Original Research

Calcium-dependent synergistic interaction of platelet activating factor and epinephrine in human platelet aggregation[1]

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KEY WORDS platelet-activating factor; epinephrine; platelet aggregation; diltiazem; verapamil; yohimbine; WEB 2086

ABSTRACT

AIM: To investigate the mechanism(s) involved in the synergistic interaction of platelet activating factor (PAF) and epinephrine. **METHODS:** Blood was obtained from healthy human subjects reported to be free of medications for at least two weeks before sampling. Aggregation was monitored at 37 °C using Dual-channel Lumi-aggregometer. The resulting aggregation was recorded for 5 min by the measurement of light transmission as a function of time. **RESULTS:** Platelet aggregation mediated by subthreshold concentrations of PAF (5-8 nmol/L) plus epinephrine (0.5-2 μmol/L) was inhibited by α_2-receptor blocker, yohimbine, and PAF receptor antagonist WEB 2086. This synergism was inhibited by calcium channel blockers, verapamil and diltiazem. In addition, platelet aggregation by co-addition of PAF and epinephrine was also inhibited by very low concentrations of phospholipase C (PLC) inhibitor (U73122; IC_{50}=0.2 μmol/L), the MAP kinase inhibitor, PD 98059 (IC_{50}=3 μmol/L), and cyclooxygenase (COX-1) inhibitors including indomethacin (IC_{50}=0.25 μmol/L), flurbiprofen (IC_{50}=0.7 μmol/L), and piroxicam (IC_{50}=7 μmol/L). However, COX-2 inhibitors, nimesulide (IC_{50}=26 μmol/L), NS-398 (IC_{50}=7 μmol/L), and etodolac (IC_{50}=15 μmol/L) were also effective in inhibiting the aggregation. The inhibitors of protein kinase C (chelerythrine) and tyrosine kinase (genistien), and phosphatidylinositol 3-kinase inhibitor (wortmannin) had no significant effect on platelet aggregation induced by PAF and epinephrine. **CONCLUSION:** The synergistic effect of PAF and epinephrine on human platelet aggregation is receptor-mediated and involves the activation of PLC/Ca^{2+}, COX and MAP kinase signalling pathways.

INTRODUCTION

In platelets, calcium serves as a second messenger and plays a pivotal role in platelet aggregation[1]. Most of the aggregating agents act largely through stimulation of G-protein coupled receptors (GPCR). Platelet activating factor (PAF), a phospholipid mediator, is a strong platelet activator and human platelets show high affinity binding sites for this agonist. It also enhances adhesion of platelets to the endothelium in the presence of activated leukocytes[2]. It is also known as a potent stimulator of thromboxane A2 (TXA2) production in human platelets. PAF, which is known to increase Ca^{2+} influx via receptor-operated Ca^{2+} channels with such an effect being independent of PLC activation[3]. PAF is known to act through stimulation of

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pertussis toxin insensitive G-proteins (Gq/11) to stimulate phospholipase C (PLC), with a resultant stimulation of protein kinase C (PKC) by diacylglycerol (DAG) and mobilization of intracellular calcium by inositol 1,4,5-triphosphate (IP_3)[4]. Both Ca^{2+} and PKC stimulate platelet aggregation and also elicit synergism in platelets[5]. Epinephrine interacts with α_2-receptors on platelets and activation of these receptors regulates the adenylyl cyclase pathway[6]. Epinephrine-mediated increase in platelet aggregation correlates well with an increase in cytosolic free Ca^{2+} and the process is known to be sensitive to Na^+ concentration[7]. The level of cytosolic Ca^{2+}-influx from outside the cell is an important determinant in the signalling cascade mediating platelet aggregation[1].

Most of the studies show that platelet aggregation can be mediated by synergism of platelet agonists added together at a subthreshold concentration[7]. Low concentrations of platelet agonist such as 5-hydroxytryptamine[8] potentiate the aggregating response of epinephrine. Such a synergism between platelet agonists was found to be mediated through activation of multiple signalling pathways. Moreover, platelet agonists are also known to regulate the arachidonic acid (AA) metabolism by cyclooxygenase (COX) pathway[9,10]. The AA metabolites particularly TXA2, play an important role in platelet aggregation[11,12]. Here we studied the signalling pathways and the role of COX in the synergistic interaction of PAF and epinephrine during human platelet aggregation.

MATERIALS AND METHODS

Materials PAF, epinephrine, yohimbine, verapamil, diltiazem, flurbiprofen, indomethacin, and etodolac were purchased from the Sigma Chemical Co (St Louis, Mo, USA). U73122 was purchased from Alexis LC Labs (UK). Nimesulide and NS-398 were purchased from Cayman Chemical Company (Ann Arbor, MI, USA). WEB 2086 was a kind gift from Boehringer-Ingleheim, Germany. 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 medications for one week. Blood samples were mixed with 3.8 % (w/v) sodium citrate solution (9:1) and centrifuged at 260 ×*g*, 20 °C for 15 min to obtain platelet rich plasma (PRP). Platelet count was determined by phase contrast microscopy and all aggregation studies were carried out at 37 °C with PRP having platelet counts between 2.5 and 3.0×10^{11}/L of plasma[13]. All experiments were performed within 2 h 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[13]. The final volume was made up to 0.5 mL with the test drug dissolved either in normal saline or appropriate vehicle known to be devoid of any effect on aggregation. Aggregation was induced with PAF and epinephrine and their subthreshold concentration were determined. To obtain the synergistic effect of PAF and epinephrine we added low concentrations of these agonists. The anti-aggregatory effects were studied by pretreatment of PRP with various inhibitors for 1 min followed by addition of the subthreshold concentrations of PAF and epinephrine. The resulting aggregation was recorded for 5 min after challenge by the change in light transmission as a function of time[8]. Once the anti-platelet activity of various inhibitors against agonists was established, dose-response curves were constructed to calculate the IC_{50} values of inhibitors. Statistical analysis was done using Student's *t*-test.

Thromboxane formation in platelets Arachidonic acid metabolism and TXA2 formation were studied using a Berthold TLC linear analyzer and chromatography data system (Model LKB 511, Berthold W, Germany) as described previously[14,15]. Protein concentration was determined by the method of Lowry *et al*[16], using human serum albumin as standard.

RESULTS

Both PAF (5-800 nmol/L) and epinephrine (EPI, 0.1-20 μmol/L) showed concentration-dependent increase in platelet aggregation. However, when low doses of PAF (5-8 nmol/L) and epinephrine (0.5-2 μmol/L) were added together, their effect was potentiated and marked aggregation was observed (Fig 1A&B). This synergistic effect of PAF and epinephrine was blocked by α_2-receptor blocker, yohimbine, and PAF-receptor antagonist, WEB 2086, with IC_{50} values of 0.16 and 0.7 μmol/L, respectively (Fig 2A&B). To investigate the possible mechanisms involved in this synergism, selective inhibitors of various signaling pathways were used. Recent studies show that βγ-subunits of activated Gi protein can also activate PLC[17]. Since PAF

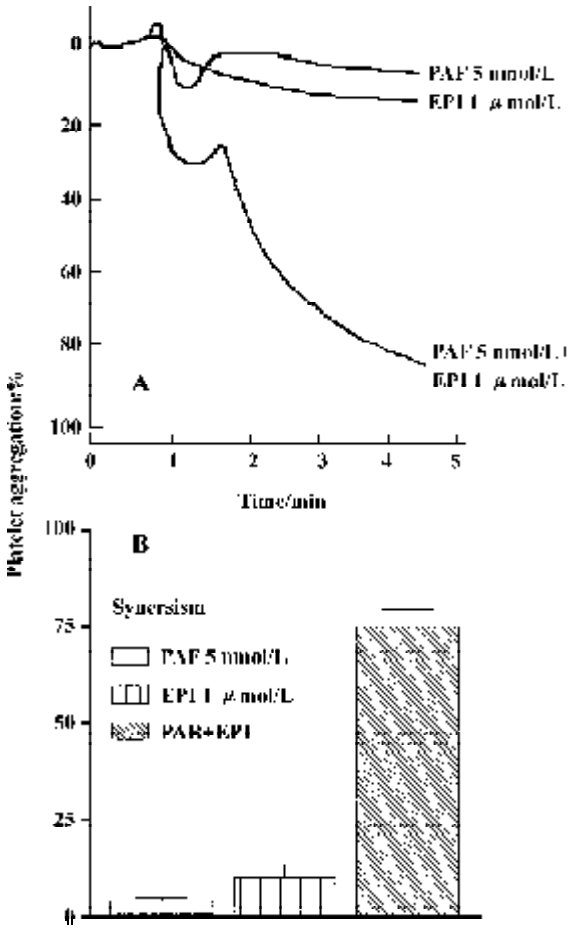

Fig 1. (A) Tracings from representative experiments showing synergism of PAF (5 nmol/L) and epinephrine (EPI, 1 μmol/L) and on human platelet aggregation. PRP was treated with agonists (PAF and epinephrine) and platelet aggregation was recorded for 5 min. (B) Quantitation of the data obtained from synergistic interaction of PAF (5 nmol/L) and EPI (1 μmol/L).

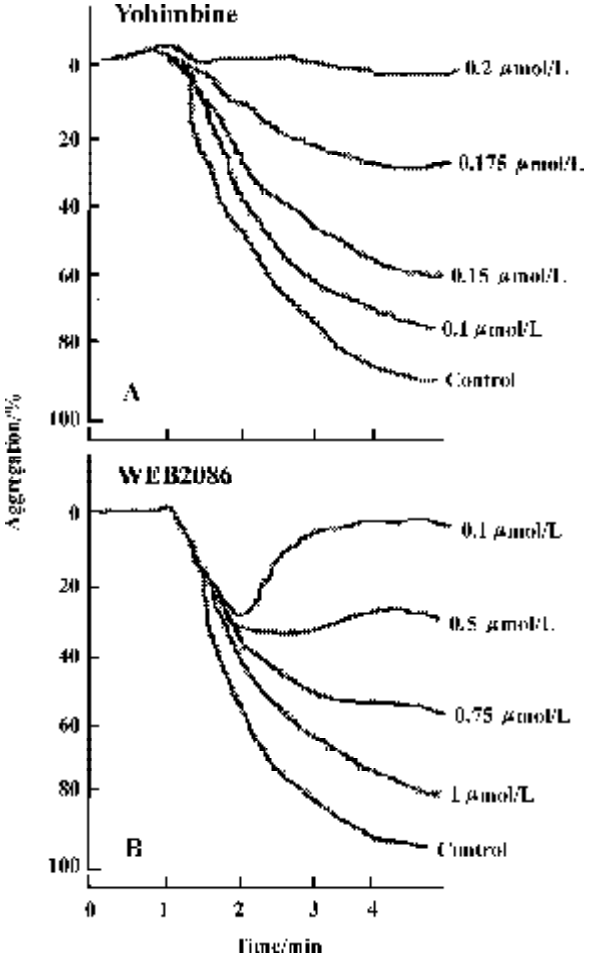

Fig 2. (A) Concentration-dependent effects of a $_2$-receptor antagonist, yohimbine and (B) PAF receptor blocker, WEB2086 on PAF and epinephrine-mediated platelet aggregation. Control means platelet aggregation induced by PAF (5 nmol/L) and epinephrine (1 μmol/L). *n*=5.

and epinephrine were reported to activate Gi protein and Gq/PLC pathway, we used PLC inhibitor U73122 to examine if PAF and epinephrine mediated effect involved activation of PLC. Pretreatment of PRP with U73122 completely inhibited synergistic effect of PAF and epinephrine with an IC_{50} value of 0.2 μmol/L (Fig 3A). Since activation of PLC led to an increase in cytosolic Ca^{2+} due to its release from internal stores by inositol triphosphate (IP_3) or through store-depleted Ca^{2+} influx[18], The effect of Ca^{2+} channel blockers (verapamil and diltiazem) on platelet aggregation was examined. The synergistic effect of PAF and epinephrine was inhibited by both verapamil and diltiazem (IC_{50}=2.5 μmol/L) and verapamil (IC_{50}=0.4 μmol/L) (Fig 3B). This indicated the possible involvement of Ca^{2+} influx in this process.

As stimulation of the G protein/Ca^{2+} cascade led to activation of mitogen-activated protein MAP kinase signaling[19], the selective MEK inhibitor PD98059 was used to examine the role of MAP kinase in PAF and epinephrine synergism. Pretreatment of PRP with PD98059 (IC_{50}=3 μmol/L) showed a strong inhibitory effect on platelet aggregation induced by co-addition of subthreshold concentration of PAF and epinephrine (Tab 1). The inhibitors of other signalling pathways such as genistein (TLCK inhibitor) and chelerythrine kinase (PKC inhibitor) had no effect on platelet aggregation.

To determine the role of cyclooxygenase, a key enzyme involved in the biosynthesis of prostaglandins, various COX-1 and COX-2 inhibitors were used. COX inhibitors (indomethacin: IC_{50}=0.25 μmol/L; flurbiprofen: IC_{50}=0.7 μmol/L; piroxicam: IC_{50}=25 μmol/L) blocked the effect of co-addition of PAF and epinephrine

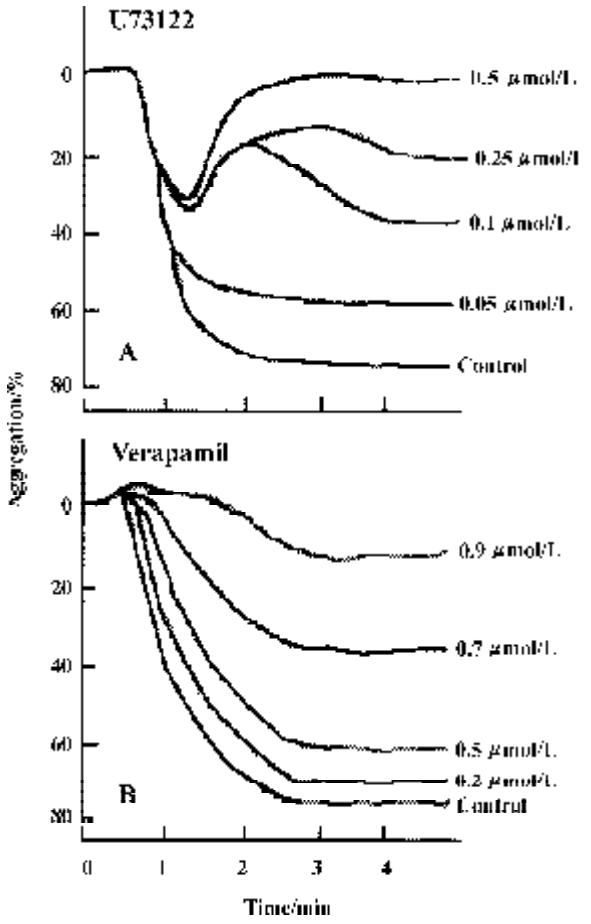

Fig 3. Effects of PLC inhibitor, U73122 (A) and (B) calcium-channel blocker, verapamil, on the synergistic interaction of PAF (5 nmol/L) and epinephrine (1 mmol/L). *n*=7.

(Tab 1). However, interestingly COX-2 inhibitors, nimesulide (IC_{50}=26 μmol/L), NS-398 (IC_{50}=7 μmol/L) and etodolac (IC_{50}=15 μmol/L), also inhibited the aggregation suggesting the possible involvement of COX-2 pathway in human platelets (Fig 4). These results suggested that the synergism between PAF and epinephrine involved the activation of COX pathway in human platelets.

DISCUSSION

The mechanism of synergism among various platelet agonists is reported to occur due to the activation of Ca^{2+} signaling cascade. The results of the present investigation show that the subthreshold concentrations of PAF and epinephrine show synergism during human platelet aggregation and this synergism is inhibited by PLC/Ca^{2+} and COX inhibitors. It has been proposed that the activation of Ca^{2+} signalling cascade with a rise in Ca^{2+} induced by first agonist primes platelets for an

Tab 1. Effects of various inhibitors on platelet aggregation mediated by synergistic interaction of PAF (5 nmol/L) and epinephrine (1 mmol/L). *n*=5-7. Mean±SD. $^bP<0.05$ *vs* control.

Inhibitors	IC_{50}/μmol·L^{-1}	95 % CI
WEB2086	0.7±0.04^b	0.66-0.73
Yohimbine	0.16±0.15^b	0.09-0.24
U73122	0.2±0.10^b	0.15-0.29
Verapamil	0.4±0.05^b	0.38-0.43
Diltiazem	2.5±0.63^b	2.21-2.86
Indomethacin	0.25±0.02^b	0.23-0.27
Flurbiprofen	0.7±0.22^b	0.58-0.81
Piroxicam	25±12^b	19.55-32.36
Nimesulide	26±23^b	14.53-37.86
NS-398	7±1.7^b	6.16-7.97
Etodolac	15±0.6^b	15.29-15.90
PD98059	3±2.0^b	1.05-4.98
Wortmannin	NE	-
Genistein	NE	-
Chelerythrine	NE	-

Data were indicated as half maximal inhibitory effect (IC_{50}) of various agents. IC_{50}=concentration (μmol/L) producing 50 % inhibition of aggregation. IC_{50} was calculated using tests done by 5-7 determinations of the inhibitors. NE=no effect.

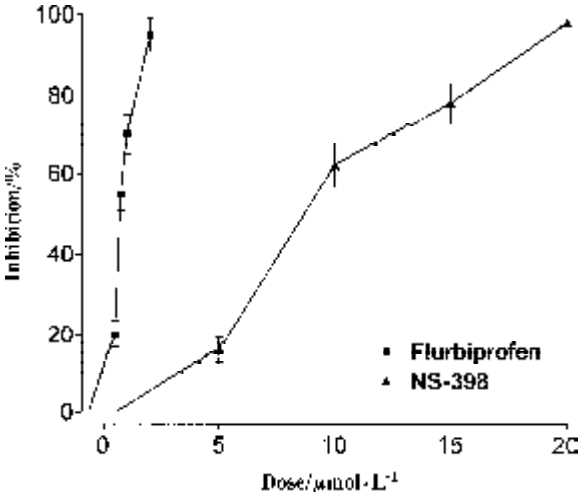

Fig 4. Cyclooxygenase (COX) inhibitor flurbiprofen and NS-398 inhibits platelet aggregation induced by co-addition of subthreshold concentrations of PAF and epinephrine. *n*=9. Mean±SD.

enhanced functional response to second agonist[7,15,20]. Interruption in the process of PLC/Ca^{2+} activation can interfere with the aggregation of platelets[1,8]. Further

support in favour of Gq/PLC pathway is provided by the recent studies in transgenic mice where it is shown that Gq-protein deficient mice lacked the ability of platelet aggregation[21].

Stimulation of Gi protein in platelets through stimulation of α_2-adrenoceptors by epinephrine causes decrease in intracellular cAMP levels, increase in Ca^{2+}-influx and stimulation of phospholipase A_2[22,6]. Further, there is a cross regulation between Gi and Gq protein linked pathways in platelets. Thus, signals originating from α_2-adrenergic receptor stimulation seem to converge with those of PAF at the level of PLC/Ca^{2+}[1,23]. Therefore, inhibition of PLC/Ca^{2+} pathway by PLC inhibitor, U73122, and Ca^{2+}-channel blockers, diltiazem, and verapamil resulted in the inhibition of platelet aggregation induced by co-activation by epinephrine and PAF. A similar mechanism has also been reported for other platelet agonists[1,8,15].

The increase in cytosolic Ca^{2+} causes activation of PLA_2 and stimulation of COX activity, thus TXA2 formation[1]. COX catalyzes the stepwise conversion of AA into reactive intermediates PGG2 and PGH2, which are the precursors of prostaglandins, prostacyclin and thromboxanes (prostanoids). COX-1 is mainly present in platelets and other tissues whereas COX-2 can be induced in other tissues[12]. Numerous studies have shown that inhibitors of COX mainly belonging to the group of non-steroidal anti-inflammatory drugs (NSAID) also inhibit platelet aggregation by inhibiting TXA2 biosynthesis[24]. Our results showed that COX-1 inhibitors, indomethacin, flurbiprofen, and piroxicam blocked the effect of synergistic interaction of epinephrine and PAF. It appears that GPCR-stimulation under our experimental conditions causes activation of COX and the inhibition of latter by selective inhibitors leads to inhibition of aggregation.

It was found that platelet aggregation mediated by co-addition of PAF and epinephrine was inhibited by nimesulide, NS-398, and etodolac, compounds known to inhibit COX-2 activity. Nimesulide inhibits platelet aggregation induced by epinephrine and also inhibits TXA2 formation in platelets[25]. In fact, recent studies have suggested that COX-1 inhibitory effects can be detected for all selective COX-2 inhibitors by a sensitive assay at low substrate concentrations. Nimesulide is reported to be more selective inhibitor for microsomal COX-2, but it is equipotent on both COX-1 and COX-2 in whole cell assay systems[26,27]. Moreover, nimesulide is also known to cause elevation of cytosolic cAMP levels, decrease in Ca^{2+} influx and PKC activity, and inhibition of TXA2 and PAF formation[25]. All these mechanisms are reported to decrease platelet aggregation[1,4]. Therefore, the possibility that the inhibitory effects of nimesulide on the synergistic interaction of PAF and epinephrine are mediated through a COX-independent mechanism can not be ignored.

MAP kinase is one of the downstream signaling molecules involved in platelet aggregation[28], that can be activated by both Gq and Gi-protein linked pathways[19]. We found that selective MEK inhibitor; PD98059 abolished the synergistic effect of PAF and epinephrine suggesting the involvement of MAP kinase, which is considered to lie distal to PLC/PKC[18,28]. However, PD98059 is also a very potent inhibitor of COX-1 activity in platelets[29]. Recent studies show that G protein $\beta\gamma$-subunits mediated α_2-receptor- and Gq/11-mediated $\alpha_1\beta$-adrenoceptor-coupled activation of MAP kinase, converge at the level of PLC[19,30,31]. In fact the $\beta\gamma$-subunit of Gi and Gq proteins have the similar efficacy in the regulation of PLC in human platelets[29]. Our results suggest that convergence of PAF and epinephrine mediated receptor activation leads to activation of multiple sites such as PLC, MAP kinase, and COX during platelet aggregation. Subthreshold concentrations of PAF and epinephrine show synergistic effect through activation of PLC/Ca^{2+} and COX pathways as the effect was blocked by selective inhibitors.

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