critical role in the regulation of various physiological functions, including aggregation and granule secretion of platelets. Stimulation of platelets with different stimuli, such as thrombin, collagen and ADP, results in an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Rink & Sage 1990; Smith et al 1992; Gibbins 2004).

In this study, we wished to determine whether dihydroginsenoside Rg3 modulates platelet aggregation, in comparison with ginsenoside Rg3. We first investigated the anti-platelet properties of dihydroginsenoside Rg3 using a platelet aggregation assay that was induced by collagen and thrombin. In addition, we attempted to determine the mechanism of dihydroginsenoside Rg3's anti-platelet action using a downstream signalling assay. We found that dihydroginsenoside Rg3 showed potent anti-platelet activity, due to the modulation of intracellular downstream components in activated platelets.

Materials and Methods

Materials

Dihydroginsenoside Rg3 and ginsenoside Rg3 (Figure 1; Lee et al 1997) were obtained from Ambo Institute (Seoul, Korea). Thrombin and Fura-2/AM were obtained from Sigma Co. (St Louis, MO, USA). Collagen was procured from the Chronolog Co (Havertown, PA, USA). Antibodies to phospho-ERK and phospho-p38 MAPK were from Cell Signaling (Beverly, MA, USA). All other chemicals were of reagent grade. The cAMP and the cGMP enzyme immunoassay kits were purchased from Cayman Co. (Ann Arbor, MI, USA).

Preparation of platelets

Blood was obtained from male Sprague–Dawley rats, 200–250 g. Rats were anaesthetized with ethyl ether and blood was collected from the abdominal aorta. The blood was anti-coagulated with acid-citrate-dextrose (ACD; 85 mM sodium citrate, 71 mM citric acid, 111 mM dextrose, pH 6.5), and was centrifuged at 120 *g* for 7 min to achieve platelet-rich plasma. To remove residual erythrocytes, the platelet-rich plasma samples were again centrifuged at 40 *g* for 7 min. To isolate the platelets, the platelet-rich plasma was centrifuged twice at 300 *g* for 7 min. The platelets of the precipitate were adjusted to the proper number (10^8/mL) for an aggregation assay with a Tyrode buffer (composition in mM: 137 NaCl, 12 $NaHCO_3$, 5.5 glucose, 2 KCl, 1 $MgCl_2$, 0.3 $NaHPO_4$, pH 7.4). All steps for platelet preparation were conducted at room temperature, and all experimental procedures and protocols were reviewed and approved by the Ethics Committee of the College of Veterinary Medicine, Kyungpook National University.

Platelet aggregation assay

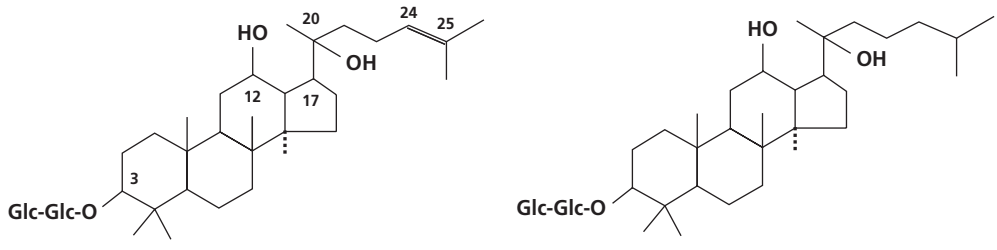
Aggregation was monitored by measuring light transmission via an aggregometer (Chronolog Co., Havertown, PA, USA). The washed platelets were pre-incubated at 37°C for 2 min with either ginsenosides or vehicles. The reaction mixture was further incubated for 5 min, stirring at 1200 rev min^{-1}, and the degree of aggregation was determined. The concentration of the vehicle was kept at <0.1% so as to exclude artificial effects.

Measurement of cAMP and cGMP

Washed platelet concentration was adjusted to 1×10^9 platelets per mL. Platelets were treated with the indicated concentration of ginsenoside either in the presence or absence of isobutyl-methyl-xanthine (IBMX), a non-selective phosphodiesterase inhibitor, in an aggregometer. After 1 min incubation at 37°C, the reactions were terminated with EDTA (100 μM), followed by immediate boiling for 2 min. After the mixture was cooled to 4°C, the reaction mixture was centrifuged at 15 000 *g* for 5 min. cAMP and cGMP contents in the supernatant were measured following the manufacturer's instructions.

Immunoblotting

Platelet extracts were prepared by the standard Laemmli method. After the platelet aggregation assay, the sample buffer (0.125 M Tris-HCl, 2% SDS, 2% β-mercaptoethanol, 20% glycerol, 0.02% bromophenol blue, pH 6.8, 2 ×) was added and the protein concentration level was determined using PRO-MEASURE (iNtRON Biotechnology, Korea).



Ginsenoside Rg3 Dihydroginsenoside Rg3

Figure 1 Chemical structure of ginsenoside Rg3 and dihydroginsenoside Rg3 used in this study.

Following centrifugation at 15 000 *g* for 5 min, 60 μg of sample proteins were resolved by 10% SDS-PAGE. The resolved proteins were electrotransferred to nitrocellulose membranes in 25 mM Tris (pH 8.5), 0.2 M glycerin and 20% methanol at 100 V for 2 h. Blots were blocked for at least 2 h with TBS-Tween-20 (TBS-T) containing 5% nonfat dry milk and were then incubated with an appropriate antibody (1:1000 dilution ratio in a blocking solution). After washing in TBS-T three times, the blot was incubated with a secondary antibody (1:5000 dilution ratio in a blocking solution) for 2 h and antibody-specific proteins were visualized by the ECL detection system (Supex Co., Pohang, Korea), according to the manufacturer's instructions.

Statistical analysis

Data values are means ± s.e.; n represents the number of independent experiments. Before tests of significance were performed, data were examined for normality and equal variance to determine whether parametric or non-parametric tests should be employed. The Kruskal–Wallis test followed by Dunn's post-hoc test were used for statistical comparisons.

Results and Discussion

Dihydroginsenoside Rg3 inhibits thrombin- and collagen-induced platelet aggregation

We first investigated whether dihydroginsenoside Rg3 inhibited rat platelet aggregation, which was induced by 0.1 U mL^{-1} of thrombin. Thrombin induced platelet aggregation in a dose-dependent manner (data not shown) and 0.1 U mL^{-1} of thrombin caused complete platelet aggregation (i.e. almost 100%). Dihydroginsenoside Rg3 inhibited thrombin (0.1 U mL^{-1})-induced platelet aggregation with an IC50 value of 18.8 ± 0.4 μM (Figure 2). Using collagen as a ligand for induction of aggregation, we found that dihydroginsenoside Rg3 also inhibited rat platelet aggregation (Figure 3). The IC50 value of dihydroginsenoside Rg3 in the collagen (2.5 μg mL^{-1})-induced platelet aggregation was 20.0 ± 0.9 μM. On the other hand, ginsenoside Rg3 inhibited thrombin (0.1 U mL^{-1})- and collagen (2.5 μg mL^{-1})-induced rat platelet aggregation with IC50 values of 40.2 ± 0.9 μM and 35.2 ± 1.2 μM, respectively. This indicated that the inhibitory potency of dihydroginsenoside Rg3 is superior to that of ginsenoside Rg3, suggesting that the aliphatic side chain of dihydroginsenoside Rg3 plays an important role in the inhibitory effect on platelet aggregation (Lee & Nah 2007).

Although both thrombin and collagen can induce platelet aggregation in a dose-dependent manner, the mode of action and binding to their respective receptors are totally different. That is, thrombin, an agonist of protease-activated receptor (PAR) 1 and 4, induces platelet aggregation via activation of typical G protein-coupled receptor (GPCR) signalling. However, it is known that collagen is an agonist of integrin-$\alpha_2\beta_1$ and glycoprotein, which are single transmembrane types of receptors (Hers et al 2000). Our results suggested that the inhibitory effect of dihydroginsenoside Rg3 is based on the modulation of downstream signalling components rather than simple blockage of the binding between the ligand and its receptor. We next decided to analyse the downstream signalling pathway as follows.

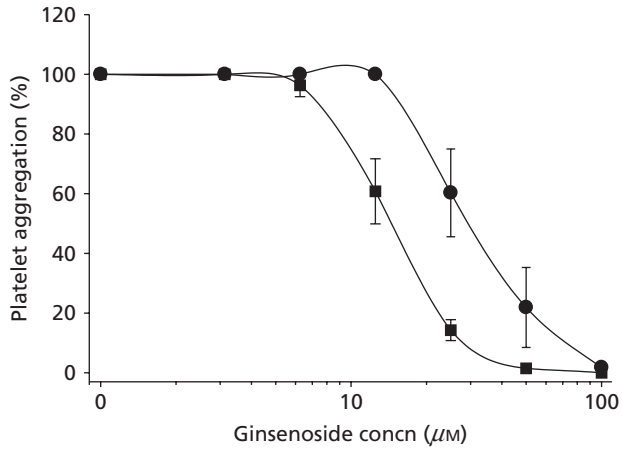


Figure 2 Effect of dihydroginsenoside Rg3 and ginsenoside Rg3 on thrombin (0.1 U mL^{-1})-induced platelet aggregation. Dose–response curves of dihydroginsenoside Rg3 (squares) and ginsenoside Rg3 (circles) on thrombin-induced platelet aggregation. The platelets were pre-incubated with 1 mM of $CaCl_2$ at 37°C for 2 min in the presence of either the vehicle or the indicated concentration of ginsenosides. The platelets were added with 0.1 U mL^{-1} of thrombin and were further incubated for 5 min. The trace was recorded during the incubation time and the degree of platelet aggregation was determined. The data are given as means ± s.e.m., n = 3.

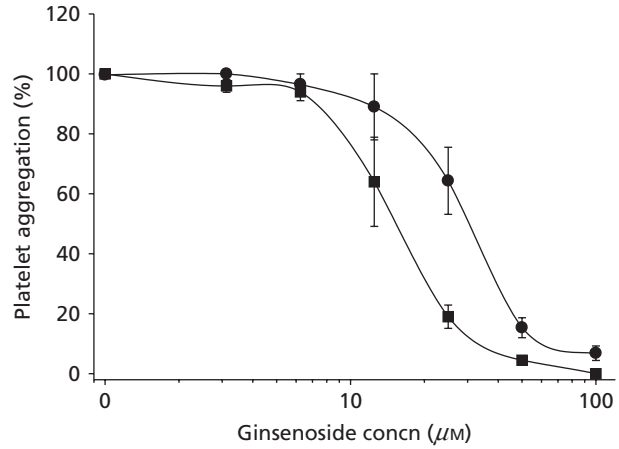


Figure 3 Effect of dihydroginsenoside Rg3 and ginsenoside Rg3 on collagen (2.5 μg mL^{-1})-induced platelet aggregation. Dose–response curves of dihydroginsenoside Rg3 (squares) and ginsenoside Rg3 (circles) on collagen-induced platelet aggregation. The platelets were pre-incubated with 1 mM of $CaCl_2$ at 37°C for 2 min in the presence of either the vehicle or the indicated concentration of ginsenosides. The platelets were combined with 2.5 μg mL^{-1} of collagen and were further incubated for 5 min. The trace was recorded during the incubation time and the degree of platelet aggregation was determined. The data are given as means ± s.e.m., n = 3.

Dihydroginsenoside Rg3 elevates cAMP production, but not cGMP, in resting platelets

Since the intracellular cyclic nucleotides cAMP and cGMP are well known anti-aggregating agents in platelets, we intended to determine whether dihydroginsenoside Rg3 affected the generation of cAMP or cGMP in resting platelets treated with either vehicle or dihydroginsenoside Rg3. Dihydroginsenoside Rg3 increased the intracellular cAMP levels in a concentration-dependent manner (Figure 4). The elevation of intracellular cAMP is due to either production of cAMP via adenylyl cyclase or decrease of cAMP degradation by phosphodiesterase such as type 3 or type 4 (Peluso 2006). Interestingly, dihydroginsenoside Rg3's effect on cAMP production was observed in the presence or absence of IBMX, a broad-spectrum phosphodiesterase inhibitor. This suggests that dihydroginsenoside Rg3 affects the cAMP production system, but not the cAMP degradation system (e.g. various types of phosphodiesterase). It is well known that cAMP acts as an anti-aggregating agent and its main target is the activation of the specific cAMP-dependent protein kinase A (PKA), which is involved in the anti-platelet activity through a reduction of Ca^{2+} availability. In addition, it has been reported that PKA phosphorylates Ser157 of the vasodilator-stimulated phosphoprotein (VASP) (Sudo et al 2003). Since phosphorylation of VASP negatively regulates its interaction with actin filaments and its localization in platelets, it may be expected that VASP-dependent regulation of platelet integrins involves an unidentified modulation of integrin–cytoskeletal linkage, resulting in a corresponding change in integrin clustering and ligand binding avidity (Walter et al 1993; Abel et al 1995; Markert et al 1996). However, we did not investigate the effect of dihydroginsenoside Rg3 treatment on the phosphorylation of VASP, which remains to be studied. Matrix metalloproteinases (MMPs), a family of zinc- and calcium-dependent proteinases, are able to degrade most components of the extracellular cell matrix and exert several effects of platelet functions such as platelet adhesion and aggregation (Santos-Martinez et al 2008). It is reported that the elevation of cAMP stimulates the production of MMP-9, which results in the anti-proliferative effect and anti-platelet activity in pulmonary artery smooth muscle cells and platelets, respectively (Growcott et al 2006; Cho et al 2007). At this moment, we cannot exclude the possibility that the anti-platelet activity of dihydroginsenoside Rg3 is due to elevation of cAMP, following the up-regulation of MMP-9 expression; this remains to be investigated. On the other hand, there was no significant increase in the level of intracellular cGMP in platelets treated with up to 100 μM dihydroginsenoside Rg3 (Figure 4).

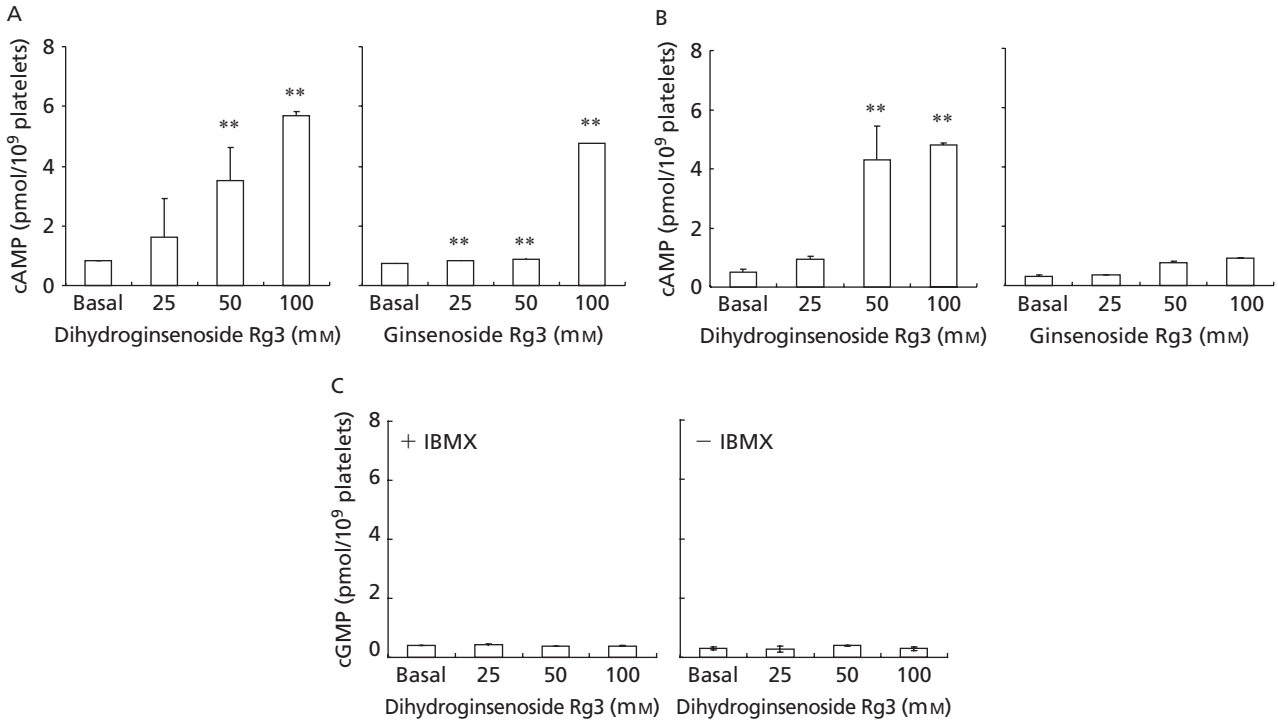


Figure 4 Effect of dihydroginsenoside Rg3 on the production of cAMP in resting platelets. After 1 min incubation at 37°C, the platelet suspension (1×10^9 platelets per mL) was challenged with various concentrations of ginsenosides in the either presence (A, C) or absence (B, C) of IBMX, a broad-spectrum phosphodiesterase inhibitor. The reaction was terminated with 100 μM of EDTA and was followed by immediate boiling for 2 min. After the mixture was cooled to 4°C, the reaction mixture was centrifuged at 15 000 *g* for 5 min. cAMP and cGMP contents in the supernatant were measured as described in the Materials and Methods per the manufacturer's instructions. Data are given as means ± s.e.m., n = 3 experiments. $^{**}P < 0.01$ vs basal.

The effect of dihydroginsenoside Rg3 on the phosphorylation of extracellular signal-regulated protein kinase 2 (ERK2) and p38 mitogen-activated protein kinase (MAPK)

The MAPK family comprises ERK1/2, p38 MAPK and c-Jun N-terminal kinase (JNK), and in this study, immune reactions of ERK1/2 and p38 MAPK were detected. Collagen (2.5 μg mL^{-1}) clearly phosphorylated ERK2 and p38 MAPK; dihydroginsenoside Rg3 inhibited ERK2 phosphorylation, but had a much less significant effect on p38 MAPK (Figure 5). It has been reported that ERKs are activated after stimulation by collagen and thrombin (Borsch-Haubold et al 1995; Mazharian et al 2005), and ERK2 activation is involved in collagen-induced platelet aggregation and secretion (Roger et al 2004). In human platelets, an ERK cascade is required in the activation of store-mediated Ca^{2+} entry and in the phosphorylation of phospholipase A2 (Rosado & Sage 2001, 2002; Garcia et al 2007). Dihydroginsenoside Rg3's inhibition of ERK2 phosphorylation seems to contribute, in part, to its anti-platelet activity in collagen-activated platelets. Both ERK1 and ERK2 are reported to be present in platelets, and we found that ERK2 was activated following stimulation by collagen in this study.

Conclusion

To summarize, ginsenoside Rg3 is known to have anti-platelet activity, but it is unstable under acidic and high-temperature conditions. To overcome this instability, a relatively stable chemical derivative of ginsenoside Rg3, dihydroginsenoside Rg3, was identified and compared for its anti-platelet effects. Dihydroginsenoside Rg3 demonstrated an inhibitory effect on collagen- and thrombin-induced platelet aggregation. In addition, dihydroginsenoside Rg3 elevated production of cAMP in resting platelets (which was not affected by phosphodiesterase-mediated degradation), but not that of cGMP. Furthermore, among MAPKs identified in platelets (i.e. ERK1/2, p38 MAPK and JNK), dihydroginsenoside Rg3 inhibited the phosphorylation of ERK2 in collagen-activated platelets. These results suggest that dihydroginsenoside Rg3 may be a valuable candidate for developing the therapeutic resources against cardiovascular disorders mediated via aberrant platelet aggregation (Figure 6) and against coagulant disorders due to a defective coagulation cascade. Further study is needed to elucidate the possible involvement of VASP and MMP-9 proteins in dihydroginsenoside-Rg3-mediated inhibition of platelet aggregation. In addition, its in-vivo effects and the clinical implications in orally anti-coagulated patients need to be addressed.

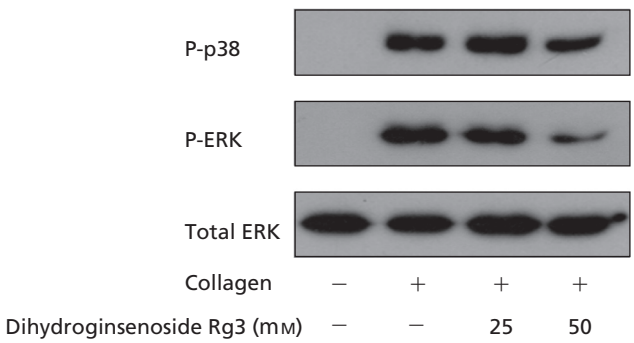


Figure 5 Effect of dihydroginsenoside Rg3 on MAPK phosphorylation in collagen-activated rat platelets. The platelets were pre-incubated with 1 mM of $CaCl_2$ at 37°C for 2 min in the presence of either the vehicle or the indicated concentration of dihydroginsenoside Rg3. The platelets were combined with collagen and were further incubated for 5 min. The reaction was terminated and sample buffer (2×) was added. The protein extraction and the protein concentration of platelets are described in the Materials and Methods section. The platelet protein (60 μg) was separated with 10% SDS-PAGE and was electrotransferred onto nitrocellulose membranes. Primary antibody and secondary antibody treatment of the membrane are described in Materials and Methods. Specific bands were visualized with an ECL kit (Supex Co).

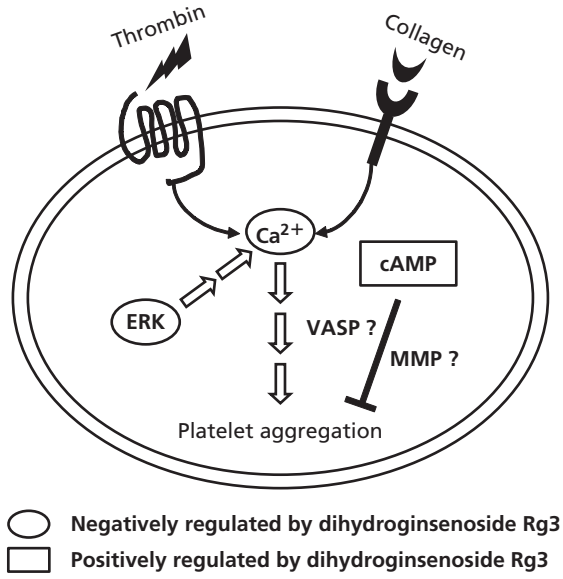


Figure 6 The proposed mechanism of inhibitory effect of dihydroginsenoside Rg3 in thrombin- and collagen-activated platelet aggregation. ERK, extracellular-regulated kinase; Ca^{2+}, intracellular Ca^{2+}; VASP, vasodilator-stimulated phosphoprotein; MMP, matrix metalloproteinase.

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