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DICENTRINE, A NOVEL ANTIPLATELET AGENT INHIBITING THROMBOXANE FORMATION AND INCREASING THE CYCLIC AMP LEVEL OF RABBIT PLATELETS

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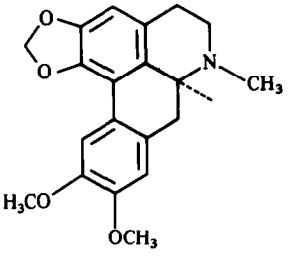
Abstract—Dicentrine is an antiplatelet agent isolated from the Chinese herb *Lindera megaphylla*. We examined the *in vitro* effects of dicentrine on various aspects of platelet reactivity. Dicentrine inhibited the aggregation and ATP release of washed rabbit platelets induced by arachidonic acid (AA), collagen, ADP, platelet-activating factor (PAF), thrombin and U46619. Dicentrine also inhibited the thromboxane B_2 formation caused by AA, collagen and thrombin in washed intact platelets or that induced by AA in lysed platelet homogenate, while prostaglandin D_2 formation caused by AA was not increased. The generation of inositol monophosphates (in the presence of indomethacin) caused by thrombin, collagen and PAF was not suppressed significantly, nor did dicentrine suppress fibrinogen-induced aggregation of elastase-treated platelets. Dicentrine inhibited the intracellular Ca^{2+} increase in quin-2/AM-loaded platelets caused by thrombin, PAF, collagen and AA. The cyclic AMP level was elevated by dicentrine in a concentration-dependent manner. These data indicate that the inhibitory effect of dicentrine on platelet aggregation and ATP release was due to the inhibition of thromboxane formation and the elevation of the level of cyclic AMP.

It is now well recognized that platelet–vessel wall interactions are important in the development of thrombosis and atherosclerosis. Platelets are activated by a wide variety of stimuli *in vivo*, including diseased arteries [1]. Mural thrombus formation can restrict the flow of blood to vital tissues or organs leading to peripheral, cerebral or coronary ischemia. Additionally, a developing thrombus may 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. In a large scale screening test, we found many biologically active compounds isolated from plant sources. Some of them possessed antiplatelet activity and inhibited the contraction of aortic smooth muscles. For example, osthole (isolated from *Angelica pubescens*) causes inhibition of platelet aggregation by inhibiting thromboxane formation and phosphoinositide breakdown [2]; denudatin B (isolated from *Magnolia fargesii*) blocks the voltage-dependent Ca^{2+} channel in rat aorta [3], and it also inhibits the aggregation and release reaction of rabbit platelets caused by platelet-activating factor (PAF§) [4]. In addition, some of the other work has shown that alkaloids of natural products possess antiplatelet activity. For example, cepharanthine (extracted from *Stephania cepharantha* Hayata) inhibits platelet activation by changing the lipid properties and inhibiting the function of the calcium channel or the susceptibility of substrate phospholipids to enzymatic hydrolysis by phospholipase A_2 [5]; *d*-corydaline, *d*-glaucine, protopine and *l*-tetrahydrocolumbamine (isolated from methanol extracts of Chinese Corydalis tuber) inhibit the platelet aggregation induced by collagen and arachidonic acid [6].



d-Dicentrine

Fig. 1. Structure of *d*-dicentrine.

Recently, we found that dicentrine, an aporphine derivative (Fig. 1) isolated from the Chinese herb *Lindera megaphylla*, possessed antiplatelet activity and also showed a strong vasorelaxant action. In

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§ Abbreviations: PAF, platelet-activating factor; AA, arachidonic acid; BSA, bovine serum albumin; PGE_1, prostaglandin E_1; PGD_2, prostaglandin D_2; RIA, radioimmunoassay; PRP, platelet-rich plasma; and DMSO, dimethyl sulfoxide.

this paper, we have tried to elucidate the mechanism of its inhibitory activity on platelet aggregation.

MATERIALS AND METHODS

Materials. Dicentrine was isolated from *L. megaphylla* as described previously [7]. Collagen (Type 1, bovine achilles tendon), obtained from the Sigma Chemical Co. (U.S.A.), was homogenized in 25 mM acetic acid and then stored at −70°. Arachidonic acid (AA), ADP, U46619, bovine serum albumin (BSA), indomethacin, prostaglandin E_1 (PGE_1), EDTA (disodium salt), sodium citrate, luciferase-luciferin, Dowex-1 (100–200 mesh: X8, chloride), *myo*-inositol, quin-2/AM and PAF were purchased from the Sigma Chemical Co. Thrombin (bovine) was obtained from the Parke Davis Co. (U.S.A.) and dissolved in 50% (v/v) glycerol to give a stock solution of 100 NIH U/mL. *myo*-[2-^{3}H]-Inositol and cyclic AMP radioimmunoassay (RIA) kits were purchased from Amersham (U.K.). RIA kits for thromboxane B_2 and prostaglandin D_2 (PGD_2) were obtained from the New England Nuclear Co. (U.S.A.).

Platelet aggregation and ATP release reaction. Platelet-rich plasma (PRP) was obtained from blood collected from the rabbit marginal vein, anticoagulated with sodium citrate (3.8%, 14:1) and centrifuged for 10 min at 90 *g* and room temperature. Platelet suspension was obtained from EDTA-anticoagulated PRP according to the washing procedures described previously [8]. Platelet numbers were counted by a Coulter Counter (model ZM) and adjusted to 4.5×10^8 platelets/mL. The platelet pellets were finally suspended in Tyrode's solution of the following composition (mM): NaCl (136.8), KCl (2.8), $NaHCO_3$ (11.9), $MgCl_2$ (2.1), NaH_2PO_4 (0.33), $CaCl_2$ (1.0) and glucose (11.2) containing BSA (0.35%). Aggregation was measured by the turbidimetric method as described by O'Brien [9]. ATP release from platelets was detected by the bioluminescence method as described by DeLuca and McElory [10]. Both the aggregation and release of ATP were measured simultaneously by a Lumi-aggregometer (Chrono-Log Co., U.S.A.) connected to a dual channel recorder. The platelet suspension was stirred at 1200 rpm. To eliminate the effect of solvent on aggregation, the final concentration of dimethyl sulfoxide (DMSO) was fixed at 0.5% (v/v).

Thromboxane B_2 and PGD_2 assay. After challenging platelets with the aggregation inducer for 6 min, 2 mM EDTA and 50 μM indomethacin were added. Thromboxane B_2 and PGD_2 in the supernatant were centrifuged in an Eppendorf centrifuge (model 5414) for 2 min and assayed using radioimmunoassay kits according to the procedure described by the manufacturer. In some cases, platelets (4.5×10^8/mL) were homogenized by ultrasound. The lysed platelet homogenate was incubated with arachidonic acid (100 μM) for 6 min, and thromboxane B_2 formation was determined by the same procedure described above.

Labeling of membrane phospholipids and measurement of the production of [^{3}H]inositol phosphate. This method was modified from those of Huang and Detwiler [11] and Neylon and Summers [12]. EDTA-PRP was centrifuged at 500 *g* for 10 min, and the platelet pellets were suspended in 700 μL of Ca^{2+}-free and BSA-free Tyrode's solution containing 75 μCi/mL of *myo*-[^{3}H]inositol and 1 mM EDTA. After incubation for 2 hr at 37°, the platelets were collected by centrifugation (500 *g* for 4 min) and resuspended in Ca^{2+}-free Tyrode's solution. The reaction was carried out at 37° for 6 min with 1 mL of platelet suspension in a 3.5-mL cuvette with a stirring bar driven at 900 rpm. An equal volume of 10% (w/v) trichloroacetic acid was added to stop the reaction. After centrifugation at 1000 *g* for 10 min, 1 mL of supernatant was pooled and trichloroacetic acid was removed by extracting with 5 × 2 vol. of diethyl ether. The aqueous phase, containing the inositol phosphates, was adjusted to pH 7–8 and diluted to 4 mL with distilled water before its application to a Dowex-1 ion-exchange column for separation of the inositol phosphates as described previously by Neylon and Summers [12]. All the experiments were carried out in the presence of 5 mM LiCl to inhibit inositol phosphate phosphatase. Because the levels of inositol bisphosphate and inositol trisphosphate were very low, we measured the inositol monophosphate as an index of the total inositol phosphate formation.

Cyclic AMP assay. The method of Karniguian *et al.* [13] was followed. Platelet suspension was warmed at 37° for 1 min; then PGE_1 or dicentrine was added and incubated for 3 min. The incubation was stopped by adding 10 mM EDTA and immediate boiling for 5 min. After cooling to 4°, the precipitated protein was sedimented by centrifugation in an Eppendorf centrifuge (model 5414). The supernatant (400 μL) was freeze-dried and the residue was dissolved in 100 μL of distilled water. Fifty microliters of supernatant was used to determine the cyclic AMP content by radioimmunoassay kits as described by the manufacturer.

Measurement of intracellular calcium in platelets. According to the method described by Rink *et al.* [14], EDTA-anticoagulated PRP was incubated with quin-2/AM (20 μM) for 40 min and then washed and suspended in the above Tyrode's solution. Fluorescence was measured with a Hitachi Fluorescence Spectrophotometer (ex. 339 nm, em. 492 nm).

RESULTS

Effect of dicentrine on the aggregation and ATP release of platelets. In washed rabbit platelet suspension, aggregation was induced by a variety of agonists including AA (100 μM), PAF (2 ng/mL), collagen (10 μg/mL), ADP (20 μM) and thrombin (0.1 U/mL). At lower concentrations (30–150 μM) dicentrine caused a concentration-dependent inhibition of the aggregation induced by AA, while at higher concentrations (225–300 μM) it inhibited the aggregation induced by PAF, collagen, ADP, thrombin and U46619 (Table 1). The IC_{50} values of dicentrine and indomethacin on arachidonate-induced platelet aggregation were about 70 and 1 μM, respectively.

The ATP release from washed rabbit platelets

Table 1. Effect of dicentrine on the aggregation of washed rabbit platelets induced by arachidonic acid, PAF, collagen, ADP, thrombin and U46619

Inducer	Aggregation (%)					
	Control	30 μM	60 μM	150 μM	225 μM	300 μM
Arachidonic acid (100 μM)	92 ± 1 (10)	88 ± 1 (4)	60 ± 4* (9)	11 ± 7† (7)		
PAF (2 ng/mL)	92 ± 2 (4)			85 ± 3 (4)	40 ± 4‡ (4)	9 ± 8† (4)
Collagen (10 μg/mL)	94 ± 1 (8)		88 ± 2* (4)	51 ± 7† (5)	0 ± 0† (4)	
ADP (20 μM)	82 ± 3 (6)			76 ± 2* (5)	41 ± 7* (5)	4 ± 3† (3)
Thrombin (0.1 U/mL)	95 ± 2 (4)			92 ± 2 (4)	50 ± 4† (4)	0 ± 0† (4)
U46619 (1 μM)	93 ± 4 (4)			90 ± 3 (4)	43 ± 7† (4)	0 ± 0† (4)

Platelets were preincubated with various concentrations of dicentrine or DMSO (0.5%, control) at 37° for 3 min; then the inducer was added. Values are means ± SEM (N = 3–10).

*–‡ Significantly different from the respective control: * $P < 0.05$, † $P < 0.001$ and ‡ $P < 0.01$.

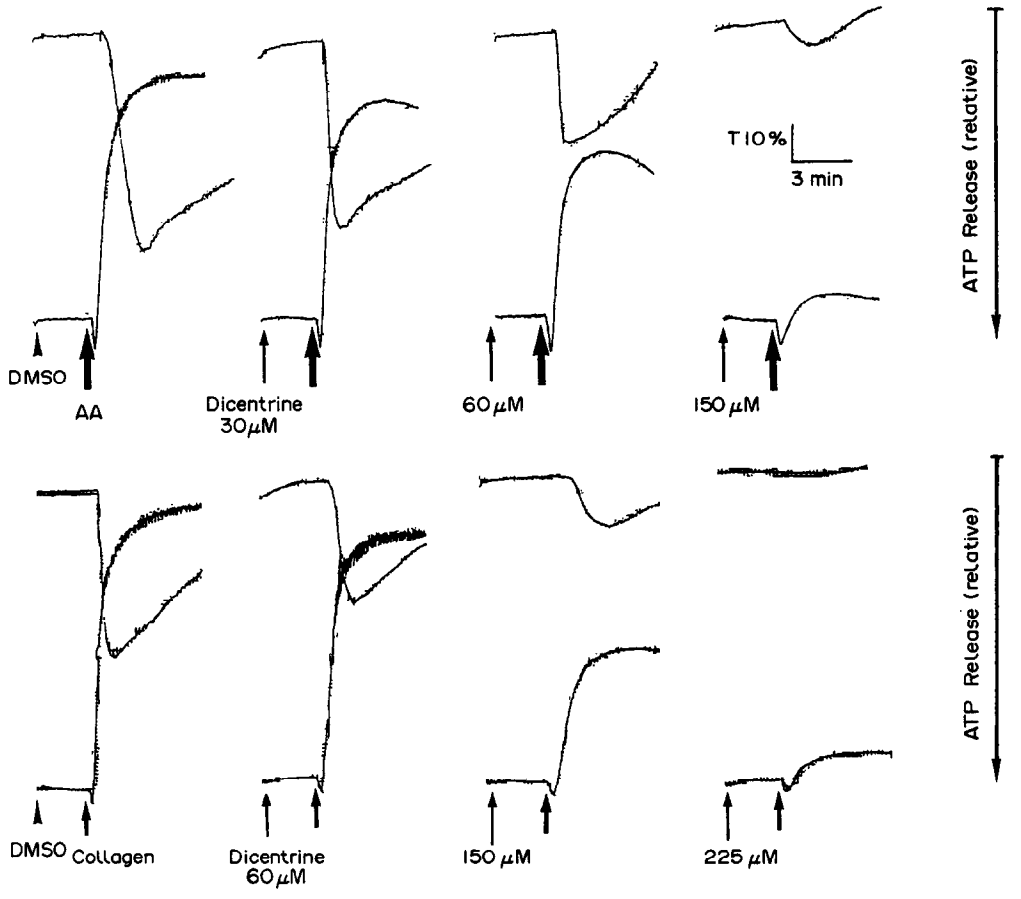

Fig. 2. Inhibitory effect of dicentrine on the platelet aggregation and ATP release induced by arachidonic acid (AA) and collagen. Washed rabbit platelets were incubated with various concentrations of dicentrine or with DMSO (0.5%, control) for 3 min; then AA (100 μM) or collagen (10 μg/mL) was added to trigger the aggregation (upward tracings) and ATP release (downward tracings).

induced by these compounds was also inhibited by dicentrine; this inhibition was concentration dependent and paralleled its inhibitory effect on aggregation (Fig. 2). Incubation of dicentrine with platelet suspension for 30 min did not cause a more pronounced inhibition than incubation for 3 min. After treating platelets with dicentrine (300 μM) for 30 min at room temperature and then washing them twice with the suspending solution, AA-induced platelet aggregation was restored (data not shown). Rabbit fibrinogen (200 μg/mL) did not antagonize the antiplatelet effects of dicentrine. Dicentrine did

Table 2. Effect of dicentrine on thromboxane B_2 formation induced by arachidonic acid, collagen and thrombin in washed rabbit platelets

	Thromboxane B_2 (ng/10^8 platelets)					
Inducer	Control	30 μM	60 μM	150 μM	225 μM	300 μM
Arachidonic acid (100 μM)	445 ± 44 (12)	250 ± 22* (4)	122 ± 67† (5)	19 ± 15† (5)		
Collagen (10 μg/mL)	478 ± 93 (8)		175 ± 29* (4)	70 ± 5‡ (4)	14 ± 6† (4)	
Thrombin (0.1 U/mL)	131 ± 25 (6)			44 ± 14* (4)		21 ± 10‡ (3)

Various concentrations of dicentrine or DMSO (0.5%, control) were preincubated with platelets at 37° for 3 min, and 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 = 3–12).

*–‡ Significantly different from the respective control: * $P < 0.05$, † $P < 0.001$ and ‡ $P < 0.01$.

Table 3. Inhibitory effect of dicentrine on thromboxane B_2 formation caused by arachidonic acid in lysed platelet homogenate

	Thromboxane B_2 (ng/10^8 platelets)
Resting	2.2 ± 0.1
DMSO + AA	31.7 ± 3.6
Indomethacin + AA	7.9 ± 0.5*
Dicentrine	
30 μM + AA	24.4 ± 1.6
60 μM + AA	16.0 ± 1.4*
150 μM + AA	13.9 ± 1.3*
225 μM + AA	8.5 ± 0.4*
DMSO + collagen	7.3 ± 1.1*

Lysed platelet homogenate was preincubated with dicentrine (30–225 μM), indomethacin (20 μM) or DMSO (0.5%) for 3 min; then arachidonic acid (AA, 100 μM) or collagen (10 μg/mL) was added and the homogenate was incubated for another 6 min. EDTA (2 mM) and indomethacin (50 μM) were used to terminate the reaction. Values are means ± SEM (N = 6).

* $P < 0.001$ compared with the control value (DMSO + AA).

Table 4. Effect of dicentrine on prostaglandin D_2 formation induced by arachidonic acid in washed rabbit platelets

	Prostaglandin D_2 (pg/10^8 platelets)
Resting	< 10
DMSO + AA	414 ± 115
Indomethacin + AA	< 10
Imidazole + AA	3958 ± 1081*
Dicentrine	
150 μM + AA	18 ± 2*
300 μM + AA	35 ± 2*

DMSO (0.5%), indomethacin (50 μM), imidazole (0.5 mM) or various concentrations of dicentrine were 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 arachidonic acid. Values are means ± SEM (N = 4).

* $P < 0.01$ compared with the control (DMSO + AA).

not suppress fibrinogen-induced aggregation of elastase-treated platelets (data not shown).

Effect of dicentrine on thromboxane B_2 formation. Thromboxane B_2 formation in washed rabbit platelets was measured at 6 min after the aggregation inducer was added. The thromboxane B_2 formation caused by arachidonic acid or collagen was inhibited by dicentrine (30–225 μM) (Table 2), and this inhibition was concentration dependent and paralleled its inhibitory effect on aggregation. However, dicentrine did not affect the basal level of thromboxane B_2 (data not shown). Thromboxane B_2 formation in rabbit platelets is not caused by ADP or PAF [8, 15]; thus, the formation in ADP- or PAF-treated platelets was not measured. Dicentrine also inhibited thromboxane B_2 formation caused by the incubation of the lysed platelet homogenate with arachidonic acid (Table 3). Under this condition, collagen did not cause marked formation of thromboxane B_2 as compared with that in the intact platelets. In washed rabbit platelets, PGD_2 formed in the presence of arachidonic acid. This PGD_2 formation was inhibited by indomethacin and dicentrine, but enhanced markedly by imidazole (Table 4).

Effect of dicentrine on the breakdown of phosphoinositides. Phosphoinositide breakdown has been observed in platelets activated by many agonists [16–18]. As shown in Fig. 3, thrombin (0.1 U/mL), collagen (10 μg/mL) and PAF (2 ng/mL) (in the presence of indomethacin) increased inositol monophosphate formation 4.2 ± 0.4-, 2.8 ± 0.2- and 2.4 ± 0.3-fold, respectively, compared with that of resting platelets. However, the formation of inositol monophosphate caused by these agonists was decreased slightly but not significantly by dicentrine (300 μM) (Fig. 3).

Effect of dicentrine on the intracellular calcium of platelets. In quin-2/AM-loaded platelets, thrombin, PAF, arachidonic acid and collagen caused an increase of intracellular free calcium. As shown in Fig. 4, the rise of intracellular Ca^{2+} caused by these four inducers was inhibited almost completely by dicentrine (300 μM).

Effect of dicentrine on the cyclic AMP level of platelets. The level of cyclic AMP in unstimulated

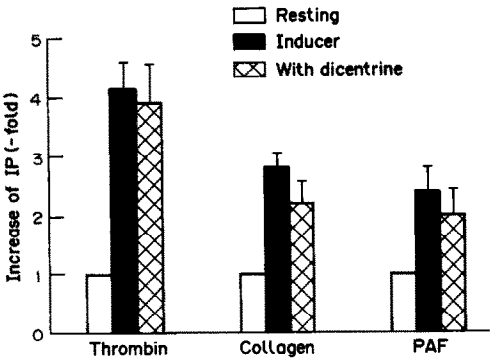

Fig. 3. Effect of dicentrine (300 μM) on the formation of inositol monophosphate caused by thrombin, collagen and PAF in washed rabbit platelets. [^{3}H]Inositol-labeled platelets were incubated with thrombin (0.1 U/mL), collagen (10 μg/mL) or PAF (2 ng/mL) in the presence of indomethacin (20 μM) for 6 min. Fold-increases of inositol monophosphate (IP) are presented as means ± SEM (N = 4). The cpm value of IP (1-fold value) was 3165 ± 100 for the resting platelets.

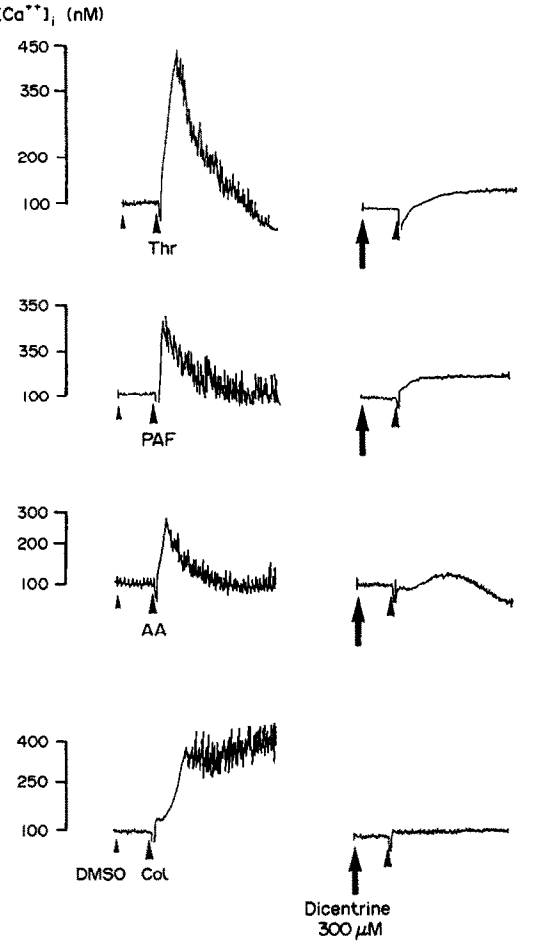

Fig. 4. Effect of dicentrine on the increase of intracellular calcium concentration ($[Ca^{2+}]_i$) in rabbit platelets caused by four inducers. Washed quin-2-loaded platelets were preincubated with dicentrine (300 μM) for 3 min at 37°, then thrombin (0.1 U/mL), PAF (2 ng/mL), AA (100 μM) or collagen (10 μg/mL) was added.

platelets was very low (0.3 ± 0.2 pmol/10^8 platelets). PGE_1 concentrations of 1 and 10 μM increased the cyclic AMP levels to 2.2 ± 0.6 and 6.0 ± 0.7 pmol/10^8 platelets, respectively. A 150 μM concentration of dicentrine had no significant effect on platelet cyclic AMP level, while higher concentrations of dicentrine (225 and 300 μM) elevated it (Table 5).

Deaggregation of platelets by dicentrine. At various times after the platelet aggregation triggered by thrombin, PAF or collagen, dicentrine (300 μM) and PGE_1 (10 μM) deaggregated the clump of platelets. The earlier the addition, the more rapid its reversal action on the platelet aggregation (Fig. 5).

DISCUSSION

The present study shows that dicentrine is a reversible inhibitor of platelet aggregation induced by a variety of agonists. Its mechanism of action includes direct inhibition of thromboxane formation and increase of cyclic AMP level.

It is well known that thromboxane A_2 is an important mediator of aggregation and release reaction of platelets [19]. The aggregation and ATP release of platelets induced by arachidonic acid are due to thromboxane A_2 formation [20]. It has been reported that phosphoinositide breakdown is another important pathway, and it may be a primary event in agonist-induced platelet activation [21, 22]. This phosphoinositide breakdown in platelets may provide a source of free arachidonate via the diglyceride lipase pathway [23] leading to thromboxane A_2 formation. Arachidonic acid also can be liberated by phospholipase A_2 from membrane phospholipids [24]. As shown in Tables 2–4 and Fig. 3, the formation of thromboxane B_2 and of PGD_2, but not phosphoinositide breakdown, was inhibited by dicentrine. These results indicate that a lower concentration of dicentrine (60–150 μM) inhibits platelet aggregation and release reaction via the

Table 5. Effect of dicentrine on platelet cyclic AMP level

	cAMP (pmol/10^8 platelets)
Resting	0.3 ± 0.2 (10)
PGE_1	
1 μM	2.2 ± 0.6* (4)
10 μM	6.0 ± 0.7† (9)
Dicentrine	
150 μM	0.3 ± 0.3 (4)
225 μM	1.7 ± 0.4* (8)
300 μM	4.2 ± 0.5† (9)

Various concentrations of PGE_1 or dicentrine or DMSO (0.5%, resting) were preincubated with platelets at 37° for 3 min; then cAMP formation was stopped by adding 10 mM EDTA and immediate boiling for 5 min. Values are means ± SEM (N = 4–10).

*,† Significantly different from the resting value: * $P < 0.05$ and † $P < 0.001$.

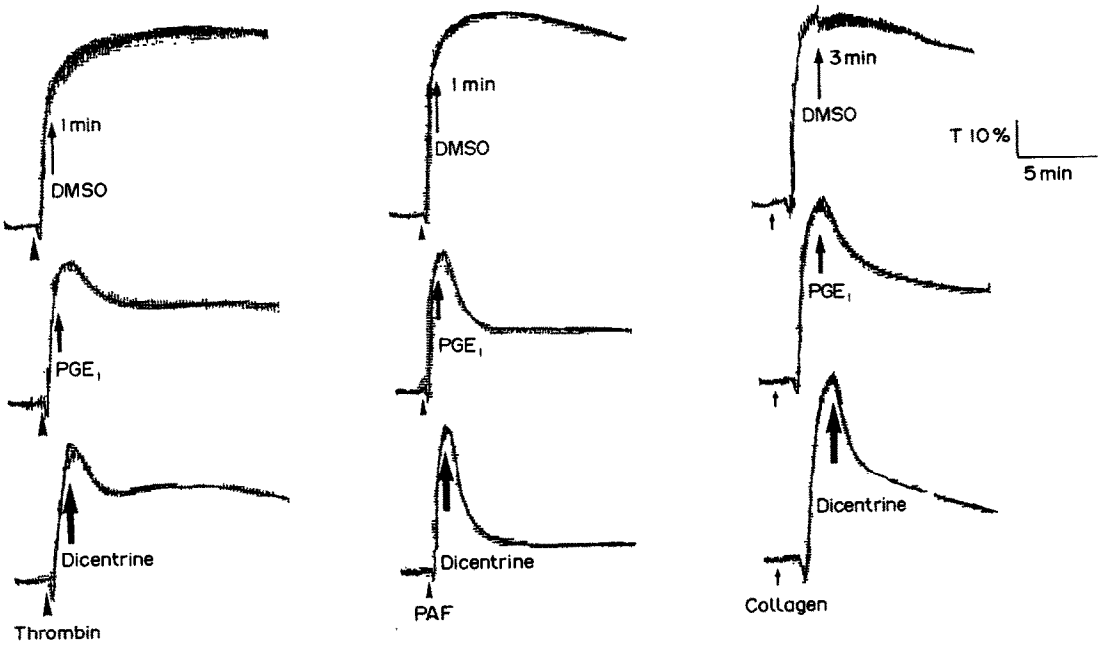

Fig. 5. Reversal effect of PGE_1 and dicentrine on the aggregation of washed rabbit platelets. Platelet aggregation was induced by thrombin (0.1 U/mL), PAF (2 ng/mL) for 1 min, or collagen (10 μg/mL) for 3 min; then DMSO (0.5%), PGE_1 (10 μM) or dicentrine (300 μM) was added.

direct inhibition of thromboxane formation. Similar to indomethacin, but not to imidazole, PGD_2 formation in the presence of arachidonic acid was inhibited by dicentrine. Thus, it may be an inhibitor of cyclooxygenase, instead of thromboxane synthetase. However, the IC_{50} of dicentrine on arachidonate-induced aggregation was about 70 μM and 70 times less potent than indomethacin. The action of dicentrine is different from that of a thromboxane A_2 receptor antagonist, such as SQ29,548 which fails to alter cyclooxygenase, thromboxane synthetase or adenylate cyclase activities [25]. The inositol monophosphate formation caused by collagen, thrombin and PAF was not inhibited significantly by dicentrine (300 μM) (Fig. 3). This result indicates that the inhibition by higher concentrations of dicentrine (225–300 μM) of platelet aggregation caused by thrombin, PAF and collagen is not due to the suppression of phosphoinositide breakdown. Nor did dicentrine suppress fibrinogen-induced aggregation of elastase-treated platelets, indicating that it did not interfere with the fibrinogen–platelet interaction.

In addition to its inhibition of thromboxane formation, the higher concentrations of dicentrine (225–300 μM) also elevated the cyclic AMP content of platelets (Table 5). The importance of cyclic AMP in modulating platelet reactivity is well established [26]. Elevated cyclic AMP inhibits most platelet responses, including a decrease of the intracellular Ca^{2+} concentration by the uptake of Ca^{2+} into the dense tubular system or extrusion of Ca^{2+} from cells [27, 28]. Our experimental data showed that dicentrine (300 μM) inhibited the rise of intracellular Ca^{2+} caused by those inducers. However, the inhibition of thromboxane formation was not due to the increase of cyclic AMP because thromboxane formation in lysed platelet homogenate incubated with arachidonic acid still could be inhibited by dicentrine. Whether the increase of cAMP formation by dicentrine is due to direct activation of adenylate cyclase or inhibition of phosphodiesterase needs further investigation. However, it is not due to the activation of adenylate cyclase by PGD_2 which was decreased by dicentrine. Dicentrine did not cause any significant lysis of platelets and its antiplatelet effect was easily washed out and was not more pronounced when the incubation time was increased. All these data seem to suggest that this inhibition was not due to cell damage and may suggest the potential use of dicentrine for preventing thrombosis in cardiovascular therapy.

It is concluded that the antiplatelet effect of dicentrine is due to the inhibition of thromboxane formation and the increase of cAMP level.

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