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Involvement of thromboxane A_2 and tyrosine kinase in the synergistic interaction of platelet activating factor and calcium ionophore A23187 in human platelet aggregation

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Abbreviations: PAF, platelet activating factor; PKC, protein kinase; PLC, phospholipase C; TXA_2, thromboxane A_2

Abstract

The present study was carried out to examine the mechanisms of the synergistic interaction of PAF and A23187 mediated platelet aggregation. We found that platelet aggregation mediated by subthreshold concentrations of PAF (5 nM) and A23187 (1 μM) was inhibited by PAF receptor blocker (WEB 2086, IC_{50} = 0.65 μM) and calcium channel blockers, diltiazem (IC_{50} = 13 μM) and verapamil (IC_{50} = 18 μM). Pretreatment of platelets with PAF and A23187 induced rise in intracellular calcium and this effect was also blocked by verapamil. While examining the role of the down stream signaling pathways, we found that platelet aggregation induced by the co-addition of PAF and A23187 was also inhibited by low concentrations of phospholipase C (PLC) inhibitor (U73122; IC_{50} = 10 μM), a cyclooxygenase inhibitor (indomethacin; IC_{50} = 0.2 μM) and inhibitor of TLCK, herbimycin A with IC_{50} value of 5 μM. The effect was also inhibited by a specific TXA_2 receptor antagonist, SQ 29548 with very low IC_{50} value of 0.05 μM. However, the inhibitors of MAP kinase, PD98059 and protein kinase C, chelerythrine had no effect on PAF and A23187-induced platelet aggregation. These data suggest that the synergism between PAF and A23187 in platelet aggregation involves activation of thromboxane and tyrosine kinase pathways.

Keywords: calcium ionophore A23187; platelet activating factor; platelet aggregation; thromboxane tyrosine kinase

Introduction

A number of platelet agonists such as epinephrine, 5-HT, ADP, and platelet activating factor (PAF) act synergistically in platelet aggregation and up to date, few studies have been carried out in human platelets on the cooperative effects of PAF and calcium ionophore A23187. It is well documented that most of the platelet agonists act largely through the stimulation of G-protein coupled receptors (GPCRs).

PAF, a phospholipid mediator, is a very strong platelet activator and human platelets show high affinity binding sites for this agonist. It also induces adhesion of platelets to the endothelium in the presence of activated leukocytes (Hirafuji and Shinoda, 1991). PAF is also known to play an important role in various pathophysiological conditions that include modulation of blood pressure, hypotension, cardiac dysfunction, cardiac anaphylaxis, hemorrhagic, traumatic and septic shock syndromes (Anderson *et al*., 1991; Montrucchio *et al*., 2000). Because of its ability to stimulate endothelial migration and angiogenesis, a potential role of PAF is also known as a potent stimulator of thromboxane A_2 (TXA_2) production in human platelets (Shah *et al*., 2001). It is reported that PAF acts through the stimulation of pertussis toxin insensitive G-proteins (Gq/11) resulting in the stimulation of phospholipase C (PLC) and thus generation of second messenger diacylglycerol (DAG) and inositol-1, 4,5-triphosphate (IP_3), which results in the activation of protein kinase C (PKC) and the mobilization of intracellular Ca^{2+}, respectively (Obberghen-Schilling and Pouyssegur, 1993). Both Ca^{2+} and PKC stimulate platelet aggregation and also elicit synergism in platelets (Crabos *et al*., 1992). Consistent with the potential involvement of G_q/PLC pathway, the deficiency of G_q protein in transgenic mice leads to impairment of agonist-induced platelet aggregation (Offermanns *et al*., 1997).

In platelets, calcium plays a pivotal role in platelet aggregation (Heemskerk and Sage, 1994). An increase in cytoplasmic Ca^{2+} can be brought about by either enhanced Ca^{2+} influx from the external medium or release from internal stores (Berridge, 1993). Ca^{2+}

ionophores, such as A23187, induce platelet aggregation. It stimulates the procoagulant activity of cells, which is thought to be mediated by scrambling of the plasma membrane phospholipids. This results in the exposure of phosphatidyl inositol serine and other negatively charged phospholipids in the outer leaflet of the plasma membrane (Williamson *et al*., 1995).

In addition, PAF also stimulates TXA_2 production in human platelets. It enhances vasoconstriction of the coronary arterioles (DeFily *et al*., 1996) and at the inflammatory coronary lesions *in vivo* by itself as well as in a synergistic manner with other agonists like epinephrine and 5-HT (Shah *et al*., 2000; Saeed and Rasheed, 2003). Because of the close interaction between many agonists and their importance in thrombosis, hypertension and atheroscelrosis, this study was conducted to examine the synergism between PAF and A23187 to elucidate the possible signaling mechanism(s) involved during this synergism.

Materials and Methods

Chemicals

PAF, calcium ionophore, A23187, diltiazem, verapamil, herbimycin A, U73122, PD98059 and chelerythrine all were purchased from the Sigma Chemical Co. (St. Louis, Mo). All other chemicals used were of the highest purity grade available.

Preparation of human platelets

Blood was taken by vein-puncture 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 for 15 min at 20°C 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 3.0×10^8 ml^{-1} of plasma (Shah and Saeed, 1995).

Measurement of platelet aggregation

Aggregation was monitored using Dual-channel Lumi-aggregometer (Model 400 Chronolog Corporation, Chicago, IL) using 0.45 ml aliquots of PRP. 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 A23187 and their sub-threshold concentrations were determined. To obtain the synergistic effect of PAF and A23187, we added low concentrations of these agonists. The anti-aggregatory effects of different compounds were studied by pretreatment of PRP with various inhibitors for one min followed by addition of the sub-threshold concentrations of PAF (5 nM) and A23187 (1 μM). The resulting aggregation was recorded for 5 min after challenge by the change in light transmission as a function of time. Once the anti-platelet activity of various inhibitors against these agonists was established, dose-response curves were constructed to calculate the IC_{50} values of inhibitors.

Thromboxane formation in platelets

Arachidonic acid metabolism and TXA_2 formation were studied with the co-addition of PAF (5 nM) and A23187 (1 μM) using radiochemical methods (Shah *et al*., 2000). For these studies, human blood platelets were routinely obtained in plastic bags containing 30-40 ml of concentrated PRP from The Aga Khan University Hospital Clinical Laboratory, Karachi. The PRP was centrifuged at 1,200 *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°C using a polytron homogenizer for 15 s. The homogenate was centrifuged at 1,200 *g* for 20 min and 300 μl of the supernatant (containing 0.4 mg of protein) was incubated with 10 μg unlabelled arachidonic acid (AA) and 0.1 μCi [1-^{14}C]-arachidonic acid in the presence and absence of the agonists. After 15 min of gentle shaking in air at 37°C, 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°C, the organic layer was separated and evaporated to dryness under nitrogen. Residues were dissolved in 40 μl ethanol and 20 μl were applied to silica gel G thin layer chromatography (TLC) plates (Analtech, Delaware). The solvent system used for the separation of TXB_2 in dried organic extracts of platelet incubates as above was ethyl acetate: isooctane: water and acetic acid (11:5:10:2, v/v, upper phase). Radioactive zones were located and quantified by use of a Berthold T.L.C. linear analyzer and chromatography data system (Model LKB 511, Berthold, W. Germany). Protein concentration was determined using human serum albumin as standard (Lowry *et al*., 1951).

Measurement of Ca^{2+} Influx

The agonist-induced influx of Ca^{2+} was measured using Fura-2 AM (Saeed *et al*., 1997). Platelets (1×10^8/ml) were suspended in Ca^{2+}-free standard medium (NaCl 145 mM, KCl 5 mM/L, $MgCl_2$ 1 mM/L, HEPES 10 mM, glucose 10 mM, pH 7.4). Fura-2 AM dissolved in DMSO was added to the platelet sus-

pension at 37°C for 45 min. The platelet suspension was centrifuged at 350 *g* for 15 min and the platelet pellet resuspended in fresh standard medium. Fura-2AM fluorescence was monitored at 340 nm and 505 nm (excitation and emission, respectively) in platelets treated with PAF (5 nM) and A23187 (1 μM).

Table 1. The effect of various inhibitors on subthreshold concentration of platelet activating factor (5 nM) and A23187 (1 μM) induced platelet aggregation.

Inhibitors	Mean IC_{50} μM ± SEM
WEB 2086	0.65 ± 0.04
Diltiazem	13 ± 2.3
Verapamil	18 ± 3.2
U73122	10 ± 0.7
Indomethacin	0.2 ± 0.03
SQ 29548	0.05 ± 0.001
Herbimycin A	5 ± 0.8
PD98056	NE
Chelerythrine	NE

Data is mean ± SEM (n = 5-7) and is indicated as half-maximal effect (IC_{50}) of the inhibitors. NE, No effect.

Data analysis

IC_{50} is the concentration (μM) producing 50% inhibition of platelet aggregation (control response taken as 100%). The 50% inhibitory concentration (IC_{50}) values were calculated as means ± SEM of 5-7 independent experiments. Differences between control and test measurements were assessed by student's *t*-test.

Results

The results demonstrated that treatment of PRP with PAF (5-800 nM) and A23187 (1 μM) showed concentration-dependent increase in platelet aggregation. However, simultaneous addition of subthreshold concentrations of PAF (5 nM) and A23187 (1 μM) exhibited a synergistic effect (Figure 1A). Such an effect was comparable to that obtained by higher concentrations of PAF (800 nM) or A23187 (10 μM) alone. The synergism between PAF and A23187 was inhibited by pre-treatment of PRP with a potent PAF antagonist, WEB 2086 (IC_{50} = 0.65 μM) (Figure 1B)

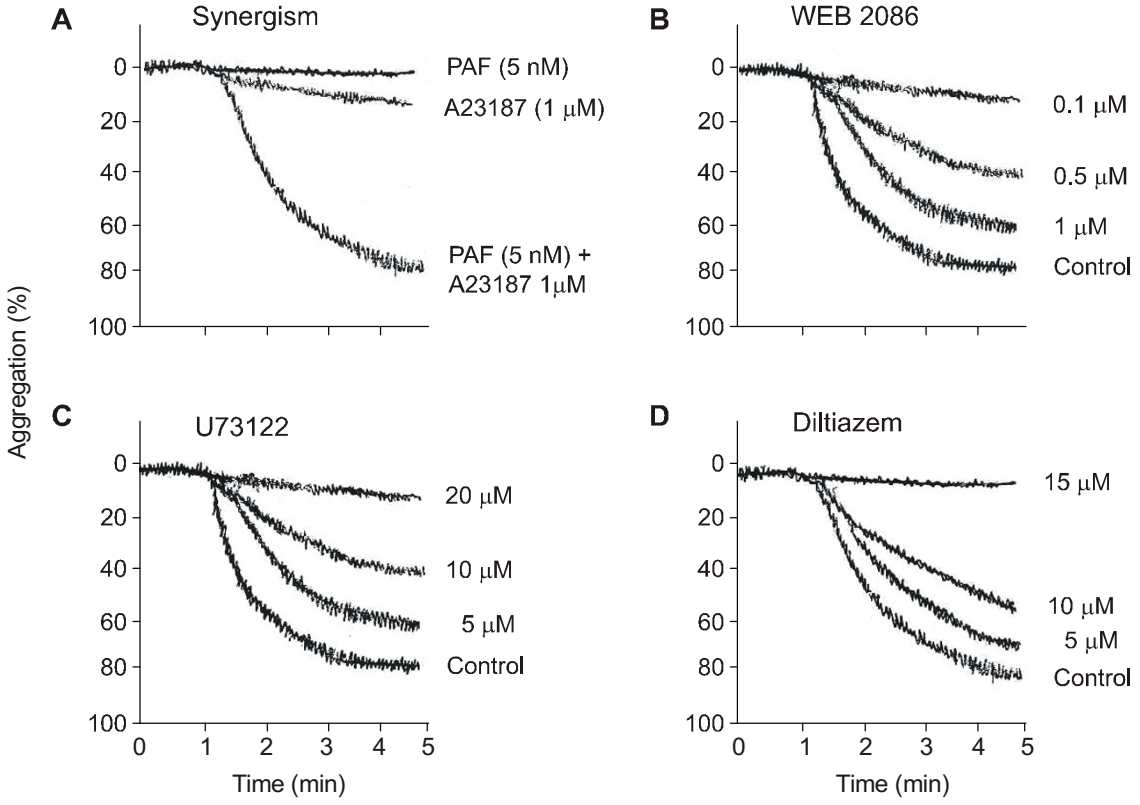

Figure 1. (A) Tracings from representative experiments showing synergism of PAF (5 nM) and A23187 (1 μM). The synergistic effect of PAF and A23187 on platelet aggregation is blocked by (B) WEB 2086, a PAF receptor antagonist, (C) U73122, a phospholipase C inhibitor and (D) diltiazem, a calcium channel blocker. Inhibitors were added one minute before the agonists. Control means platelet aggregation induced by PAF (5 nM) and A23187 (1 μM). n = 5.

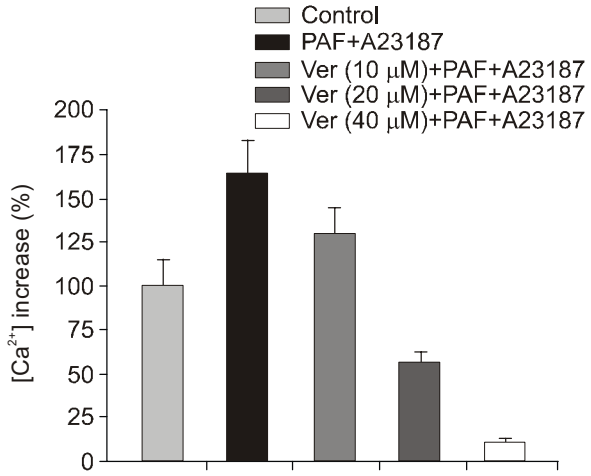

Figure 2. Effect of verapamil on PAF (5 nM) and A23187 (1 μM) induced rise in intracellular calcium [Ca^{2+}]. Platelets were loaded with Fura-2 AM and assays done as described in Materials and Methods. Control represents unstimulated platelets and is taken as 100%. *n* = 6. Mean ± SEM.

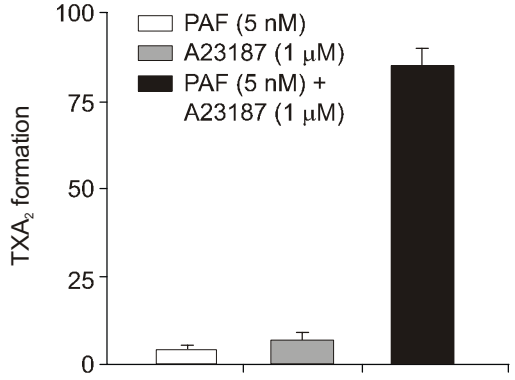

Figure 4. Effects of subthershold concentrations of PAF and A23187 on thromboxane A_2 (TXA_2) formation in human platelets. *n* = 6.

indicating that the effect is receptor mediated. We used PLC inhibitor; U73122 to examine if PAF and A23187 mediated effects involved the activation of PLC. Results show that pretreatment of PRP with U73122 completely inhibited the synergistic effect of PAF and A23187 with an IC_{50} of 10 μM (Figure 1C). We also tested the effect of Ca^{2+} channel blockers on platelet aggregation and found that the synergistic effect of PAF and A23187 was inhibited by both verapamil and diltiazem (Figure 1D) with IC_{50} values of 18 and 13 μM respectively.

Similar inhibitory effect of verapamil was also obtained using Fura-2 AM assay for the measurement of Ca^{2+} release as shown in Figure 2. To determine the role of cyclooxygenase, we used indomethacin (Figure 3A) which inhibited PAF and A23187 induced aggregation with IC_{50} value of 0.2 μM while, SQ

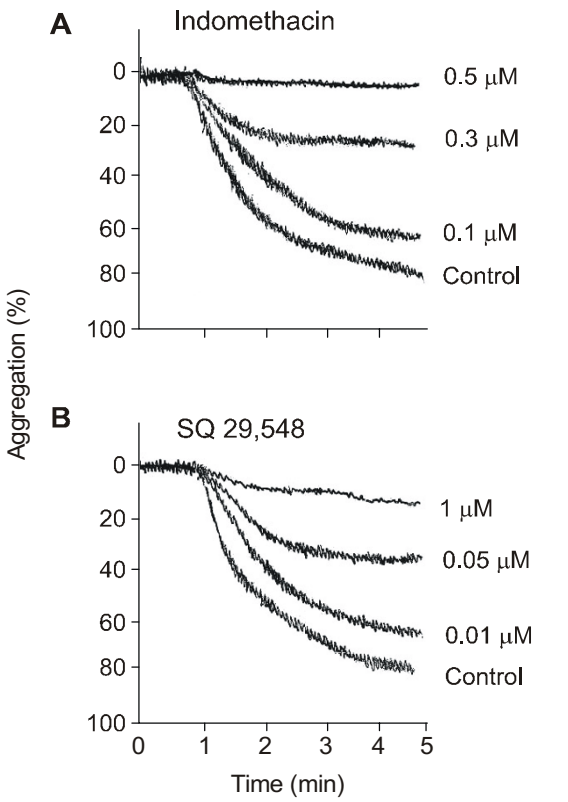

Figure 3. (A) Concentration-dependent effects of cyclooxygenase inhibitor, indomethacin and (B) TXA_2 receptor antagonist, SQ 29,548 on platelet aggregation induced by co-addition of PAF and A23187. Control means platelet aggregation induced by PAF (5 nM) and A23187 (1 μM). *n* = 7.

29548 a specific TXA_2 receptor antagonist also inhibited platelet aggregation with IC_{50} value of 0.05 μM (Figure 3B). Both PAF and A23187 in subthreshold concentrations also stimulated TXA_2 formation in platelets up to many folds (Figure 4). The results of SQ, 29548 and TXA_2 stimulation clearly explain the involvement of thromboxane/COX pathway in PAF and A23187 induced platelet aggregation.

Herbimycin A, a specific inhibitor of tyrosine kinase also inhibited PAF and A23187-induced aggregation with IC_{50} of 5 μM indicating the involvement of tyrosine kinase in this cascade (Figure 5).

As stimulation of the G-protein/Ca^{2+} cascade leads to mitogen activated protein (MAP) kinase signaling (Heemskerk and Sage, 1994), we used the selective MEK inhibitor PD98059 against PAF and A23187 synergism. Results show that pretreatment of PRP with PD98059 did not show any inhibitory effect on platelet aggregation by co-addition of subthreshold concentration of PAF and A23187. Similarly, the inhibitor of protein kinase C (chelerythrine; 20 μM) had no effect.

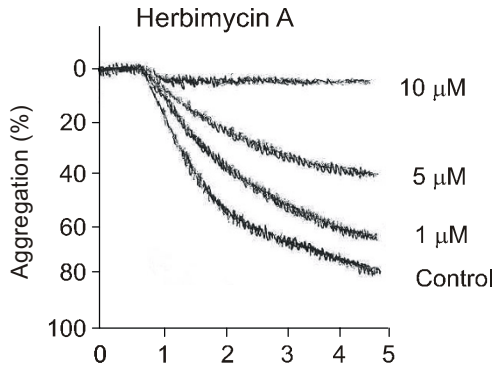

Figure 5. Dose-response curve of specific tyrosine kinase inhibitor, herbimycin A on the synergistic interaction of subthreshold concentrations of PAF and A23187. *n* = 7

Discussion

In human platelets PAF causes the stimulation of G*q* proteins by binding with specific transmembrane PAF receptors. The second messengers, Ca^{2+} and PKC generated in response to G*q*/PLC activation bring about coordinated changes leading towards platelet aggregation (Crabos *et al*., 1992; Heemskerk and Sage, 1994). This indicates that both PAF and Ca^{2+} play a pivotal role in aggregation. Similarly our study shows that both PAF and A23187 in subthershold concentrations show synergism when used exogenously. While studying mechanism of PAF and A23187 mediated aggregation we found that different inhibitors exhibited variable response against the synergism.

Our data show that WEB2086, a PAF receptor antagonist inhibited PAF and A23187 induced aggregation at very low IC_{50} values showing that the effect is receptor mediated.

PAF causes the stimulation of G*q* protein followed by the activation of PLC. This explains why U73122, a selective inhibitor of PLC, shows strong inhibitory effects as platelet aggregation induced by co-activation of these agonists. Further support in favour of G*q*/PLC pathways is provided by the recent studies in transgenic mice where it is shown that G*q* protein deficient mice lacked the ability of platelet aggregation (Offermanns *et al*., 1997). Some of the investigators reported that platelets lack L-type voltage dependent calcium channels but contain receptors operated calcium channels. The antiplatelet effects of calcium antagonists have been extensively studied *in vitro*, but such studies may involve high concentrations of the drugs. Verapamil is well-documented calcium antagonist with regard to antiplatelet effects having the most varied possible mechanisms of action (Hjemdahl and Wallen, 1997). Our previous studies show that synergistic effect of various platelet agonists is blocked by calcium-channel blockers, verapamil and diltiazem in very low concentration (Saeed *et al*., 1997; Shah *et al*., 1999; Saeed *et al*., 2003). Similarly the present findings also show that PAF and A23187 mediated platelet aggregation is also blocked by low concentration of verapamil or diltiazem. It is also supported by other studies that calcium channel blockers inhibit platelet activation induced by various agonists through different intracellular mechanisms (Valone, 1987).

Results with the channel blockers are consistent with the enduring proposal that calcium influx causes aggregation (Ware *et al*., 1986; Vinge *et al*., 1988). The increase in cytosolic Ca^{2+} causes activation of PLA_2 and stimulation of COX activity, thus TXA_2 formation (Heemskerk and Sage, 1994).

COX catalyzes the stepwise conversion of AA into reactive intermediates PGG_2 and PGH_2, which are the precursors of prostaglandins, prostacyclin and thromboxanes (prostanoids). COX-1 is mainly present in platelets and in other tissues (Piomelli, 1993). Numerous studies have shown that inhibitors of COX mainly belonging to the group of non-steroidal anti-inflammatory drugs (NSAIDs) also inhibit platelet aggregation by inhibiting TXA_2 biosynthesis. TXA_2 in an antocrine fashion binds with TXA_2 receptors coupled with Gq protein and stimulate PLC and further enhance platelet aggregation. MAP kinase is one of the down stream signaling molecules involved in platelet aggregation (Shah *et al*., 2000). That can cultivate both G*q* and G*i*-protein linked pathways (Della Rocca, *et al*., 1999).

Many studies show that activation of platelets by some agonists increase the level of tyrosine phosphorylation resulting in the appearance of a new set of tyrosine-phosphorylated proteins (Ferrel and Martin, 1988; Golden and Yamamura, 1989). Increase in the phosphorylation of tyrosine residues is an early event in the signal transduction pathway for stimulation of platelets by PAF (Animesh *et al*., 1990). To investigate the involvement of tyrosine kinase in present study we used herbimycin A, a known and specific inhibitor of tyrosine kinase which block PAF and A23187-induced aggregation in a concentration- dependent manner (IC_{50} = 15 μM) showing that the synergism may also be due to the TLCK activation.

We found that MAPK inhibitor; PD98059 did not exhibit any inhibitory effect showing that MAP kinase pathway was inactive in PAF and A23187 mediated aggregation. However the role of PKC in the present study was changed as PKC inhibition had no effect on the synergism of PAF and A23187 in platelets In conclusion, our study show that the synergistic interaction of PAF and A23187 in human platelet aggregation seems to follow the activation of thromboxane and tyrosine kinase pathways.

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