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The Inhibitory Effect of Cinchonine On Human Platelet Aggregation Due to Blockade of Calcium Influx

Bukhtiar H. Shah, Zafar Nawaz, Salim S. Virani, Imran Q. Ali, Sheikh A. Saeed and Anwar H. Gilani*

Department of Physiology and Pharmacology, The Aga Khan University, Karachi-74800, Pakistan

ABSTRACT. The Cinchona bark contains alkaloids like quinine, quinidine, cinchonine and cinchonidine. These agents are effective antimalarial drugs and have been used clinically in malaria caused by *Plasmodium falciparum*. Previous studies show that quinine and quinidine exert effects on cardiovascular system. This study was conducted to examine the effect of cinchonine on human platelet aggregation. The results show that cinchonine inhibited platelet aggregation mediated by platelet agonists, epinephrine (200 μM), ADP (4.3 μM), platelet activating factor (PAF; 800 nM) and collagen (638 nM) but had no effect on arachidonic acid (AA; 0.75 mM). Cinchonine was most effective in inhibiting aggregation induced by platelet activating factor and epinephrine with IC_{50} values of 125 and 180 μM respectively, however, higher concentrations of cinchonine were required to inhibit aggregation mediated by ADP or collagen (IC_{50}; 300 μM). Pretreatment of platelets with cinchonine inhibited aggregation caused by Ca^{2+} ionophore, A-23187 (6 μM), in a dose-dependent manner (IC_{50}; 300 μM) indicating an inhibitory effect on Ca^{2+}-signaling cascade. This was supported by measuring $[Ca^{2+}]i$ in platelets loaded with Fura-2AM where cinchonine inhibited the rise in cytosolic Ca^{2+} mediated by A-23187 (6 μM) or collagen (638 nM). Results show that cinchonine (20 μM) also inhibited aggregation when platelets were pretreated with protein kinase C (PKC) activator, phorbol myristate acetate (PMA; 0.1 μM) in combination with low doses of platelet activating factor (80 nM). Cinchonine, however, had no effect on AA-induced platelet aggregation and thromboxane A_2 (TXA_2) synthesis in platelets. These results suggest that antiplatelet effects of cinchonine are mediated mainly through inhibition of Ca^{2+}-influx and protein kinase C pathways in platelets. BIOCHEM PHARMACOL **56**;8:955–960, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. calcium channel blocker; human platelets; cinchonine; platelet aggregation; calcium ionophore; calcium influx

The Cinchona bark contains alkaloids like quinine, quinidine, cinchonine and cinchonidine. These agents have been used as effective antimalarial drugs [1]. Due to emergence of drug resistance against these drugs, one of the approaches toward malaria treatment employs the use of combination therapy of these alkaloids or with other drugs [2–4]. Besides quinine and quinidine also exhibit K^+ channel blocking and antiarrhythmic activities [1, 5]. The other unrelated pharmacological effects of these alkaloids include reversal of multidrug resistance in different types of tumors [6]. Cinchonine in this regard has much lower toxicity and greater activity compared to other quinine-related compounds [7].

Previous studies have shown that quinine and quinidine cause conformational changes in platelet function and thus alter their reactivity [8, 9]. Our recent studies have shown that medicinal plants possess antihypertensive and antiplatelet activities mainly through blockade of Ca^{2+} channels [10–13]. While cinchonine was found to exhibit antihypertensive effect possibly through blockade of α-adrenoceptor and Ca^{2+}-channels [11], its effect on platelet have not been studied. The first step in the response of platelets to vascular injury is irreversible attachment to the altered surfaces followed by platelet aggregation. This mainly takes place by the action of endogenous agonists such as AA†, PAF, ADP, collagen and through adhesiveness of platelets to the site of injury. This study was conducted to examine the effect of cinchonine in human platelet aggregation. We show that cinchonine is a potent inhibitor of human platelet aggregation and the effect is mediated mainly through inhibition of calcium signaling cascade.

MATERIALS AND METHODS

Chemicals

ADP, AA, PAF, collagen, epinephrine and cinchonine were purchased from the Sigma Chemical Co. Fura-2 AM

* Corresponding author. Tel. (92) 21 4930051/ext 4565; FAX (92) 21 4934294; E-mail: bukhtiar.shah@aku.edu

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† *Abbreviations*. AA, arachidonic acid; DAG, diacylglycerol; IP_3, inositol triphosphate; PAF, platelet activating factor; PKC, protein kinase C; PLC, Phospholipase C; PMA, phorbol myristate acetate; PRP, platelet-rich plasma; RAFTK, related adhesion focal tyrosine kinase; and TXA_2, thromboxane A_2.

was from Calbiochem Co. (UK). All other chemicals were of the highest purity grade available.

Preparation of Human Platelets

Blood was taken by veinpuncture from normal human volunteers reported to be free of medication for 1 week. Blood samples were mixed with 3.8% (w/v) sodium citrate solution (9:1) and centrifuged at 260 *g* for 15 min at 20° to obtain PRP. Experiments were performed within 2 hr of PRP preparation.

Measurement of Platelet Aggregation

Aggregation was monitored using a Dual-channel Lumi-aggregometer (Model 400 Chronolog Corporation, Chicago, USA) using 0.45-mL aliquots of PRP [14, 15]. The final volume was made up to 0.5 mL with test drug dissolved either in normal saline or appropriate vehicle known devoid of any effect on aggregation. Aggregation was induced with ADP (4.3 μM), AA (0.75 mM), epinephrine (200 μM), PAF (800 nM) or collagen (638 nM). The anti-aggregatory effects of cinchonine were studied by pretreatment of human platelets with cinchonine for one min and then by adding the agonists in varying doses. Like all other dilutions, cinchonine was diluted in normal saline. The resulting aggregation was recorded at 5 min challenge by the change in light transmission as a function of time. To test the calcium channel blocking activity of the cinchonine, platelets were treated with alkaloid for 1 min and then exposed to Ca^{2+}-ionophore, A-23187 (6 μM). Once the anti-platelet activity of the cinchonine against Ca^{2+}-ionophore and other agonists was established, dose-response curves were constructed to calculate the IC_{50} values of the drug.

Meaurement of Cytosolic Ca^{2+}

The agonist-induced influx of Ca^{2+} was measured using Fura-2 AM as described previously [16]. Briefly platelets (1×10^8/mL) were suspended in Ca^{2+}-free standard medium (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes, 10 mM glucose, pH 7.4) and loaded with Fura-2 AM dissolved in DMSO. The cell suspension was centrifuged at 350 *g* for 15 min and cell pellet was resuspended in fresh standard medium. Fura-2 fluorescence was monitored at 340 and 505 (excitation and emission).

AA Metabolism by Platelets

The formation of TXA_2 in platelets was estimated as described previously [17]. The PRP was centrifuged at 1200 *g* for 20 min and the sedimented platelets were washed twice with an ice-cold phosphate buffer (50 mM, pH 7.4) containing sodium chloride (0.15 M) and EDTA (0.2 mM). After centrifugation, washed platelets were resuspended in the same buffer without EDTA and homogenized at 4° using a polytron homogenizer for 15 sec. The homogenate was centrifuged at 1200 *g* for 20 min and 300 μL of the supernatant (containing 0.4 mg of protein) was incubated with 10 μg of unlabelled AA and 0.1 μCi [1-^{14}C]-AA in the presence and absence of the test compound. After 15 min with gentle shaking in air at 37°, the reaction was stopped by adding 0.4 mL of citric acid (0.4 M) and ethyl acetate (7.0 mL). After mixing and centrifuging at 600 *g* for 5 min at 4°, the organic layer was separated and evaporated to dryness under nitrogen. Residues were dissolved in 40 μL of ethanol and 20 μL were applied to silica gel G TLC plates (Analtech). The AA, TXB_2 (a stable degradation product of TXA_2) and 12-HETE standards were spotted separately. The plates were developed in ether/petroleum ether [boiling range 40–60°C/acetic acid (50:50:1 by volume)] to a distance of 17 cm. Radioactive zones were located and quantified by use of a Berthold TLC linear analyzer and chromatography data system (Model LKB 511, Berthold, Germany).

RESULTS

The platelet agonists, PAF, ADP, epinephrine, AA and collagen induced platelet aggregation in a dose-dependent manner in human PRP (not shown). Pre-treatment of platelets with cinchonine for one minute blocked the platelet aggregation mediated by ADP (4.3 μM), epinephrine (200 μM), collagen (638 nM) and PAF (800 nM) in a dose-dependent manner (Fig. 1). However, there was no inhibitory effect of cinchonine up to 800 μM on platelet aggregation induced by AA (0.75 nM) (not shown). Cinchonine showed different half-maximal inhibitory concentrations (IC_{50}) against platelet aggregation induced by various agonists (Table 1). The order of effectiveness of cinchonine in blocking the platelet aggregation induced by these agonists was; PAF > epinephrine > collagen = ADP. The cinchonine was distinctly potent against PAF and epinephrine.

While investigating the mechanism of cinchonine action, platelets pre-treated with cinchonine were exposed to calcium ionophore, A-23187 (6 μM). Cinchonine decreased the aggregatory effect of A-23187 in a concentration-dependent manner as shown in Fig. 2. The IC_{50} of cinchonine against A-23187-induced aggregation was 340 μM. We also measured the changes in the cytosolic Ca^{2+} in platelets stimulated with A23187 and collagen. Results show that pretreatment of platelets with cinchonine reduced the A23187-induced rise in $[Ca^{2+}]i$ as shown in Fig. 3.

PKC has several isoforms, the α-, β- and σ-PKC isoforms being most sensitive to physiological agonists in platelets [18, 19]. Activation of PKC is known to stimulate platelet aggregation in human PRP [10, 16]. Phorbol esters like PMA (an exogenous PKC activator) are frequently used in monitoring effects of PKC in aggregation [20]. Pretreatment of PRP with PMA (0.1–1 μM) for 5 min. resulted in potentiation of aggregation by subthreshold concentration of PAF (80 nM) and such an effect was blocked by low doses (10 μM) of cinchonine (Fig. 4).

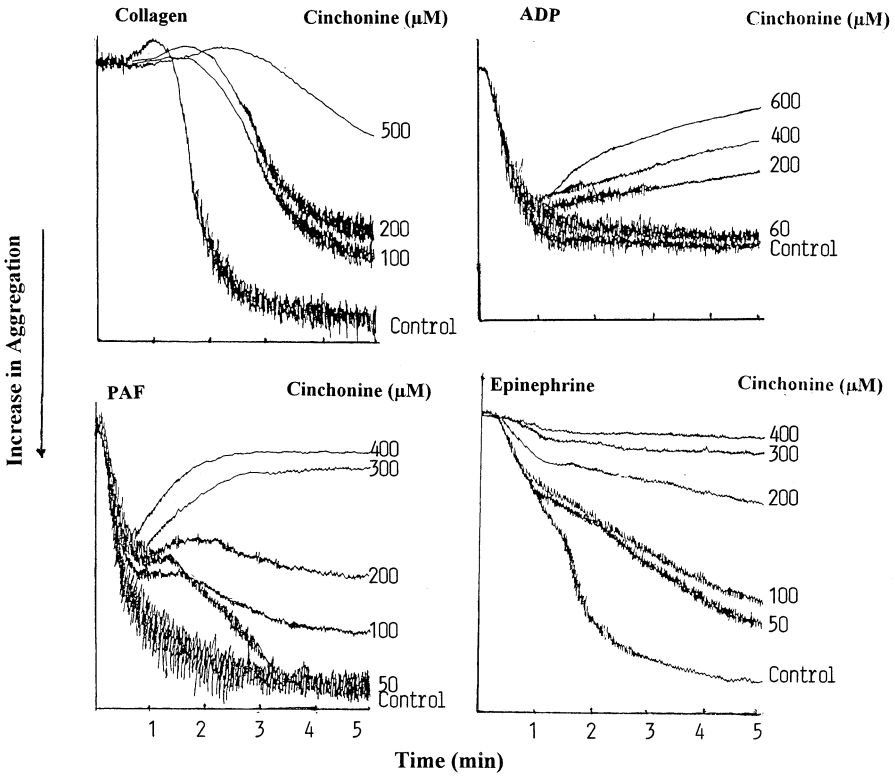

FIG. 1. Cinchonine inhibits platelet aggregation induced by various platelet agonists in a concentration-dependent manner. Data from a typical experiment is presented. The other experiments gave the similar pattern of results. Control represents the aggregating dose of PAF (800 nM), ADP 4.3 (μM), epinephrine (200 μM), and collagen (638 nM). The platelets pretreated with different concentrations of cinchonine for 1 min were challenged with the agonist and the effect recorded for 5 min.

Because platelet activation following AA treatment occurs through production and release of TXA_2 which interacts with receptors on platelets, we measured the effects of cinchonine on TXA_2 production in platelets stimulated with AA. Cinchonine had no effect on TXA_2 formation (data not shown).

DISCUSSION

The Cinchona alkaloids are structurally similar but show quantitatively different effects on various physiological parameters, e.g. effects on neutrophil function [21], multidrug resistance [6, 7], phospholipid synthesis [22] and K^+-channels and cardiac arrhythmias [1]. Quinine specifically interferes with the synthesis of phospholipids in Jurkat T cells [22]. Here we show that cinchonine inhibits platelet aggregation through interference with Ca^{2+} signaling without affecting synthesis of TXA_2.

TABLE 1. Inhibitory effects of cinchonine on human platelet aggregation induced by various platelet agonists

Agonists	Concentration	IC_{50} of cinchonine (μM)
PAF	(800 μM)	125
Epinephrine	(200 μM)	180
Collagen	(638 nM)	300
ADP	(4.3 μM)	310
A23187	(6 μM)	340
AA	(0.75 μM)	NE

IC_{50} values of cinchonine are mean of 5 experimetns for each agonist treatment. NE indicates no effect.

Platelet aggregation is mediated through the action of endogenous agonists like ADP, PAF, epinephrine and collagen and through adhesiveness of platelets to the site of injury. Numerous reports suggest the involvement of Ca^{2+} in major cellular processes including vascular disorders and platelet activation [23–25]. We show that the sensitivity of platelet agonists varied to the inhibitory effect of the cinchonine, PAF and epinephrine mediated aggregation being the most sensitive. Both these agonists directly or indirectly increase cytosolic Ca^{2+} levels [24, 26]. Cinchonine also showed inhibition of platelet aggregation mediated by calcium ionophore, A-23187 in a dose-dependent manner indicating the likely possibility of an effect produced through impairment of Ca^{2+}-influx.

A rise in cytosolic Ca^{2+} levels accompanies platelet activation through stimulation of the enzymes which are not fully functional at the low Ca^{2+} concentration present in the resting platelets [24, 27]. In platelets multiple signaling mechanisms cause an increase in cytosolic Ca^{2+}. These include stimulation of PLC, activation of inhibitory G-protein (Gi) and opening of receptor operated Ca^{2+}-channels [28–30]. Agonists like PAF activate the pertussis toxin-insensitive G-proteins (Gq family) leading to generation of second messenger IP_3 and DAG which in turn cause release of Ca^{2+} from dense tubular system and activation of PKC, respectively [24, 32–35]. Multiple isoforms of both IP_3 receptor and PKC are present in platelets [18, 19, 36]. Defects at any of the signaling cascade can impair the platelet response to agonists. For example Gq protein deficient mice lack platelet aggregation capabilities [37]. Nevertheless, our findings indicate an inhibitory

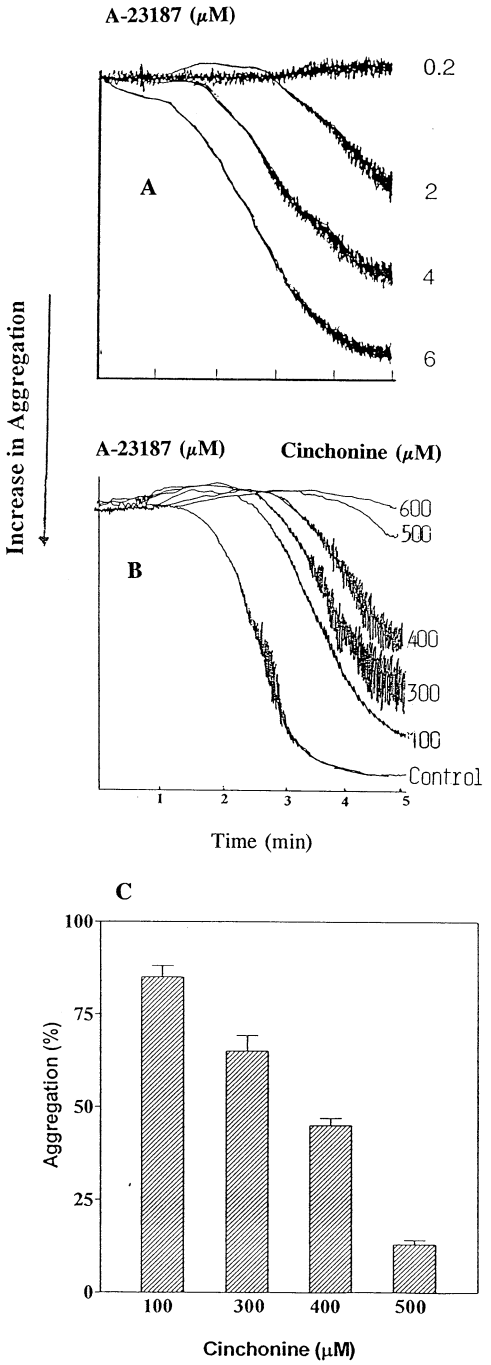

FIG. 2. (A) Dose-response effect of Ca^{2+} ionophore, A-23187 in platelet aggregation. (B) A representative experiment showing the inhibitory effect of cinchonine against A23187-induced aggregation. Platelets pre-treated with cinchonine with varying concentrations as shown in the figure were subjected to treatment with A-23187 (6 μM) which is indicated as control. (C) The cinchonine mediated inhibition of aggregation is quantitated (Mean ± SD; N = 5). A-23187 (6 μM) induced platelet aggregation is taken as 100%.

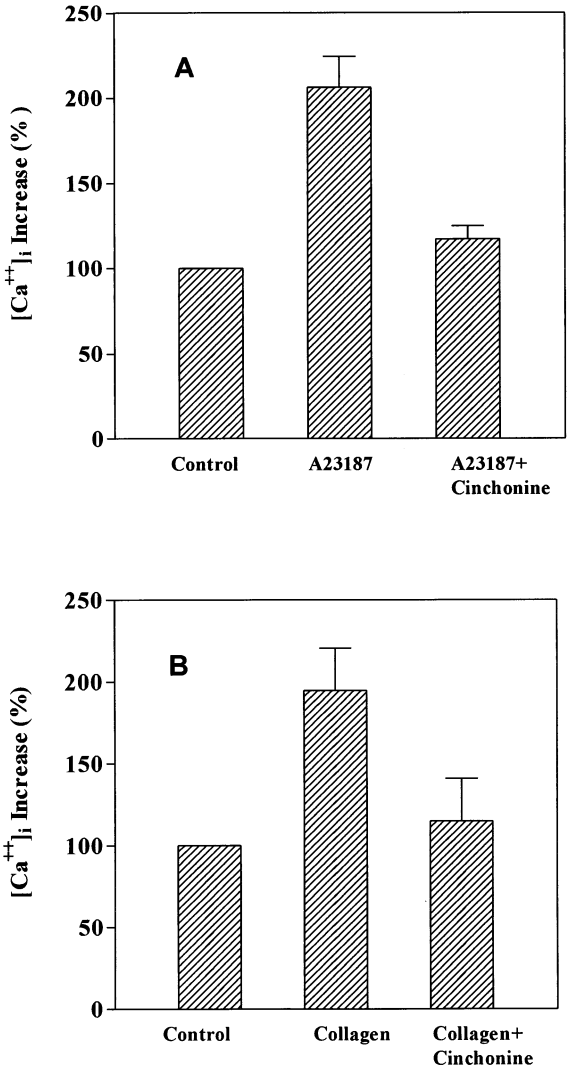

FIG. 3. Effect of cinchonine on A-23187 (6 μM) and collagen (638 nM) induced rise in $[Ca^{2+}]i$. The platelets were loaded with Fura-2 AM and assays done as described in Methods (mean ± SD, N = 3).

action of cinchonine at points distal to any specific receptor or G protein, most likely at Ca^{2+} and PKC activated signaling cascades. Recent studies have shown that PKC acts in synergy with Ca^{2+} to potentiate the aggregation response [10, 18]. Cinchonine blocked the aggregation induced by synergistic interaction of PMA and PAF. This indicates the possible role of PKC in cinchonine-mediated inhibition of aggregation and suggests the presence of some PKC inhibitory activity of cinchonine. Because PKC isoforms in platelets show Ca^{2+} dependency [18], it is likely that PKC inhibition and thus antiplatelet action of cinchonine is mediated indirectly through an effect on Ca^{2+}-signals. Our recent studies show that the synergistic effect of PMA and PAF can also be blocked by diltiazem and nitric oxide donor (SNAP) suggesting the involvement of multiple pathways (unpublished data).

ADP causes a transient increase in IP_3-mediated Ca^{2+} levels and it may eventually lead to store-depleted influx of Ca^{2+} through calcium channels [24, 38, 39]. However, PAF which is known to activate PLC can enhance aggregation through increase in Ca^{2+}-influx via receptor operated Ca^{2+}-channels and such an effect at low concentrations of

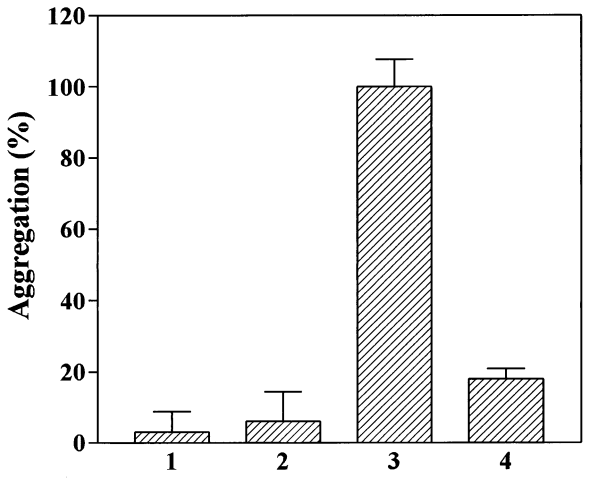

FIG. 4. Cinchonine inhibits platelet aggregation induced by PMA (1 μM) plus low concentrations of PAF (80 nM). 1; PAF (80 nM), 2; PMA (1 μM), 3; PAF (80 nM) + PMA (1 μM), 4; 3 + cinchonine (10 μM). Platelets were pretreated with PMA for 5 min and then other agonists and inhibitors. The maximum aggregation response induced by PAF plus PMA was kept at 100% for comparison with other treatments (mean ± SD, N = 4).

PAF (picomolar) is independent of PLC activation [40]. Our studies provide an indication that cinchonine inhibits the Ca^{2+}-influx in platelets as demonstrated through inhibition of platelet aggregation induced by various agonists and calcium ionophore, A23187.

Cinchonine also inhibited platelet aggregation induced by epinephrine. Epinephrine through interaction with α_2-adrenoceptors, activates Gi protein and inhibits adenylyl cyclase activity, thus causes a decrease in intracellular cAMP levels in platelets [28, 31, 38]. Multiple studies have shown that agents which decrease cAMP levels stimulate platelet aggregation [41, 42]. In fact epinephrine effects in platelets may involve multiple pathways; stimulation of IP_3 production, increase in Ca^{2+}-influx and activation of some other proteins like Syk and RAFTK [43–45]. Besides epinephrine, other platelet agonists like ADP, collagen and A-23187 also induce RAFTK phosphorylation which is regulated by Ca^{2+} and is dependent on PKC. It may be interesting to examine the effect of cinchonine on Syk and RAFTK.

Our recent studies have shown that plant materials contain potent Ca^{2+} channel blockers [9–13] and one of these also possesses PKC inhibitory activity [10]. The role of Ca^{2+} in platelet activation is well established [24–30]. Interruption in the process of Ca^{2+} activation at any point of the cascade can inhibit platelet aggregation. For example, receptor blockers for various platelet agonists (ADP, PAF etc), inactivation or deficiency of Gi and/or Gq protein [37], Ca^{2+}-channel blockers like verapamil and diltiazem [12, 16] or IP_3 receptor antagonist, heparin [36] can interfere in the activation of platelets. *In vitro* experiments have shown that Ca^{2+}-channel blockers interfere with the exflagellation and gaemetogenesis of malaria parasite, *P. falciparum* [46, 47]. Recent efforts to enhance the effectiveness of these antimalarial compounds in patients suffering from resistant malaria include the combination therapy with Ca^{2+}-channel blockers like verapamil and diltiazem [48–49].

Using platelets as a model system, our studies show that cinchonine impairs the Ca^{2+} signaling and further that all the agonists sensitive to the action of cinchonine are linked directly or indirectly with the Ca^{2+} mobilization process [31, 41]. Cinchonine had no effect on AA-induced aggregation and AA metabolism. Moreover, the agonists acting through G-protein linked signaling cascade (epinephrine and PAF) were more sensitive than others like collagen. In conclusion, we show that cinchonine inhibits platelet aggregation and this effect is primarily mediated through an inhibitory effect on Ca^{2+}-signaling cascade in human platelets.

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