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INHIBITION OF CYCLOOXYGENASE ACTIVITY AND INCREASE IN PLATELET CYCLIC AMP BY GIRINIMBINE, ISOLATED FROM *MURRAYA EUCHRESTIFOLIA*

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Abstract—Girinimbine is an antiplatelet agent isolated from *Murraya euchrestifolia*. In washed rabbit platelets, it inhibited arachidonic acid (AA)-, collagen-, U46619- and platelet-activating factor (PAF)-induced aggregation and ATP release in a concentration-dependent manner with IC_{50} values of 9.1 ± 1.5, 16.7 ± 1.7, 60.0 ± 5.1 and 71.9 ± 5.6 μM, respectively. However, it did not apparently affect thrombin-induced aggregation and ATP release even when a concentration of 80 μM was used. In citrated human platelet-rich plasma (PRP), girinimbine selectively inhibited secondary aggregation and ATP release without appearing to affect the primary aggregation induced by epinephrine and ADP. The formation of both platelet thromboxane B_2 (TxB_2) and prostaglandin D_2 (PGD_2) caused by AA was inhibited by girinimbine concentration dependently, with a maximal effect at 20 μM. Girinimbine also inhibited cyclooxygenase activity as reflected by the attenuation of prostaglandin E_2 (PGE_2) formation after incubation of sheep vesicular gland microsomes with arachidonic acid. In *myo*-[^{3}H]inositol-labeled and fura-2-loaded platelets, [^{3}H]inositol monophosphate generation and the increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) stimulated by AA and collagen, but not that stimulated by U46619, PAF and thrombin, were inhibited by girinimbine (20 μM). Platelet cyclic AMP levels were elevated by high concentrations of girinimbine (20 and 80 μM). These data indicate that the antiplatelet effect of girinimbine is due to the inhibition of cyclooxygenase activity and elevation of the cyclic AMP level.

Key words: girinimbine; cyclooxygenase; thromboxane; cAMP; *Murraya euchrestifolia*; platelet

Platelet–vessel wall interactions are important in the development of thrombosis and atherosclerosis [1, 2]. Circulating platelets in normal subjects probably do not adhere to "normal" endothelium. However, at sites of endothelium injury, platelets adhere to the subendothelium, form TxA_2§ and release granule contents [3]. These may, in turn, be responsible for the adhesion of more platelets at the site of injury, increasing the size of platelet aggregates. The morphologic evidence that arterial thrombi are composed predominantly of platelet aggregates (white thrombi) [4] has led many investigators to postulate that platelet aggregation is the major pathogenetic mechanism in arterial thrombosis. Mural thrombus formation not only can restrict the flow of blood to vital tissues or organs, leading to peripheral, cerebral or coronary ischemia, but it may also embolize with potentially lethal consequences. Thus, inhibition of platelet function may be a promising approach for the prevention of thrombosis.

Medicinal plants have been used as traditional remedies in oriental countries for hundreds of years. Some of the alkaloids from these medicinal plants possess antiplatelet activity. For example, protopine (isolated from methanol extracts of Chinese Corydalis tuber) inhibits platelet aggregation by inhibiting platelet Tx formation and phosphoinositide breakdown [5]; cepharanthine (extracted from *Stephania cepharantha* Hayata) inhibits platelet activation by changing lipid properties and inhibiting the function of the calcium channel or the susceptibility of substrate phospholipids to enzymatic hydrolysis by phospholipase A_2 [6]. *Murraya euchrestifolia* (Rutaceae) has proved to be a rich source of carbazole alkaloids [7]. In a screening test, we found that girinimbine, a carbazole alkaloid isolated from *M. euchrestifolia*, possessed antiplatelet activity. In this paper, we have tried to elucidate the mechanism of its inhibitory activity on platelet aggregation.

MATERIALS AND METHODS

Materials. Girinimbine (Fig. 1) was isolated from the plant *M. euchrestifolia*, as described previously [7]. Bovine thrombin, from the Parke Davis Co., was dissolved in 50% (v/v) glycerol for a stock solution of 100 NIH units/mL. Collagen (type I, bovine Achilles tendon), from the Sigma Chemical Co., was homogenized in 25 mM acetic acid and stored (1 mg/mL) at −70°. PAF (1-*O*-alkyl-2-acetyl-*sn*-glycerol-3-phosphorylcholine), purchased from Sigma, was dissolved in chloroform and diluted into

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§ Abbreviations: Tx, thromboxane; PG, prostaglandin; AA, arachidonic acid; PAF, platelet-activating factor; EIA, enzyme immunoassay; RIA, radioimmunoassay; PRP, platelet-rich plasma; TCA, trichloroacetic acid; and $[Ca^{2+}]_i$, intracellular free calcium concentration.

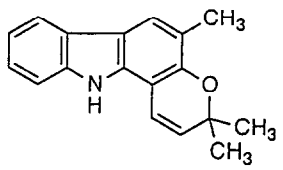

Fig. 1. Chemical structure of girinimbine.

0.1% BSA-saline solution immediately prior to use. AA, BSA, indomethacin, imidazole, epinephrine, DMSO, TCA, fura-2/AM, EDTA (disodium salt), EGTA, sodium citrate, luciferase-luciferin, Dowex-1 (100–200 mesh: x8, chloride) resin and *myo*-inositol were purchased from Sigma. U46619 (9,11-dideoxy-9α,11α-methanoepoxy$PGF_{2\alpha}$) and the cyclooxygenase activity assay kit were obtained from Biomol. TxB_2, PGD_2, and cyclic AMP EIA kits were purchased from the Cayman Chemical Co. *myo*-[2-^{3}H]Inositol (10–20 Ci/mmol) and a PGE_2 RIA kit were purchased from Amersham, U.K.

Preparation of platelets. PRP was obtained from blood collected from healthy human volunteers, who did not take any medication for 2 weeks prior to collection, or from rabbit marginal vein, anticoagulated with sodium citrate (3.8%; 1:9 v/v) and was centrifuged for 10 min at 90 *g* and room temperature. Platelet suspension was prepared from EDTA-anticoagulated PRP according to washing procedures described previously [8]. Platelets were counted by Hemalaser 2 (Sebia, France) and adjusted to a concentration of 3×10^8 platelets/mL. Platelet pellets were finally suspended in Tyrode's buffer (pH 7.4) of the following composition: NaCl (136.8 mM), KCl (2.8 mM), $NaHCO_3$ (11.9 mM), $MgCl_2$ (2.1 mM), NaH_2PO_4 (0.33 mM), $CaCl_2$ (1 mM), glucose (11.2 mM) containing 0.35% BSA.

Platelet aggregation and ATP release reaction. Aggregation was measured by the turbidimetry method as described by O'Brien [9]. ATP released from platelets was detected by the bioluminescence method of DeLuca and McElory [10]. Both aggregation and ATP release were measured simultaneously in a Lumi-aggregometer (model 1020B, Payton, Canada) connected to two dual-channel recorders. Platelet preparations were stirred at 900 rpm. When DMSO was used as solvent, its final concentration was fixed at 0.5% (v/v) to eliminate the effect of the solvent.

To determine whether the inhibitory effects of girinimbine on aggregation and ATP release were reversible, platelets were incubated with girinimbine (40 μM) for 3 min. Following that, platelets were sedimented by centrifugation and resuspended in fresh Tyrode's solution. Platelet aggregation and ATP release were then performed as described above.

TxB_2 and PGD_2 assay. After 6 min of platelet incubation with the inducer, EDTA (2 mM) and indomethacin (50 μM) were added to halt Tx and PGD_2 formation. After centrifugation in an Eppendorf microcentrifuge (model 5415 C) for 2 min, TxB_2 and PGD_2 in the supernatant were assayed by EIA.

Labeling of membrane phospholipids and measurement of the production of [^{3}H]inositol phosphate. The method employed for labeling platelet membrane phospholipids with *myo*-[2-^{3}H]inositol was the same as described previously [11]. Phosphoinositide breakdown was initiated by addition of aggregation inducers to [^{3}H]inositol-labeled platelet suspension (1 mL) in a 3.5-mL cuvette with a stirring bar driven at 1200 rpm. Incubation was continued for 6 min at 37°. An equal volume of 10% (v/v) TCA was then added to stop the reaction. After centrifugation at 1000 *g* for 10 min, 1 mL of supernatant was aspirated, and TCA was removed by washing with 4×3 vol. of diethyl ether. The aqueous phase containing inositol phosphates was adjusted to pH 7–8, diluted to 4 mL with distilled water, and applied to a Dowex-1 ion-exchange column for separation of inositol phosphates as described by Neylon and Summers [12]. All experiments were carried out in the presence of LiCl (5 mM) to inhibit inositol monophosphate phosphatase. Because concentrations of inositol bisphosphate and inositol trisphosphate were very low, we measured inositol monophosphate as an index of the formation of total inositol phosphates.

Measurement of intracellular calcium in platelets. The method of Pollock and Rink was followed [13]. Platelets (3×10^8/mL) were incubated with fura-2/AM (5 μM) at 37° for 40 min and then centrifuged at 500 *g*; the resultant pellet was washed with EDTA (1 mM)-containing Tyrode's solution. After centrifugation, platelets were resuspended in Tyrode's solution containing 1 mM Ca^{2+}. Fluorescence (ex. 339 nm, em. 500 nm) was measured with a Hitachi Fluorescence Spectrophotometer (model F4000). At the end of the experiment, the cells were treated with 0.1% Triton X-100 followed by the addition of 10 mM EGTA to obtain the maximal and minimal fluorescence, respectively. $[Ca^{2+}]_i$ was calculated as described for fura-2 using the Ca^{2+}-dye dissociation constant of 224 nM [14].

Cyclic AMP assay. The method of Karniguian *et al.* [15] was followed. Platelet suspension (10^9/mL) was warmed at 37° for 1 min, and then PGE_1 or girinimbine was added with incubation for 3 min. The reaction was stopped by adding 10 mM EDTA followed immediately by boiling for 5 min. Upon cooling to 4°, precipitated protein was sedimented by centrifugation in an Eppendorf microcentrifuge (model 5415 C). The supernatant was used to assay for cyclic AMP content by EIA.

Assay of cyclooxygenase activity. The effect of girinimbine on cyclooxygenase activity was determined using a cyclooxygenase activity assay kit (Biomol). In brief, sheep vesicular gland microsomes (0.2 mg/mL; 100 μL) were incubated with DMSO (2 μL), indomethacin (0.01 mg/2 μL) or various concentrations of girinimbine (2 μL) at 4° for 3 min. Reaction cofactor (epinephrine, tryptophan, hydroquinone and GSH; 10 μL) was then added and incubated at 25° for 3 min with shaking. After that, arachidonic acid (2 μg/2 μL) was added and the mixture incubated for another 3 min. $FeCl_2$ (25 mM, 10 μL) was then added to halt the reaction, and the reaction mixture was allowed to stand at room

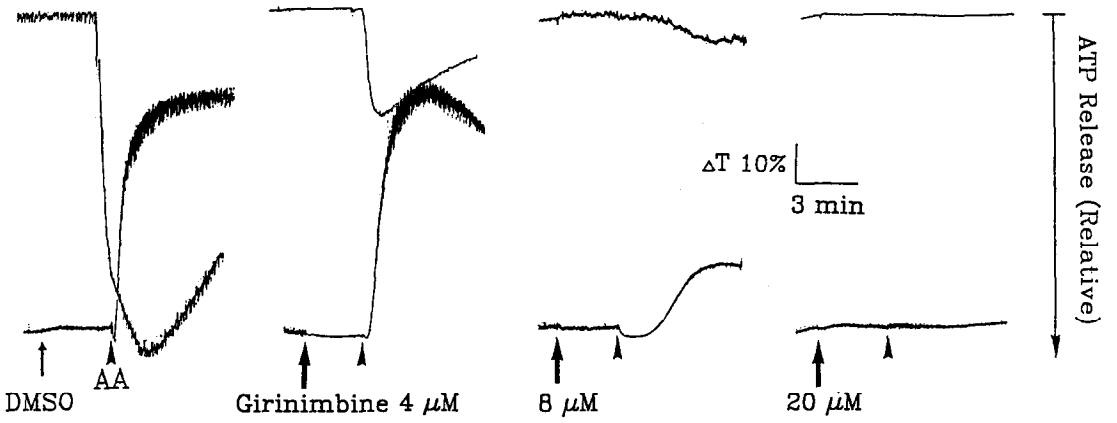

Fig. 2. Inhibitory effect of girinimbine on platelet aggregation and ATP release induced by AA. Washed rabbit platelets were incubated with DMSO (0.5%) or various concentrations of girinimbine at 37° for 3 min; then AA (100 μM) was added to trigger aggregation (upward tracings) and ATP release (downward tracings).

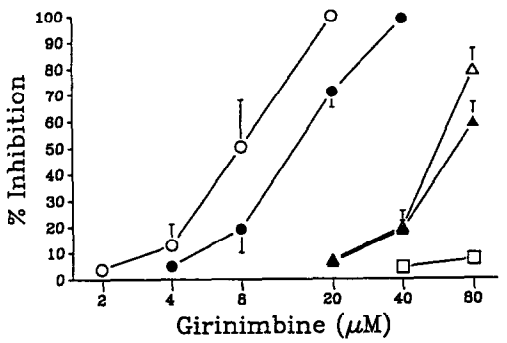

Fig. 3. Concentration-dependent inhibition of girinimbine on platelet aggregation induced by AA (100 μM; ○), collagen (10 μg/mL; ●), U46619 (1 μM; △), PAF (3.6 nM; ▲) or thrombin (0.1 U/mL; □). Washed rabbit platelets were incubated with DMSO (0.5%) or various concentrations of girinimbine at 37° for 3 min; then the inducer was added to trigger aggregation. Values are means ± SEM (N = 6).

temperature for 15 min. After centrifugation at 3000 *g* and 4° for 10 min, PGE_2 in the supernatant was assayed by RIA. Cyclooxygenase activity was reflected by the production of PGE_2.

RESULTS

Effects of girinimbine on the aggregation and ATP release reaction of washed rabbit platelets. AA (100 μM), collagen (10 μg/mL), U46619 (1 μM), PAF (3.6 nM) and thrombin (0.1 U/mL) all caused 85–90% aggregation of washed rabbit platelets. Girinimbine inhibited AA- and collagen-induced platelet aggregation and ATP release in a concentration-dependent manner (Figs. 2 and 3). The IC_{50} values of girinimbine on AA- and collagen-induced platelet aggregation were 9.1 ± 1.5 and 16.7 ± 1.7 μM, respectively. A high concentration of girinimbine (80 μM) markedly inhibited U46619- and PAF-induced platelet aggregation and ATP release without affecting those caused by thrombin (Fig. 3). Indomethacin (0.1 to 2 μM) and aspirin (10 to 100 μM) also inhibited AA-induced aggregation in a concentration-dependent manner with IC_{50} values of about 0.5 and 25 μM, respectively. When a concentration of 20 μM was used, indomethacin caused only a slight inhibition of collagen-induced platelet aggregation (19.3 ± 2.8% inhibition, N = 5) without affecting the aggregation caused by U46619, PAF and thrombin.

The inhibitory effect of girinimbine on platelet aggregation was time dependent. Incubation of girinimbine with washed rabbit platelets for 30 min caused a more pronounced inhibition than incubation for 3 min (data not shown). After treating platelets with girinimbine (40 μM) for 3 min at room temperature and then washing them with Tyrode's solution, AA- and collagen-induced platelet aggregation and ATP release were restored (data not shown).

Effects of girinimbine on ADP- and epinephrine-induced aggregation and ATP release in human platelet-rich plasma. ADP (5 μM) and epinephrine (5 μM) caused biphasic aggregation in human PRP (Fig. 4). Secondary aggregation and ATP release were suppressed by girinimbine, while primary aggregation was still not abolished at a concentration of 400 μM. Indomethacin (20 μM) also selectively blocked the secondary aggregation and ATP release without affecting the primary aggregation of ADP and epinephrine (Fig. 4).

Effects of girinimbine on platelet TxB_2 and PGD_2 formation. The TxB_2 level of resting platelets was 0.5 ± 0.1 ng/3 × 10^8 platelets. In rabbit platelets, U46619 (1 μM) failed to raise the level of TxB_2 significantly, whereas PAF (3.6 nM) caused only slight thromboxane formation. However, AA

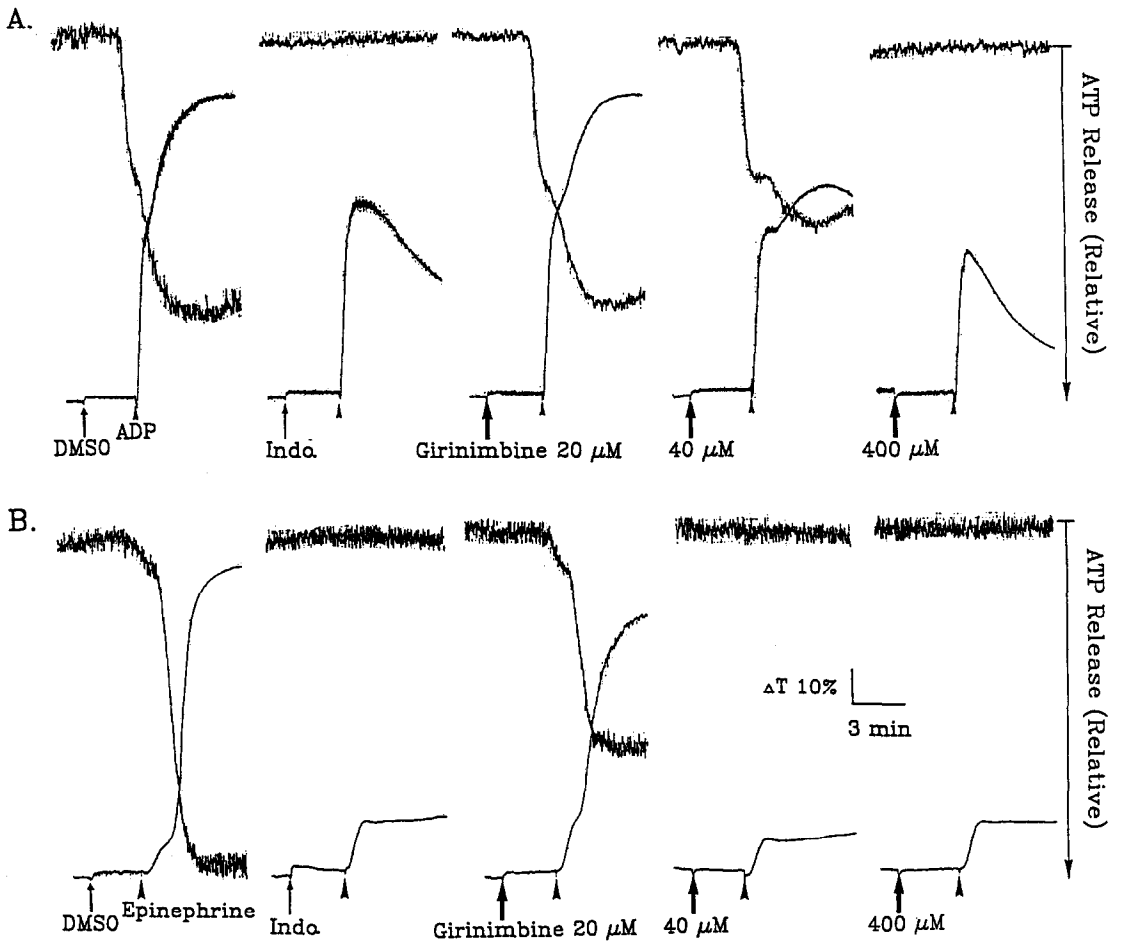

Fig. 4. Effects of girinimbine on the aggregation and ATP release induced by ADP (A) and epinephrine (B) in human platelet-rich plasma. Platelet-rich plasma was incubated with DMSO (0.5%, control), indomethacin (Indo. 20 μM) or various concentrations of girinimbine at 37° for 3 min, and then ADP (5 μM) or epinephrine (5 μM) was added to trigger aggregation (upward tracings) and ATP release (downward tracings).

Table 1. Effects of girinimbine, indomethacin and imidazole on the formation of thromboxane B_2 in washed rabbit platelets caused by arachidonic acid, collagen, U46619, PAF and thrombin

	Thromboxane B_2 (ng/3 × 10^8 platelets)				
	Arachidonic acid (100 μM)	Collagen (10 μg/mL)	U46619 (1 μM)	PAF (3.6 nM)	Thrombin (0.1 U/mL)
Control	571 ± 74 (6)	339 ± 16 (5)	1.0 ± 0.3 (5)	5.3 ± 2.6 (5)	10 ± 2 (6)
Girinimbine, 4 μM	350 ± 74* (6)				
8 μM	153 ± 36† (6)				
20 μM	33 ± 6† (6)	2 ± 1† (5)	1.0 ± 0.3 (5)	0.2 ± 0.1† (5)	1.0 ± 0.4† (6)
Indomethacin, 2 μM	20 ± 3† (6)				
Imidazole, 1 mM	88 ± 23† (6)				

DMSO (0.5%, control), indomethacin, imidazole or girinimbine (various concentrations) was preincubated with platelets at 37° for 3 min; then the inducer was added. Aggregation and thromboxane formation were terminated by EDTA (2 mM) and indomethacin (50 μM) 6 min after the addition of the inducer. Values are means ± SEM (N).

*,† Significantly different from the respective control: *$P < 0.05$, and †$P < 0.001$.

Table 2. Effects of girinimbine, indomethacin and imidazole on prostaglandin D_2 formation induced by arachidonic acid in washed rabbit platelets

	Prostaglandin D_2 (ng/3 × 10^8 platelets)
Resting	0.09 ± 0.01
DMSO + AA	1.58 ± 0.34
Girinimbine + AA	0.17 ± 0.02*
Indomethacin + AA	0.33 ± 0.03*
Imidazole + AA	93.40 ± 10.30*

DMSO (0.5%), girinimbine (20 μM), indomethacin (2 μM) or imidazole (1 mM) was preincubated with platelets at 37° for 3 min; then arachidonic acid (AA, 100 μM) was added. Aggregation and prostaglandin D_2 formation were terminated by EDTA (2 mM) and indomethacin (50 μM) 6 min after the addition of AA. Values are means ± SEM (N = 6).

* $P < 0.001$, as compared with the control (AA, 100 μM).

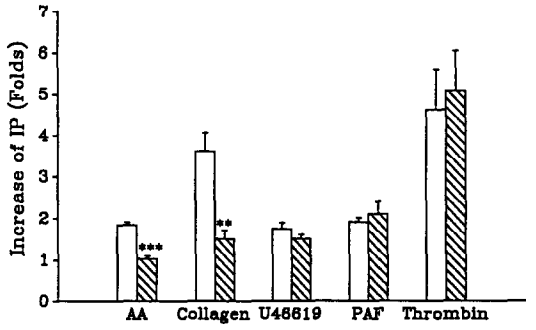

Fig. 5. Inhibitory effects of girinimbine on the formation of inositol monophosphate caused by some aggregation inducers in washed rabbit platelets. [^{3}H]Inositol-labeled platelets were incubated with DMSO (0.5%, control; □) or girinimbine (20 μM; ▨) at 37° for 3 min; then AA (100 μM), collagen (10 μg/mL), U46619 (1 μM), PAF (3.6 nM) or thrombin (0.1 U/mL) was added for another 6 min. Indomethacin (2 μM) was present in the medium except in those experiments challenged by AA and collagen. The [^{3}H]inositol monophosphate level of resting platelets was 630 ± 100 cpm/3 × 10^8 platelets. Increases (folds) in inositol phosphate (IP) are presented as means ± SEM (N = 4–5). Key: (**) $P < 0.01$, and (***) $P < 0.001$ as compared with the respective control.

(100 μM), collagen (10 μg/mL) and thrombin (0.1 U/mL) caused marked TxB_2 formation in washed rabbit platelets. Girinimbine significantly inhibited AA-, collagen-, PAF- and thrombin-induced TxB_2 formation. Indomethacin (2 μM) and imidazole (1 mM) also inhibited AA-induced TxB_2 formation significantly (Table 1). The PGD_2 level of resting platelets was low (0.09 ± 0.01 ng/3 × 10^8 platelets). However, PGD_2 formed in the presence of arachidonic acid. This PGD_2 formation was inhibited by both girinimbine and indomethacin, but enhanced significantly by imidazole (1 mM) (Table 2).

Effects of girinimbine on phosphoinositide breakdown. In [^{3}H]inositol-labeled washed rabbit platelets, U46619 (1 μM), PAF (3.6 nM) and thrombin (0.1 U/mL) increased [^{3}H]inositol monophosphate formation 1.7 ± 0.1-, 1.9 ± 0.1- and 4.6 ± 1.0-fold, respectively, in the presence of indomethacin (2 μM). AA (100 μM) and collagen (10 μg/mL), in the absence of indomethacin, also increased [^{3}H]inositol monophosphate formation 1.8 ± 0.1- and 3.6 ± 0.5-fold, respectively. Girinimbine (20 μM) significantly inhibited AA- and collagen-induced [^{3}H]inositol monophosphate formation without affecting that caused by U46619, PAF and thrombin (Fig. 5).

Effects of girinimbine on the intracellular calcium of platelets. In fura-2-loaded platelets, U46619, PAF and thrombin caused an increase of intracellular free calcium in the presence of indomethacin (2 μM). AA and collagen also increased intracellular free calcium in the absence of indomethacin. The rise was only short-lived, and apparent intracellular free calcium declined towards the resting level within a few minutes. This was due to the aggregates interfering with the fluorescence signal and constitutes a limitation of the technique. As shown in Fig. 6, only the increases of intracellular Ca^{2+} caused by AA and collagen were inhibited by girinimbine.

Effect of girinimbine on cyclooxygenase activity. Cyclooxygenase activity was reflected by PGE_2 production. After incubation of sheep vesicular gland microsomes with AA (2 μg) at 25° for 3 min, the PGE_2 formation was 40.1 ± 11.0 ng/mL. Both girinimbine and indomethacin significantly inhibited this PGE_2 formation (Fig. 7).

Effect of girinimbine on the cyclic AMP level of platelets. The level of cyclic AMP in unstimulated platelets was very low (0.8 ± 0.1 pmol/10^9 platelets). A 1 μM concentration of PGE_1 increased the cyclic AMP level to 20.8 ± 3.4 pmol/10^9 platelets. Girinimbine at 20 and 80 μM also caused slight but significant increases of the platelet cyclic AMP level (Table 3).

DISCUSSION

The present study shows that girinimbine is a reversible inhibitor of platelet aggregation and ATP release induced by a variety of inducers. Its mechanisms of action include direct inhibition of platelet cyclooxygenase activity and increase of the cyclic AMP level.

TxA_2 is an important mediator of release reaction and aggregation of platelets [16]. The aggregation and ATP release of platelets induced by AA are due to TxA_2 formation [17]. In human platelet-rich plasma, ADP and epinephrine cause biphasic aggregation with secretion accompanying the second phase. Secondary aggregation and ATP release are mediated by TxA_2 formation and inhibited by cyclooxygenase inhibitors, such as aspirin [18]. Phosphoinositide breakdown is observed in platelets activated by many agonists, and may be a primary event in the agonist-induced activation [19, 20]. This phosphoinositide breakdown in platelets may provide a source of free arachidonate via the diglyceride lipase pathway or phosphatidic acid-specific phospholipase A_2 leading to TxA_2 formation [21, 22]. Arachidonic acid also can be liberated from membrane phospholipids by phospholipase A_2, which is

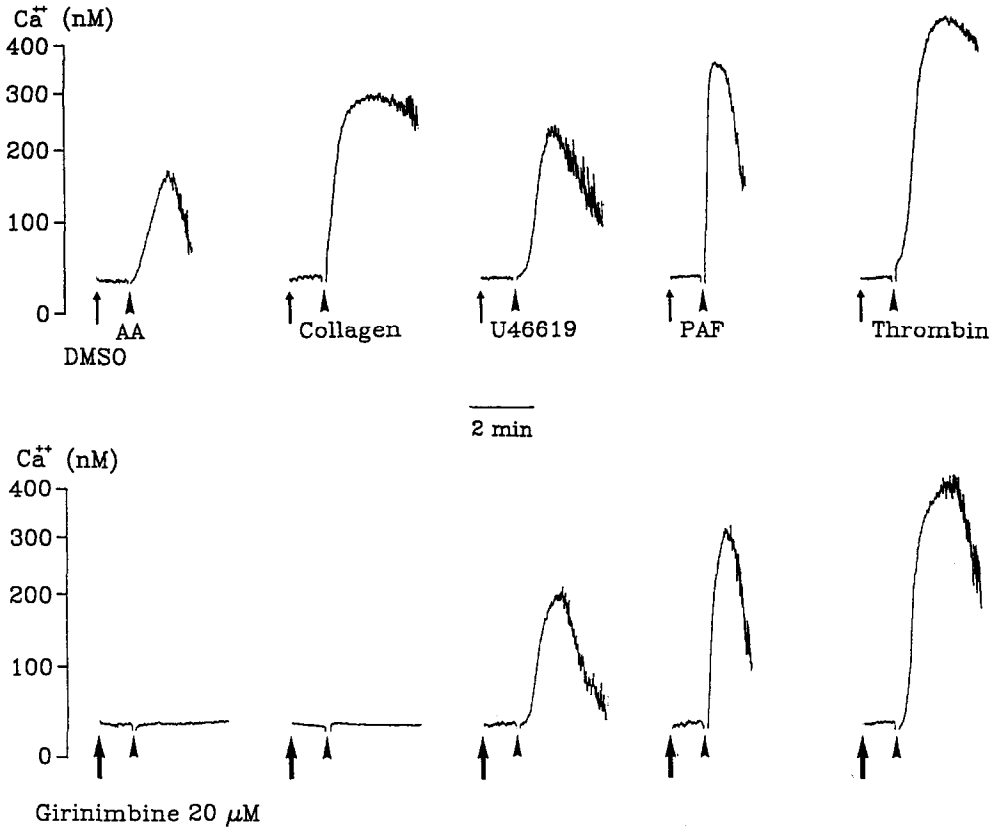

Fig. 6. Effects of girinimbine on the increase of intracellular calcium concentration caused by some aggregation inducers in fura-2-loaded platelets. Fura-2-loaded washed rabbit platelets were preincubated with DMSO (0.5%) or girinimbine (20 μM) at 37° for 3 min; then AA (100 μM), collagen (10 μg/mL), U46619 (1 μM), PAF (3.6 nM) or thrombin (0.1 U/mL) was added. Indomethacin (2 μM) was present in the medium except in those experiments challenged by AA and collagen.

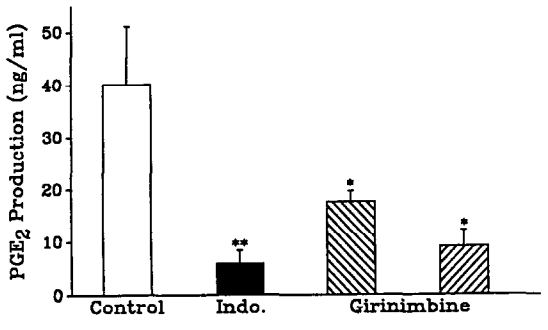

Fig. 7. Inhibitory effect of girinimbine on cyclooxygenase activity. Sheep vesicular gland microsomes (SVM) were incubated with DMSO (0.5%, □), indomethacin (0.01 mg, ■) or girinimbine (8 μM, ▧; 20 μM, ▨) at 4° for 3 min; then the cofactors (epinephrine, tryptophan, hydroquinone and GSH) were added and the mixture was incubated at 25° for another 3 min. After AA (2 μg) was added to trigger the PGE_2 production at 25° for 3 min, the reaction was stopped by $FeCl_2$ (25 mM, 10 μL). Cyclooxygenase activity was reflected by PGE_2 production (ng/mL), which is presented as means ± SEM (N = 4). Key: (*) $P < 0.05$, and (**) $P < 0.01$ as compared with the control.

Table 3. Effects of girinimbine and prostaglandin E_1 on platelet cyclic AMP level

	Cyclic AMP (pmol/10^9 platelets)
Resting	0.8 ± 0.1
Girinimbine, 20 μM	1.7 ± 0.2*
80 μM	2.0 ± 0.4*
Prostaglandin E_1, 1 μM	20.8 ± 3.4†

DMSO (0.5%, resting), girinimbine or prostaglandin E_1 was preincubated with platelets at 37° for 3 min; then cyclic AMP formation was stopped by adding 10 mM EDTA and immediate boiling for 5 min. Values are means ± SEM (N = 4).

*,† Significantly different from the resting value: *$P < 0.05$, and †$P < 0.01$.

activated by inositol 1,4,5-trisphosphate-triggered Ca^{2+} release [23]. Girinimbine (20 μM) inhibited AA-, collagen, PAF- and thrombin-induced thromboxane formation (Table 1). It also inhibited AA-induced phosphoinositide breakdown and the rise of intracellular Ca^{2+} without affecting those caused by U46619, PAF and thrombin (Figs. 5 and 6). In

human platelet-rich plasma, girinimbine inhibited only secondary aggregation and ATP release without affecting the primary aggregation of ADP and epinephrine (Fig. 4). These results indicate that girinimbine inhibits platelet aggregation and release reaction via inhibition of thromboxane formation. Similar to indomethacin, but not to imidazole, girinimbine inhibited PGD_2 formation in the presence of AA (Table 2). In addition, it also inhibited cyclooxygenase activity reflected by the attenuation of PGE_2 production after incubation of sheep vesicular gland microsomes with AA (Fig. 7). Thus, girinimbine is an inhibitor of cyclooxygenase. The action of girinimbine is different from that of a TxA_2 receptor antagonist, such as SQ 29,548, which fails to alter cyclooxygenase, Tx synthase or adenylate cyclase activity [24].

High concentrations of girinimbine (40 and 80 μM) inhibited platelet aggregation induced by collagen completely and those by U46619 and PAF markedly. However, indomethacin (20 μM) caused only a slight inhibition of collagen-induced platelet aggregation without affecting aggregation caused by U46619, PAF and thrombin. Inhibition of platelet thromboxane formation cannot explain why the aggregation caused by collagen, U46619 and PAF was inhibited by girinimbine. Elevation of the cyclic AMP level, either by stimulation of adenylate cyclase or by inhibition of cyclic AMP-dependent phosphodiesterase, is one of the most potent mechanisms of inhibition of platelet functions [25]. Elevated cyclic AMP inhibits most platelet responses, including aggregation, ATP release and rise of intracellular Ca^{2+} concentration. In our experimental data, girinimbine (20 and 80 μM) did increase the platelet cyclic AMP content, which may be involved in girinimbine inhibition of collagen-induced platelet responses. However, platelet cyclic AMP levels raised by girinimbine (1.7 ± 0.2 and 2.0 ± 0.4 pmol/10^9 platelets for 20 and 80 μM, respectively) were much smaller than the cyclic AMP level raised by PGE_1 (20.8 ± 3.4 pmol/10^9 platelets for 1 μM). Furthermore, 80 μM girinimbine did not increase platelet cyclic AMP more than 20 μM did, whereas 80 μM girinimbine caused more pronounced inhibition of U46619- and PAF-induced aggregation than 20 μM. Thus, the platelet cyclic AMP level elevated by girinimbine may not be involved in girinimbine inhibition of U46619- and PAF-induced platelet aggregation. The mechanism of girinimbine inhibition of U46619- and PAF-induced aggregation is not known at present. Whether the increase of platelet cyclic AMP formation by girinimbine is due to direct activation of adenylate cyclase or to inhibition of phosphodiesterase needs further investigation.

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