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Antiplatelet Effect of Marchantinquinone, Isolated from *Reboulia hemisphaerica*, in Rabbit Washed Platelets

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Abstract

Platelet activation is involved in serious pathological situations, including atherosclerosis and restenosis. It is important to find efficient antiplatelet medicines to prevent fatal thrombous formation during the course of these diseases.

Marchantinquinone, a natural compound isolated from *Reboulia hemisphaerica*, inhibited platelet aggregation and ATP release stimulated by thrombin ($0{\cdot}1\,\text{units}\,\text{mL}^{-1}$), platelet-activating factor (PAF; $2\,\text{ng}\,\text{mL}^{-1}$), collagen ($10\,\mu\text{g}\,\text{mL}^{-1}$), arachidonic acid ($100\,\mu$M), or U46619 ($1\,\mu$M) in rabbit washed platelets. The IC50 values of marchantinquinone on the inhibition of platelet aggregation induced by these five agonists were $62{\cdot}0 \pm 9{\cdot}0$, $86{\cdot}0 \pm 7{\cdot}8$, $13{\cdot}6 \pm 4{\cdot}7$, $20{\cdot}9 \pm 3{\cdot}1$ and $13{\cdot}4 \pm 5{\cdot}3\,\mu$M, respectively. Marchantinquinone inhibited thromboxane B_2 (TxB_2) formation induced by thrombin, PAF or collagen. However, marchantinquinone did not inhibit TxB_2 formation induced by arachidonic acid, indicating that marchantinquinone did not affect the activity of cyclooxygenase and thromboxane synthase. Marchantinquinone did inhibit the rising intracellular Ca^{2+} concentration stimulated by the five platelet-aggregation inducers. The formation of inositol monophosphate induced by thrombin was inhibited by marchantinquinone. Platelet cAMP and cGMP levels were unchanged by marchantinquinone.

The results indicate that marchantinquinone exerts antiplatelet effects by inhibiting phosphoinositide turnover.

Platelets can be activated by many agonists such as thrombin, collagen, platelet-activating factor (PAF), arachidonic acid or adrenaline. When platelets are activated by aggregation agonists, they undergo shape changes, release reactions and subsequently express fibrinogen receptors on their membrane surface (Bennet & Vilaire 1979). The fibrinogen binding to its specific platelet receptors associated with glycoprotein IIb/IIIa complex appears to be a prerequisite for platelet aggregation (Ruggeri et al 1982).

The activation of platelets is counter-regulated by processes that attenuate or prevent agonist-induced responses. Agents can inhibit platelet activation by many mechanisms. There are receptor antagonist mechanisms that are intrinsic to the platelet, produced by normal agonist-induced signal pathways possibly to attenuate or terminate the initiating stimulus. These include the lipoxygenase metabolites, lipocortin and protein kinase C. Moreover, elevated concentrations of platelet cyclic nucleotides (cAMP and cGMP) can dampen platelet aggregation, ATP release and calcium mobilization. These inhibitory actions seem to be dependent on the effects of protein kinase A or protein kinase G (Walter et al 1993). Agents interfering with G-protein and phospholipase C or affecting calcium mobilization by platelet agonists also inhibit platelet activation.

The physiological and pathological roles of platelets have been well established (Arocha-Pinango et al 1999). Platelets are involved in normal haemostatic processes and in cerebral vascular diseases such as transient ischaemic attack (Genton et al 1977; Sherman & Hart 1986), ischaemic heart diseases such as myocardial infarction (Trip et al 1990) and peripheral vascular diseases (Genton et al 1986). Consequently, proper inhibition of platelet activation by medicines is thought to be a useful method for the prophylaxis and treatment of these

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diseases (Karsten 1996). Whilst studying anti-platelet agents we have found that marchantinquinone, isolated from the liverwort plant, *Reboulia hemisphaenica*, inhibited platelet aggregation and ATP release. In this study we have assessed the inhibitory activity of marchantinquinone on platelet aggregation.

Materials and Methods

Materials

Marchantinquinone (Figure 1) was isolated from the liverwort, *Reboulia hemisphaerica* (Linn.) Raddi, as described previously (Wei & Wu 1991) and was dissolved in dimethylsulphoxide (DMSO). Collagen (type I, bovine Achilles tendon; Sigma Chemical Co., St Louis, MO) was homogenized in 25 mM acetic acid and stored at −20°C at a concentration of 1 mg mL^{-1}. Thrombin (bovine; Parke Davis and Co., Detroit, MI) was dissolved in 50% glycerol to give a stock solution of 100 NIH units mL^{-1}. Platelet-activating factor (PAF; Sigma) was dissolved in chloroform and diluted into 0·1% bovine serum albumin saline (BSA) solution immediately before use. Arachidonic acid, ethylenediaminetetraacetic acid (EDTA, disodium salt), luciferase-luciferin, DMSO, Dowex-1 (100–200 mesh: × 8, chloride) resin, myoinositol, indomethacin, U46619, and trichloroacetic acid were purchased from Sigma. Thromboxane B_2 (TxB_2), cAMP, and cGMP enzyme immunoassay kits were obtained from Cayman Chemical Co. (Ann Arbor, MI). Myo-[2-^{3}H]inositol (10–20 Ci $nmol^{-1}$; 1 Ci = 37 GBq) was obtained from Amersham (Arlington Heights, IL).

Platelet preparation

Blood was collected from the marginal ear vein of New Zealand White rabbits. The blood was then anticoagulated with sodium citrate (3·8%, 1 : 14) and centrifuged for 10 min at 90 *g* at room temperature; platelet-rich plasma (PRP) was then obtained from the upper portion. The platelet suspension was obtained from EDTA-anticoagulated PRP according to the washing procedure described by Teng et al (1987). Platelet numbers were counted by a cell counter (Hemalaser 2, Sebia, France) and adjusted to 3×10^8 platelets mL^{-1}. The platelet pellets were resuspended in Tyrode solution of 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%). All glassware was siliconized.

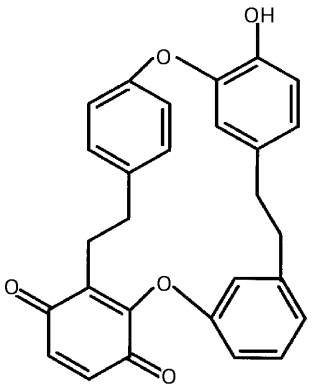

Figure 1. Chemical structure of marchantinquinone.

Platelet aggregation and ATP release

Platelet suspension stirred at 900 rev min^{-1} was incubated with solvent or inhibitor at 37°C for 3 min. The aggregation inducer was then added to trigger the aggregation. Aggregation was measured by the turbidimetric method (Born & Cross 1963). The absorbance of the platelet suspension was taken as 0% aggregation and that of the Tyrode solution as 100% aggregation. ATP released from platelets was detected by a bioluminescence method (DeLuca et al 1979) using a known ATP concentration to calibrate. The aggregation and release of ATP were simultaneously measured by a Lumiaggregometer (model 1020, Payton, Stouffville, Ontario, Canada) connected to two dual-channel recorders. When DMSO was used as the solvent the final concentration was fixed at 0·5% (v/v) to eliminate the effect of the solvent.

Thromboxane B_2 assay

Six minutes after the challenge of platelets with the aggregation inducer, 2 mM EDTA and 50 mM indomethacin were added to stop thromboxane formation. After centrifugation in an Eppendorf microcentrifuge (model 5414) for 2 min, the TxB_2 content of the supernatant was assayed by enzyme immunoassay kits.

Measurement of intracellular calcium ($[Ca^{2+}]i$) in platelets

The method of Pollock & Rink (1986) was followed. Platelets (3×10^8 platelets mL^{-1}) were incubated with fura-2/AM (5 μM) at 37°C for 45 min and centrifuged at 500 *g*. The resultant pellets were washed with Tyrode solution containing 1 mM EDTA. After centrifugation, platelets were resuspended in Tyrode solution containing Ca^{2+} (1 mM). Fluorescence (excitation 339 nm, emission 500 nm) was measured with a Hitachi

fluorescence spectrophotometer (model F4000; Tokyo, Japan) at 37°C. At the end of the experiment, the cells were treated with Triton X-100 (0·1%) followed by the addition of EGTA (10 mM) 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 224 nM.

cAMP and cGMP assay

Platelet suspension (10^9 platelets mL^{-1}) was warmed at 37°C for 1 min. Various agents were then added to the suspension with incubation for 45 s. The reaction was stopped by the addition of 10 mM EDTA followed immediately by boiling for 2 min. Upon cooling to 4°C, precipitated protein was sedimented by centrifugation in an Eppendorf microcentrifuge (mode 5414). The supernatant was assayed for cAMP or cGMP content by enzyme immunoassay.

Labelling of membrane phospholipids and measurement of [^{3}H]inositol monophosphate production

The method used was modified from those of Huang & Detwiler (1986) and Neylon & Summers (1987). EDTA-PRP was centrifuged at 500 *g* for 10 min; the platelet pellets were suspended in 1 mL Ca^{2+}-free and BSA-free Tyrode solution containing 75 μCi mL^{-1} [^{3}H]inositol and 1 mM EDTA. After incubation for 2 h at 37°C, the platelets were collected by centrifugation (500 *g* for 4 min) and suspended in Tyrode solution containing 1 mM Ca^{2+}. The reaction was carried out at 37°C for 6 min with 1 mL platelet suspension in a 3·5-mL cuvette with a stirring bar driven at 900 rev min^{-1}. 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 supernatant was aspirated and trichloracetic acid was removed by extracting with 5 × 2 vols 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 (Neylon & Summers 1987). All the experiments were carried out in the presence of 5 mM LiCl to inhibit monophosphate phosphatase. The levels of inositol bisphosphate and inositol trisphosphate were very low, and so the level of inositol monophosphate was measured as an index of the total inositol phosphate formation.

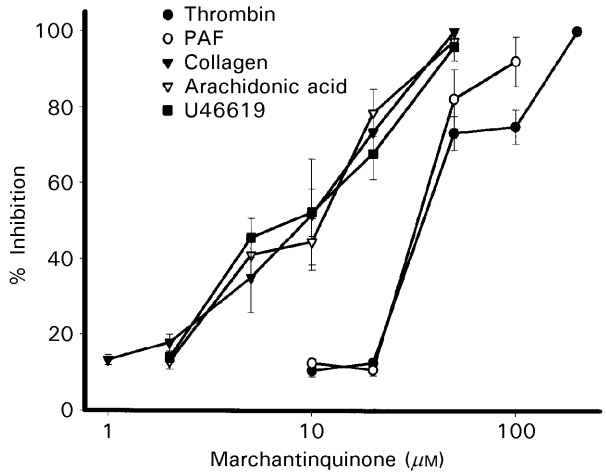

Figure 2. Concentration-dependent inhibition by marchantinquinone of platelet aggregation induced by thrombin (0·1 units mL^{-1}), PAF (2 ng mL^{-1}), collagen (10 μg mL^{-1}), arachidonic acid (100 μM) or U46619 (1 μM). Washed rabbit platelets were incubated with DMSO (0·5%, control) or various concentrations of marchantinquinone at 37°C for 3 min, then the inducer was added to trigger the aggregation. Values are expressed as percent inhibition of the aggregation and are presented as means ± s.e.m. (n = 6).

Results

Effects of marchantinquinone on platelet aggregation and ATP release

Thrombin (0·1 units mL^{-1}), PAF (2 ng mL^{-1}), collagen (10 μg mL^{-1}), arachidonic acid (100 μM), and U46619 (1 μM) caused approximately 90% aggregation in rabbit washed platelets. Marchantinquinone inhibited these effects in a concentration-dependent manner, with IC50 values of 62·0 ± 9·0, 86·0 ± 7·8, 13·6 ± 4·7, 20·9 ± 3·1 and 13·4 ± 5·3 μM, respectively (Figure 2). Marchantinquinone also inhibited ATP release induced by the five inducers. Thrombin induced approximately 90% platelet aggregation and ATP release, but the addition of marchantinquinone inhibited both responses in a concentration-dependent manner and inhibition of ATP release was parallel to the inhibition of platelet aggregation (Figure 3).

Effects of marchantinquinone on TxB_2 formation

TxB_2 formation in rabbit platelet suspension was 0·5 ± 0·1 ng mL^{-1} in the unstimulated condition. Table 1 gives the TxB_2 formation of platelets. U46619 failed to raise the TxB_2 level significantly, whereas arachidonic acid, collagen, thrombin, and PAF increased TxB_2 formation to 294·5 ± 22·6, 63·7 ± 9·2, 58·9 ± 11·0, and 73·4 ± 22·4 ng mL^{-1}, respectively. Marchantinquinone inhibited TxB_2 formation caused by collagen, thrombin and PAF in a concentration-dependent manner. However,

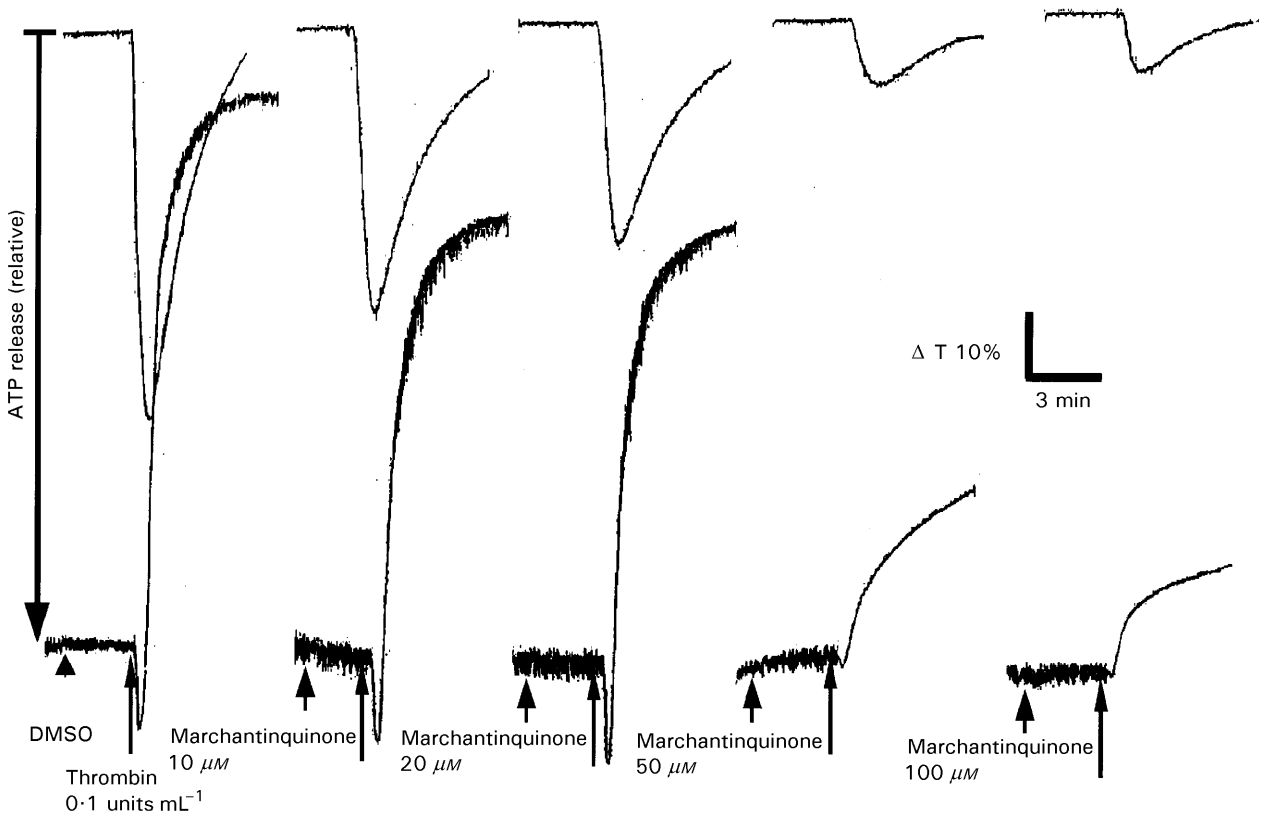

Figure 3. Inhibitory effect of marchantinquinone on thrombin-induced platelet aggregation and ATP release. Washed platelets were pre-incubated with various concentrations of marchantinquinone or DMSO (0·5%, control) at 37°C for 3 min, then thrombin (0·1 units mL^{-1}) was added to trigger the aggregation (upward tracings) and ATP release (downward tracings).

marchantinquinone did not inhibit TxB_2 formation caused by arachidonic acid. Imidazole and indomethacin were chosen as reference drugs (Table 1).

Effects of marchantinquinone on cAMP and cGMP formation

In the unstimulated condition, cAMP and cGMP formation in rabbit platelet suspension was $1·7 \pm 0·5$ and $1·5 \pm 0·5$ pmol mL^{-1}, respectively. 3-Isobutyl-1-methylxanthine (IBMX, 300 μM) increased cAMP and cGMP levels to $7·9 \pm 2·1$ and $8·6 \pm 1·9$ pmol mL^{-1}, respectively. Prostaglandin E_1 (PGE_1; 1 μM) and sodium nitroprusside (10 μM) also increased cAMP and cGMP formation to $20·1 \pm 3·1$ and $169·9 \pm 48·9$ pmol mL^{-1}, respectively. Moreover, IBMX markedly potentiated PGE_1-induced and sodium nitroprusside-induced

Table 1. Effects of marchantinquinone on thromboxane B_2 formation of washed rabbit platelets induced by arachidonic acid, collagen, thrombin, PAF, or U46619.

Group	Thromboxane B_2 (ng mL^{-1})				
	Arachidonic acid	Collagen	Thrombin	PAF	U46619
Control	294·5 ± 22·6	63·7 ± 9·2	58·9 ± 11·0	73·4 ± 22·4	0·5 ± 0·1
Marchantinquinone					
10 μM	306·9 ± 108·6	33·1 ± 8·4*	24·7 ± 7·7*	49·8 ± 24·4	–
20 μM	280·4 ± 64·2	23·1 ± 4·5*	15·4 ± 6·5***	53·7 ± 25·7	–
50 μM	257·3 ± 43·9	8·4 ± 0·2**	5·6 ± 0·9***	3·4 ± 0·9***	1·4 ± 0·5
100 μM			5·1 ± 1·6***	3·5 ± 0·8***	–
Imidazole (1 mM)	54·7 ± 17·6***				
Indomethacin (1 μM)	0·7 ± 0·2***				

The thromboxane B_2 level of resting platelet was $0·5 \pm 0·1$ ng mL^{-1}. Values are presented as means ± s.e.m. (n = 5 or 6). *$P < 0·05$, **$P < 0·01$, ***$P < 0·001$ compared with the respective control. Final concentration: arachidonic acid, 100 μM; collagen, 10 μg mL^{-1}; thrombin, 0·1 units mL^{-1}; PAF, 2 ng mL^{-1}, U46619, 1 μM.

cAMP and cGMP formation (Table 2). Marchantinquinone did not significantly stimulate cAMP or cGMP formation in rabbit washed platelets (Table 2). IBMX did not potentiate marchantinquinone-induced cAMP or cGMP formation (Table 2).

Effects of marchantinquinone on intracellular calcium mobilization

In fura-2-loaded platelets, thrombin, PAF, collagen, arachidonic acid and U46619 caused increased intracellular Ca^{2+} concentration by $170{\cdot}5 \pm 10{\cdot}8$, $107{\cdot}9 \pm 12{\cdot}6$, $78{\cdot}9 \pm 7{\cdot}8$, $65{\cdot}3 \pm 4{\cdot}5$ and $66{\cdot}8 \pm 9{\cdot}5$ nM, respectively (Figure 4). The rise was short-lived, and apparent intracellular Ca^{2+} concentration 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 Figure 4B, marchantinquinone (100 μM) markedly or completely inhibited intracellular calcium mobilization induced by the five platelet inducers.

Effects of marchantinquinone on phosphatidylinositol breakdown

In myo-[^{3}H]inositol-labelled platelets, thrombin (0·1 units mL^{-1}) increased [^{3}H]inositol monophosphate formation to $10{\cdot}7 \pm 1{\cdot}4$-fold. Marchantinquinone (100 μM) decreased [^{3}H]inositol monophosphate formation induced by thrombin ($2{\cdot}3 \pm 0{\cdot}3$-fold).

Discussion

Marchantinquinone inhibited platelet aggregation, ATP release and intracellular calcium mobilization caused by thrombin, PAF, collagen, arachidonic acid or U46619. According to our data, marchantinquinone did not interfere with the interactions between aggregation inducers and their receptors on the platelet membrane surface. Judging from its chemical structure, it is not possible that marchantinquinone is a disintegrin. Thus it may interfere with signal transductions that are commonly activated by these platelet activators.

The aggregation and ATP release of platelets induced by exogenous arachidonic acid are mainly due to TxA_2 formation (Hamberg et al 1975). The half-life of TxA_2 is very short, and so the amount of TxB_2, a stable metabolite of TxA_2, was measured as an index of TxA_2 formation. The aggregation and ATP release caused by exogenous arachidonic acid was inhibited by marchantinquinone, however, the TxB_2 formation was not suppressed. This data indicated that marchantinquinone did not inhibit the activity of cyclooxygenase or thromboxane synthase. Therefore, marchantinquinone-inhibited arachidonic acid-induced platelet activation might be due to the inhibition of TxA_2-induced platelet activation. The data from the marchantinquinone inhibition of platelet activation caused by U46619, a TxA_2 analogue, supports this theory. However, the TxB_2 formation caused by thrombin, collagen, or PAF was inhibited by marchantinquinone. This implies that the possible mode of action of marchantinquinone occurred before the arachidonic acid metabolic process.

PGE_1, sodium nitroprusside or IBMX inhibited platelet aggregation and ATP release caused by thrombin, PAF, collagen, arachidonic acid or U46619 (data not shown). They also caused disaggregation after aggregation was induced by thrombin, PAF, collagen, arachidonic acid or U46619. Elevated cyclic nucleotides (cAMP and cGMP) inhibit most platelet responses, including modulation of the intracellular calcium concentration. Calcium is essential to active myosin light chain kinase for inducing shape change, phospholipase A_2 activation for arachidonic acid release

Table 2. Effects of marchantinquinone, prostaglandin (PGE_1), sodium nitroprusside, and IBMX on the cAMP and cGMP levels of washed rabbit platelets.

Group	cAMP (pmol mL^{-1})	cGMP (pmol mL^{-1})
Resting	$1{\cdot}7 \pm 0{\cdot}5$	$1{\cdot}5 \pm 0{\cdot}5$
IBMX (300 μM)	$7{\cdot}9 \pm 2{\cdot}1$**	$8{\cdot}6 \pm 1{\cdot}9$**
PGE_1 (1 μM)	$20{\cdot}1 \pm 3{\cdot}1$**	–
Sodium nitroprusside (10 μM)	–	$169{\cdot}9 \pm 48{\cdot}9$***
IBMX (300 μM) + PGE_1 (1 μM)	$640{\cdot}4 \pm 62{\cdot}6$***	–
IBMX (300 μM) + sodium nitroprusside (10 μM)	–	$247{\cdot}7 \pm 44{\cdot}6$***
Marchantinquinone 50 μM	$5{\cdot}2 \pm 2{\cdot}2$	$1{\cdot}8 \pm 0{\cdot}4$
Marchantinquinone 100 μM	$2{\cdot}7 \pm 0{\cdot}4$	$1{\cdot}9 \pm 0{\cdot}6$
IBMX (300 μM) + marchantinquinone (100 μM)	$19{\cdot}2 \pm 2{\cdot}6$***	$10{\cdot}3 \pm 2{\cdot}6$***

Values are presented as means ± s.e.m. (n = 6). *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$ compared with the respective resting value.

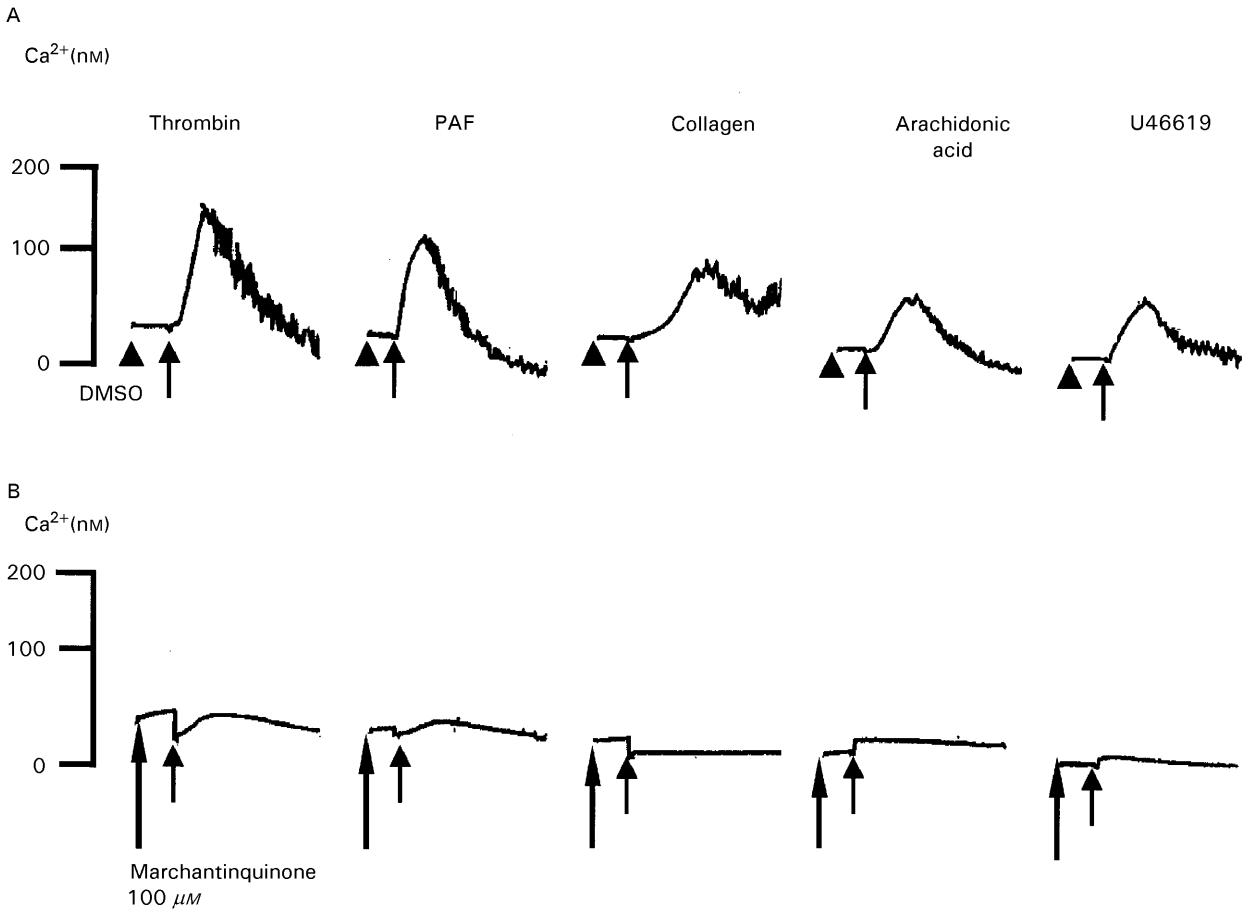

Figure 4. Effect of marchantinquinone on the increase in intracellular calcium concentration in platelets caused by some aggregation inducers. Fura-2-loaded platelets were pre-incubated with (A) DMSO (0·5%) or (B) marchantinquinone (100 μM) at 37°C for 3 min, then thrombin (0·1 unit mL^{-1}), PAF (2 ng mL^{-1}), collagen (10 μg mL^{-1}), arachidonic acid (100 μM) or U46619 (1 μM) was added.

and platelet aggregation. Marchantinquinone inhibited platelet and ATP release and decreased the intracellular calcium concentration caused by thrombin, PAF, collagen, arachidonic acid or U46619. This phenomenon indicated that marchantinquinone may increase the cAMP or cGMP levels in platelet. Marchantinquinone alone did not cause an increase in cyclic nucleotides, neither did it show synergetic effects with IBMX. This data implied that marchantinquinone did not activate adenylate cyclase or guanylate cyclase. We can not exclude that marchantinquinone may have minor inhibitory activity on phosphodiesterase. However, marchantinquinone did not disaggregate clumped platelets (data not shown). This data showed that the inhibitory effect of marchantinquinone on platelet activation was not due to an increase in cyclic nucleotides. According to this result, marchantinquinone did not affect the activity of phosphodiesterase and its inhibitory effect on the intracellular calcium concentration caused by platelet agonists was not due to an increase in cyclic nucleotides.

Another factor affecting platelet intracellular calcium is IP_3 release. IP_3 is a product from the hydrolysis of phosphatidylinositides by phospholipase C and binds to its receptor on dense tubular system to release intracellular calcium (O'Rouke et al 1985). IP_3 release is a common pathway for many platelet activators, therefore, agents that inhibit IP_3 formation can inhibit platelet activation. The short half-life for IP_3 meant that the level of IP, a metabolite of IP_3, was measured as an index of phosphatidylinositide breakdown. In our past experiments, thrombin, PAF, collagen, arachidonic acid or U46619 all increased IP formation (Liao et al 1996). Of the platelet activators studied, thrombin was the most potent agent to increase IP formation, and we found that marchantinquinone inhibited the IP formation caused by thrombin. This

can be interpreted as marchantinquinone inhibiting the increased intracellular calcium caused by thrombin, collagen, PAF, arachidonic acid or U46619.

The results indicated that inhibition of phosphatidylinositide breakdown by marchantinquinone was the mechanism for inhibition of intracellular calcium mobilization and this was also the reason for inhibition of platelet aggregation and ATP release.

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