PKC inhibitors RO 31-8220 and Gö 6983 enhance epinephrine-induced platelet aggregation in catecholamine hypo-responsive platelets by enhancing Akt phosphorylation

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Impaired responsiveness of platelets to epinephrine (epi) and other catecholamines (CA) has been reported in approximately 20% of the healthy Korean and Japanese populations. In the present study, platelet aggregation induced by epi was potentiated by RO 31-8220 (RO) or Gö 6983 (Gö). Phosphorylated Akt (p-Akt) was very low in epi-stimulated PRP from CA-hypo-responders (CA-HY), whereas it was detected in those from CA-good responders (CA-GR). RO and Gö increased p-Akt, one of the major downstream effectors of phosphoinositol-3 kinase (PI3K), in epi-stimulated PRP from both groups. Wortmannin, a PI3K inhibitor, attenuated the RO or Gö-induced potentiation of p-Akt in epi-stimulated PRP, suggesting positive effects for RO and Gö on PI3K. TXA$_2$ formation was increased by the addition of either RO or Gö in epi-stimulated platelets. The present data also suggest that impaired Akt phosphorylation may be responsible for epinephrine hypo-responsiveness of platelets. [BMB reports 2011; 44(2): 140-145]

INTRODUCTION

Circulating platelets are exposed to diverse tissue-releasing substances (1) and play a pivotal role in haemostasis and thrombosis (2). When platelets are activated by agonists such as adenosine diphosphate (ADP), U46619 (9, 11-dieoxy-11α, 9α-methanoepoxyprostaglandin Fα; thromboxane A$_2$ mimetic), collagen, or thrombin, they aggregate and release their granule contents. Secreted granule contents such as ATP, ADP, serotonin, and calcium ion from dense granules as well as TXA$_2$ formation are essential for the induction of secondary platelet aggregation (3, 4). The majority of platelet agonists are also

known to activate phosphoinositol-3 kinase (PI3K) in platelets, and inhibitors of PI3K block fibrinogen binding and platelet aggregation. The lack of phosphorylated Akt, one of the major downstream effectors of the PI3K pathway, was reported to cause impaired platelet aggregation (5).

Epinephrine (epinephrine) induces heterogeneous responses on human platelets. Impaired responsiveness of platelets to epinephrine and other catecholamines (CA) has been reported in approximately 20% of samples from healthy normal Korean and Japanese individuals (6, 7). The degrees of aggregation in response to other aggregation-inducing agents (AA, ADP, and U46619) are also significantly lower in platelets that are hypo-responsive to CA (8, 9). Elevated plasma concentrations of NO and cGMP have been observed in CA-hypo-responders (CA-HY) compared to CA-good responders (CA-GR) (10).

In this study, use of a widely used PKC inhibitor, RO 31-8220 (RO) increased epinephrine-induced platelet aggregation in platelet-rich plasma (PRP) from CA-GR. In addition, RO induced platelet aggregation in epinephrine-stimulated PRP from CA-HY, who are normally characterized by impaired platelet aggregation. The present study was undertaken to understand the effect of RO on epinephrine-stimulated human platelet aggregation and especially to identify the mechanism of their upregulatory effect on epinephrine-induced PRP from CA-HY.

RESULTS

RO 31-8220 and Gö 6983 potentiate platelet aggregation

The effects of five different commercially available PKC inhibitors were investigated on epinephrine-induced platelet aggregation in PRP obtained from both CA-GR and CA-HY. As shown in Fig. 1A and 1B, two PKC inhibitors, RO 31-8220 (RO), a non-selective inhibitor, and Gö 6983 (Gö), a PKC α, β, δ, γ, and ζ inhibitor, potentiated 0.1 uM epinephrine-induced platelet aggregation in PRP from CA-GR in a dose-dependent manner; RO and Gö alone did not induce platelet aggregation. Moreover, both RO and Gö induced platelet aggregation in 1 uM epinephrine-treated PRP from CA-HY. Normally, epi-

A CA-GR

Light Absorbance

RO 10
epi 0.1

RO 0.1 + epi 0.1

RO 1 + epi 0.1
RO 10 + epi 0.1

1 min

Go 10
epi 0.1

Go 0.1 + epi 0.1

Go 1 + epi 0.1
Go 10 + epi 0.1

B CA-HY

Light Absorbance

RO 10

epi 1

RO 0.1 + epi 1

RO 1 + epi 1

RO 10 + epi 1

1 min

Go 10

epi 1

Go 0.1 + epi 1
Go 1 + epi 1

Go 10 + epi 1

C CA-GR

Light Absorbance

Rottlerin 10 + epi 1
Rottlerin 50 + epi 1

Rottlerin 1 + epi 1

epi 1

1 min

Go 6976 50 + epi 1

Go 6976 10 + epi 1
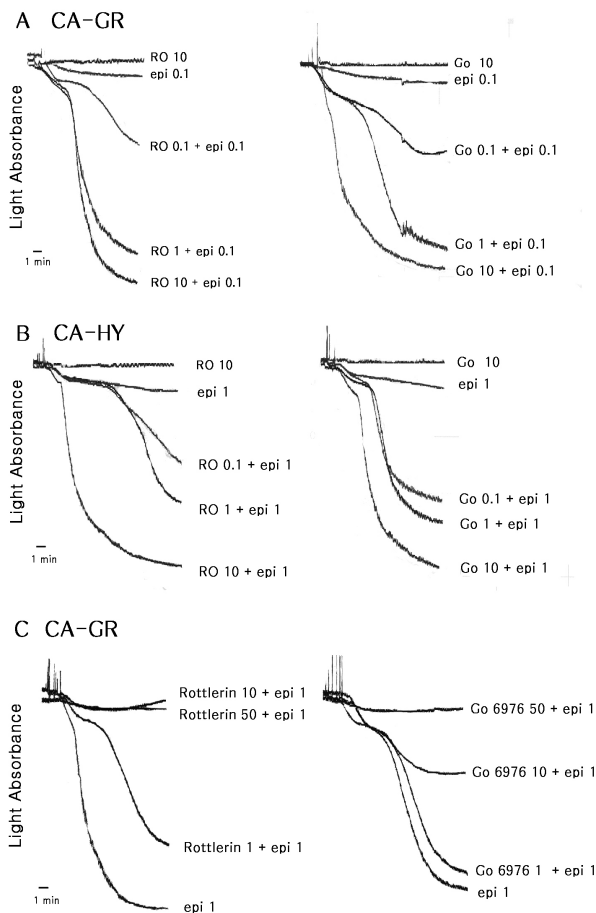
Go 6976 1 + epi 1
epi 1

Fig. 1. Representative tracings of the effects of RO 31-8220, Gö 6983, rottlerin, and Gö 6976 on epinephrine-induced human platelet aggregation. (A) PRP of CA-GR was stimulated by 0.1 uM epinephrine with or without RO 31-8220 (0.1, 1, and 10 uM) or Gö 6983 (0.1, 1, and 10 uM). (B) PRP of CA-HY was stimulated by 1 uM epinephrine with or without RO 31-8220 (0.1, 1, and 10 uM) or Gö 6983 (0.1, 1, and 10 uM). (C) PRP of CA-GR was stimulated by 1 uM epinephrine with or without rottlerin (1, 10, or 50 uM) and Gö 6976 (1, 10, or 50 uM). The tracings are representative figures of a minimum of three tests each of PRP from three individuals.

nephrine (1-100 uM) alone fails to induce aggregation in PRP from CA-HY (6, 7). On the other hand, RO and Gö suppressed platelet aggregation induced by PMA, a PKC activator, in PRP from both groups. In contrast, RO and Gö presented neither potentiatory nor inhibitory effects on other agonists such as ADP, collagen, and U46619, a TXA$_2$ mimic, although U46619-stimulated aggregation in the presence of aspirin was inhibited by RO (data not shown), as previously reported (11). Rottlerin (a PKC δ inhibitor) and Gö 6976 (a PKC α and β inhibitor) did not augment epinephrine-induced aggregation but rather inhibited the extent of aggregation in PRP from CA-GR (Fig. 1C).

Effect of RO 31-8220 and Gö 6983 on platelet aggregation is blocked by wortmannin

The effect of wortmannin, a PI3K inhibitor, was evaluated on epinephrine-induced as well as RO or Gö-potentiated platelet aggregation to determine the involvement of the PI3K pathway. Epinephrine (1 uM)-induced platelet aggregation in PRP from CA-GR was be inhibited by the addition of wortmannin (10 uM), as shown in Fig. 2A. Epinephrine (0.1 or 1 uM)-induced aggregation potentiated by either RO or Gö was also suppressed by wortmannin in PRP from both CA-GR and CA-HY (Fig. 2A and B).

RO 31-8220 and Gö 6983 augment phosphorylation of Akt

The effect of RO or Gö on the level of phosphorylated Akt, the major downstream effector of PI3K, in PRP was evaluated. In PRP from CA-GR, epinephrine upregulated phosphorylation of Akt, whereas no detectable effects were observed with either RO or Gö alone (Fig. 3A and B). In PRP from CA-HY, neither epinephrine, RO, nor Gö induced phosphorylation of Akt. However, the level of phosphorylated Akt was increased upon the addition of either RO or Gö in epinephrine-stimulated PRP from both groups, as shown in Fig. 3A, B and C.

RO 31-8220 or Gö 6983 increases ATP secretion

The level of secreted ATP was measured to determine the effects of RO and Gö on granule secretion during platelet aggregation. ATP was not detected in either 0.1 uM epinephrine-stimulated PRP from CA-GR or 1 uM epinephrine-stimulated PRP from CA-HY, whereas negligible platelet aggregation was observed (Fig. 4A and B). Secretion of ATP was increased by RO and Gö in 0.1 uM epinephrine-stimulated PRP from both CA-GR (0.10 ± 0.04 and 0.11 ± 0.03 nmol, respectively) and 1 uM epinephrine-stimulated PRP from CA-HY (0.42 ± 0.04 and 0.13 ± 0.08 nmol, respectively), and the degree of platelet aggregation was approximately equal to that of full platelet aggregation in 1 uM epinephrine-stimulated PRP from CA-GR. However, the amount of secreted ATP was much lower than that (1.66 ± 0.07 nmoles) observed in 1 uM epinephrine-stimulated PRP from CA-GR.

RO 31-8220 and Gö 6983 potentiate TXA$_2$ formation

TXA$_2$ generation is essential for the induction of secondary platelet aggregation. The effects of RO or Gö on TXA$_2$ production were observed during platelet aggregation. The formation of TXA$_2$ was determined by measuring TXB$_2$, the stable metabolite of TXA$_2$. As shown in Fig. 4C, TXA$_2$ production was increased by the addition of 1 uM epinephrine to PRP from CA-GR, whereas TXA$_2$ formation was negligibly increased compared to control platelets in 0.1 uM epinephrine-stimulated PRP from both CA-GR and 1 uM epinephrine-stimulated PRP from CA-HY. Upon addition of RO or Gö, TXA$_2$ formation was enhanced in 0.1 uM epinephrine-activated PRP from both CA-GR (141.32 ± 0.3550 or 146.32 ± 0.0135 vs 46.321 ±
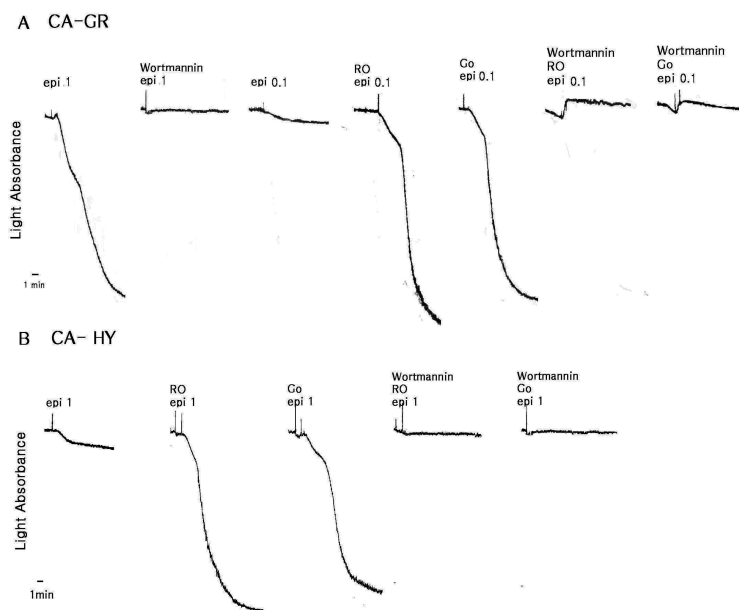
A CA–GR

B CA– HY

Fig. 2. Representative tracings of the effects of wortmannin on epinephrine-induced platelet aggregation with or without RO 31-8220 or Gö 6983. (A) PRP of CA-GR was stimulated by 1 uM epinephrine or 0.1 uM epinephrine with RO 31-8220 (10 uM) or Gö 6983 (10 uM). (B) PRP of CA-HY was stimulated by 1 uM epinephrine with or without RO 31-8220 (10 uM) or Gö 6983 (10 uM). Wortmannin (10 uM) or vehicle was added 30 sec prior to the stimulation. The tracings are representative figures of a minimum of three tests each of PRP from three individuals.

0.1355 pg/ml) and 1 uM epinephrine-stimulated PRP from CA-HY (140.50 $\pm$ 0.0024 or 148.30 $\pm$ 0.0156 vs 44.654 $\pm$ 0.0812 pg/ml). These levels were comparable to TXA$_2$ formation (134.67 $\pm$ 0.0071 pg/ml) in 1 uM epinephrine-stimulated PRP from CA-GR, wherein full platelet aggregation was observed.

DISCUSSION

The α_2-adrenoceptors in platelets are coupled to G$_z$/G$_i$ protein and are known to mediate aggregation by reducing the level of cyclic AMP (cAMP) and increasing intracellular Ca^{+2} release (12), finally leading to exposure of fibrinogen receptors (13). Next to G$_i$ protein-mediated cAMP reduction, activation of the PI3K-mediated Akt pathway is also known to be essential to the signaling of epinephrine-induced platelet aggregation and for the potentiation of other agonists (14, 15). Moreover, TXA$_2$ production through the arachidonic acid metabolic pathway can enhance α_2-adrenoceptor-mediated platelet activation (16, 17).

The PKC inhibitors RO and Gö potentiated epinephrine-induced platelet aggregation in a dose-dependent manner (Fig. 1A and B) in PRP from both CA-GR and CA-HY. Other PKC inhibitors, Gö 6976, rottlerin, and chelerythrine, did not augment epinephrine-induced aggregation, although chelrythrine was previously reported to potentiate the aggregation response of low-dose epinephrine (18). Since rottlerin is known to be a PKC δ inhibitor, although controversial observations have been reported (19), and Gö 6976 is a selective inhibitor of PKC α and β, the observed potentiation effects of RO and Gö on epinephrine-induced platelet aggregation are suspected to affect PKC isoforms α, β and δ. Although PKCs are recognized

as essential signaling mediators in platelet activation and aggregation, the elucidated roles that various PKC isoforms play have been complicated with conflicting data regarding the regulation of platelet stimulation and platelet procoagulant functions (20, 21). The enhancing effects of RO and Gö suggest that certain PKC isoforms suppress epinephrine-stimulated platelet aggregation, which implies that PKC inhibitors potentiate it. This should be further investigated in relation to future advancements in specific agonists and antagonists of each PKC isoform. Epinephrine-mediated α_{2A}-adrenoceptor-coupled G$_z$ signaling alone is known to be incapable of inducing full platelet aggregation, and activation of P$_2$Y$_{12}$ receptor-coupled G$_i$ signaling, which leads to inhibition of adenylyl cyclase, is required (22-24). An elevated plasma level of NO has been observed in CA-HY compared to CA-GR (10), and NO is known to raise cAMP levels in platelets (25). However, it is consistent with previous reports that inhibition of cAMP accumulation by epinephrine was not significantly different between epinephrine hypo-responders and epinephrine normal responders (9, 10, 26, 27). Nakahashi *et al.* suggested that the reduced platelet response to epinephrine and ADP in CA-HY is most likely due to the impaired signal transduction of both α_{2A}-adrenoceptor and P$_2$Y$_{12}$ purinoceptor, indicating that platelet hypo-responses to epinephrine in CA-HY are not due to failure of adenyl cyclase inhibition by G$_i$ protein coupling (9). Activation of Akt has been demonstrated to be another downstream event in G$_i$ signaling in platelets, and PI3K has been shown to be an upstream regulator of Akt (28). The PI3K inhibitor, wortmannin, was observed to suppress both epinephrine-induced as well as RO or Gö-potentiated platelet aggregation (Fig. 2). Our immunoblotting data indicate that RO and Gö augmented the phosphorylation of Akt in epinephrine-stimulated PRP from
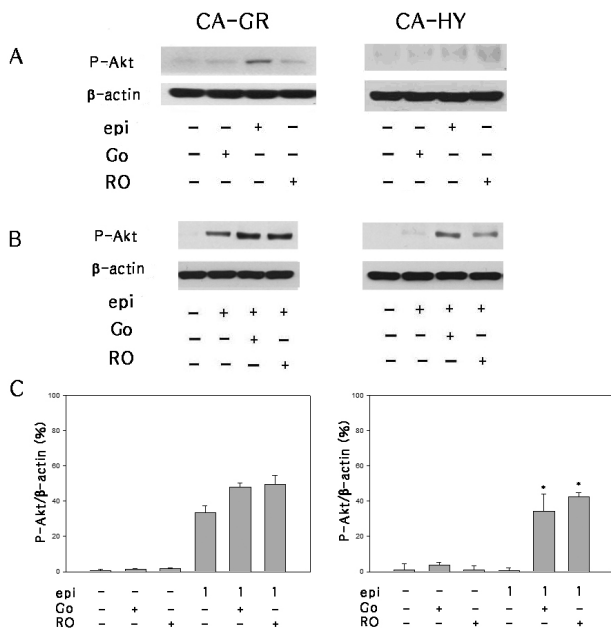
Fig. 3. Effects of RO 31-8220 or Gö 6983 on epinephrine-induced phosphorylation of Akt. PRP from either CA-GR (left panel) or CA-HY (right panel) was stimulated with either (A) 1 uM epinephrine or RO 31-8220 (10 uM) or Gö 6983 (10 uM) alone, (B) 1 uM epinephrine alone or with RO 31-8220 (10 uM) or Gö 6983 (10 uM). The tracings are representative figures of a minimum of three tests each of PRP from three individuals. (C) The results are expressed as the mean ± S.D. Data are based on a minimum of three tests each of PRP from three individuals, n = 3. *P < 0.05; significantly different from 1 uM epinephrine-induced aggregation.

Fig. 4. Effects of RO 31-8220 and Gö 6983 on epinephrine-induced platelet aggregation, ATP secretion, and TXB$_2$ formation. (A and B) Lumereagent was added to PRP before initiation of platelet aggregation (black) to detect the secretion of ATP (grey). (A) PRP from CA-GR was stimulated by 1 uM epinephrine or 0.1 uM epinephrine with or without RO 31-8220 (10 uM) or Gö 6983 (10 uM), n = 5. (B) PRP of CA-HY was stimulated by 1 uM epinephrine with or without RO 31-8220 (10 uM) or Gö 6983 (10 uM), n = 5. (C) Formation of TXA$_2$ was determined by measuring TXB$_2$, the stable metabolite of TXA$_2$. PRP from CA-GR (black and line) was stimulated by 1 uM epinephrine or 0.1 uM epinephrine, whereas PRP from CA-HY (white) was stimulated by 1 uM epinephrine with or without RO 31-8220 (10 uM) or Gö 6983 (10 uM), n = 3. (A) *P < 0.05; significantly different from 0.1 uM epinephrine-induced aggregation, #P < 0.05; significantly different from 0.1 uM epinephrine-induced ATP secretion. (B) *P < 0.05; significantly different from 1 uM epinephrine-induced aggregation, #P < 0.05; significantly different from 1 uM epinephrine-induced ATP secretion. (C) *P < 0.001; significantly different from control.

both CA-GR and CA-HY (Fig. 3A and B), which paralleled the degree of aggregation (Fig. 1A and B). The upregulation of Akt phosphorylation was supposed to induce aggregation in epinephrine-stimulated platelets of CA-HY (Fig. 1B) and potentiate aggregation in platelets from CA-GR (Fig. 1A). The above results suggest that the impaired phosphorylation of Akt might be responsible for the hypo-responsiveness of PRP from CA-HY. It is most likely that the downregulation of both PI3K and Akt phosphorylation impaired the G$_i$-mediated signal transduction pathways for α_{2A}-adrenoceptor in PRP from CA-HY.

Platelet aggregation, ATP secretion, and TXA$_2$ production were all negligible when PRP from either CA-GR was stimulated with 0.1 uM epinephrine or PRP from CA-HY was treated with 1 uM epinephrine, whereas almost full aggregation was induced by the addition of RO or Gö (Fig. 4A and B). On the other hand, RO and Gö increased the amount of TXA$_2$ formation in 0.1 uM epinephrine-stimulated PRP from both CA-GR and CA-HY (Fig. 4C) to a level approximately equal to that observed in 1 uM epinephrine-activated fully aggregated PRP from CA-GR. The above results suggest that TXA$_2$ generation plays an important role in RO- or Gö-mediated potentiation of epinephrine-induced platelet aggregation. On the
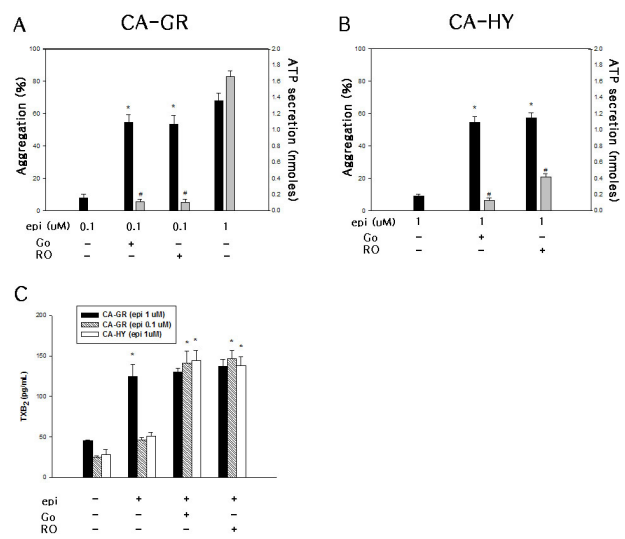
other hand, ATP secretion may only play a complimentary role, and the potentiation of platelet aggregation by RO or Gö was not secondary to the release of dense granule releases. The dense granule release may be increased secondary to the result of the increased TXA$_2$ production and subsequent increased platelet aggregation.

In summary, the present results demonstrate that RO and Gö upregulated Akt phosphorylation in α_{2A} receptor-coupled G$_i$-mediated signal transduction, resulting in increased TXA$_2$ production and augmented platelet aggregation. The present data suggest that impaired Akt phosphorylation may be responsible for the epinephrine-mediated hypo-responsiveness of platelets in PRP from CA-HY.

MATERIALS AND METHODS

Materials and instruments

(-)-Epinephrine, rottlerin, chelerythrine, RO 31-8220 (RO) methanesulfonate salt, Gö 6976, and Gö 6983 (Gö) were pur-

chased from Sigma-Aldrich Co. (St. Louis, MO, USA). Anti-phospho (Ser[473])-Akt antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). Thromboxane B_2 (TXB_2) EIA assay kit was purchased from GE Healthcare (Buckinghamshire, UK).

The platelet count was determined using a hematology analyzer (Exell TM 18, Drew Scientific Inc., Dallas, TX, USA). Platelet aggregation was detected using a four-channel platelet aggregometer (Model 490-X, Chrono-Log Corp., Havertown, PA, USA) interfaced with a personal computer. The amount of ATP secretion was measured on a Lumi-aggregation system (Model 500-VS, Chrono-Log Corp., Havertown, USA).

Subjects
Ten healthy Korean volunteers (male/female, 5/5), ranging in age from 20 to 33 years (mean $\pm$ SD; 27.5 $\pm$ 2.7 years) and who had not taken any medicine for at least two weeks prior to the study, were recruited. They were given information concerning the experimental purpose. The research protocol was approved by the Institutional Review Board of Seoul National University Hospital, Korea.

Blood collection and preparation of platelet-rich plasma (PRP) and platelet-poor plasma (PPP)
Whole blood was collected from the antecubital veins of the subjects using a 21G needle into a disposable syringe and then immediately mixed with 3.8% sodium citrate (9:1, v/v). PRP was prepared by centrifugation of the citrated blood at 200 $\times$ g for 10 min at room temperature. PPP was obtained from the residue by centrifugation at 1,500 $\times$ g for 20 min. Platelet numbers were adjusted to 300-350 $\times$ 10^6/ml by mixing PRP and PPP with the aid of a platelet counter. The adjusted PRP was used for the subsequent experiments.

Platelet aggregation assay
Platelet aggregation responses were monitored by a turbidimetric method using an optical aggregometer (29). PRP (500 μl) was incubated at 37°C with continuous stirring at 1,200 rpm. The degree of platelet aggregation was determined after the final addition of an aggregating agent and was standardized by assuming that PPP represented 100% light transmission and PRP represented 0% light transmission. Reduction in turbidity of PRP was observed as platelet aggregation processed. PRP was equilibrated at 37°C for 3 min prior to the initiation of each experiment. Five microliters of agent or vehicle was then added at 30 sec intervals. The individuals whose PRP underwent aggregation in response to (-)-epinephrine (10 μM), (-)-norepinephrine (10 μM), and epinine (100 μM) were classified as CA good-responders (CA-GR). Those whose PRP did not respond to any of the above-mentioned CA were classified as CA hypo-responders (CA-HY) (7, 10).

Luminescent assay measuring ATP secretion
The amount of ATP secretion from dense granules upon plate-let aggregation was measured by the lumi-aggregation system. Lume reagent (buffered firefly luciferin-luciferase extract) became luminescent in the presence of ATP secreted during dense granule release, and the system provided a voltage output proportional to the ATP that was secreted (29). The voltage was plotted as a function of time using a pen recorder. PRP (450 μl) was treated as described above with 50 μl of Lume reagent, which was added before the addition of epinephrine.

Measurement of TXB_2
The amount of TXB_2 produced following platelet aggregation was measured. The aggregation was terminated at 12 min by the addition of indomethacin (50 uM), and the reaction mixture was stored at -20^oC until the assay was conducted. After shaking the mixture (250 ul) with acetone (500 ul) for 2 min, the mixture was centrifuged at 12,000 rpm and 4°C for 2 min. The supernatant (500 ul), after shaking with hexane (500 ul), was centrifuged at 12,000 rpm and 4°C for 2 min, and the upper hexane layer was discarded. After adjusting the pH of the lower layer to 3-4 with 1 M citric acid, TXB_2 was extracted with chloroform (2 x 500 ul). The solvent was evaporated, and the amount of TXB_2 was measured using an enzyme immunoassay kit according to the manufacturer's instructions.

Western blotting
Five microliters of agent or vehicle was added at 30 sec intervals to the adjusted PRP (500 ul) to stimulate platelet aggregation. The aggregation mixture was placed in ice to terminate the reaction at 12 min, followed by centrifugation at 11,000 rpm for 5 min at room temperature and washing of the precipitated platelets with PBS twice. The platelets were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% TritonX-100, 50 mM NaF, 2 mM EDTA, 100 uM Na-orthovanadate, 1 mM PMSF, 5 ug/ml of leupeptin, and 1 uM pepstatin A). The lysate was centrifuged at 13,000 rpm for 15 min at 4°C, and the supernatant was resolved for detection of phosphorylated Akt by SDS-PAGE. Specific phosphorylated antibodies for Akt were used for the analysis. β-actin was used to indicate equal protein loading. The result was analyzed by Western blotting using a PVDF membrane (Millipore) for the transfer step and Western blotting detection reagent (Roche or ELPIS Biotech) to detect the chemifluorescent signal.

Statistical analysis
The results are presented as means $\pm$ SD (standard deviation). The data were analyzed by Student's t-test from the Sigmaplot to determine whether or not the means were significantly different from the control. P values below 0.05 were considered significant.

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