Signaling mechanisms mediated by G-protein coupled receptors in human platelets[1]

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KEY WORDS platelet aggregation; epinephrine; arachidonic acid; cyclooxygenase; phospholipase C; calcium channel blockers; nitric oxide; MAP kinase

ABSTRACT

AIM: The present study deals with the investigation of mechanisms involved in the synergistic interaction between epinephrine and arachidonic acid (AA). **METHODS**: Venous blood was taken from healthy human volunteers reported to be free of medications for one week. Platelet aggregation was monitored at 37 ºC 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:** The data show that a synergism in platelet aggregation mediated by subthreshold concentrations of epinephrine (1 μmol/L) and AA (0.2 μmol/L) was inhibited by the α_2-receptor antagonist (yohimbine, IC_{50}=0.6 μmol/L) and an inhibitor of AA-cyclooxygenase (COX), indomethacin (IC_{50}=0.25 μmol/L). In examining receptor influence on intraplatelet signalling pathways, it was found that the synergistic effect was inhibited by calcium channel blockers, verapamil (IC_{50}=0.4 μmol/L) and diltiazem (IC_{50}=2.5 μmol/L), as well as by low concentrations of inhibitors of phospholipase C (PLC) (U73122; IC_{50}=0.2 μmol/L) and mitogens activated protein kinase (MAPK) (PD 98059; IC_{50}=3.8 μmol/L). Herbimycin A, a specific inhibitor of tyrosine light chain kinase (TLCK), showed inhibition at IC_{50} value of 15 μmol/L, whereas chelerythrine, a protein kinase C (PKC) inhibitor, had no effect up to 20 μmol/L. **CONCLUSION:** These data suggest that synergism between epinephrine and AA in platelet aggregation is triggered through receptors coupled to G-protein, which in turn, activate PLC, COX, and MAP kinase-signaling pathways.

INTRODUCTION

Platelet agonists, such as epinephrine, thromboxane, adenosine diphosphate (ADP), platelet activating factor (PAF), and thrombin interact with transmembrane receptors coupled to GTP binding proteins (G-proteins) on platelets. G-proteins mediate a variety of intracellular processes by affecting other effector molecules, including adenylyl cyclase, inositol phospholipid-specific phospholipase C (PLC) or ion channels[1]. In human platelets, activation of Gi/adenylyl cyclase (eg, by epinephrine) decreases cAMP levels and increases platelet aggregation[1]. However, stimulation of platelet receptors coupled with phosphoinositide C-linked G-proteins, Gq (eg, by 5-HT, PAF or thrombin) and Gi (eg, epinephrine and histamine) activates PLC and gen-

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erates second messengers, diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3). These second messengers activate protein kinase C (PKC) and mobilize intracellular Ca^{2+}, respectively[2]. Both Ca^{2+} and PKC act in synergy to enhance platelet aggregation[3,4], whereas deficiency of Gq protein impairs it[5].

A number of agonists have been reported to act synergistically in platelet aggregation[6]. However, few study has done in human platelets on the cooperative effects of epinephrine and arachidonic acid (AA)[7]. In platelet membrane AA is the precursor for the synthesis of thromboxane A_2. Interactions of different stimuli could be physiologically important *in vivo* and in isolated platelet defects. It is now well documented that most platelet agonists act by stimulating G-protein coupled receptors (GPCRs). TXA_2, produced by the action of cyclo-oxygenase (COX) on AA, is a potent vasoconstrictor and mediator of platelet aggregation[8]. It induces aggregation by binding to specific receptors on the platelet membrane[9]. TXA_2 receptor stimulation activates phospholipase C and increases [Ca^{2+}] via G-protein of the Gq/11 family; this leads to aggregation by Ca^{2+} influx[10]. It has been postulated that the main proag-gregatory effects of TXA_2 are mediated by inhibition of an adenylate cyclase/cAMP complex[11].

Epinephrine interacts with α_2-adrenergic receptors on platelets; activation of these receptors inhibits the adenylyl cyclase pathway[12]. Epinephrine-mediated increase in platelet aggregation correlates with an increase in cytosolic free Ca^{2+}, a process sensitive to Na^+ concentration[12]. Inward cytosolic Ca^{2+}-influx is an important determinant in the signaling cascade mediating platelet aggregation[13].

This study was conducted to examine the interaction between epinephrine and AA to elucidate the possible intracellular signaling mechanism(s) involved during synergism. We further evaluated the involvement of multiple signaling pathways including PLC/Ca^{2+}, cyclooxygenase and mitogen activated protein kinase (MAPK) pathways in epinephrine and AA mediated platelet aggregation.

MATERIALS AND METHODS

Chemicals and drugs Arachidonic acid, epinephrine, yohimbine, indomethacin, diltiazem, verapamil, genistein and chelerythrine were obtained from the Sigma Chemical Co (St Louis, Mo, USA). U73122 was obtained from Alexis LC Labs (UK). Herbimycin A was purchased from RBI (Natick, MA. USA). All other chemicals used were of the highest purity grade available.

Preparation of human platelets Venous blood was taken from healthy human subjects of either sex, aged (25±4) year and a body weight of 60±7 kg, reported to be free of medication 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 ºC to obtain platelet rich plasma (PRP) and at 1200×*g* for 10 min to obtain platelet poor plasma (PPP). Aggregation studies were carried out at 37 ºC with PRP having platelet counts between 2.5 and 3.0×10^9/L of plasma[14].

Measurement of platelet aggregation Aggregation was monitored in 0.45 mL aliquots of PRP using a Dual-channel Lumi-aggregometer (Model 400 Chronolog Corporation, Chicago, USA)[14]. The final volume was made up to 0.5 mL with test drug dissolved either in normal saline or appropriate vehicle known to be devoid of any effect on aggregation. A sub-threshold concentration for aggregation by epinephrine was determined. To examine the synergistic effect of epinephrine and AA, we added low concentrations of these agonists (1 mmol/L and 0.2 mmol/L respectively). Anti-aggregatory effects of various inhibitors were studied by pretreating PRP with an inhibitor for 1 min, followed by addition of sub-threshold concentrations of epinephrine and AA. The resulting aggregation was recorded for 5 min after challenge by change in light transmission as a function of time. Once the antiplatelet activity of various inhibitors against agonists was established, dose-response curves were constructed to calculate 50 % inhibitory concentration of the inhibitors.

Thromboxane formation in platelets TXA_2 formation was studied with the co-addition of epinephrine (1 μmol/L) and AA (0.2 μmol/L) using radiochemical methods described previously[15]. For these studies, human blood platelets having counts between 2.5 and 3.0×10^9/L of plasma were routinely obtained in plastic bags from The Aga Khan University Hospital Clinical Laboratory, Karachi. The PRP was centrifuged at 260×*g* for 20 min and the sedimented platelets were washed twice with an ice-cold phosphate buffer (50 mmol/L, pH 7.4) containing sodium chloride (0.15 mol/L) and edetci acid (0.2 mmol/L). After centrifugation, washed platelets were resuspended in the same buffer without edetic acid and homogenized at 4 ºC using a polytron homogenizer for 15 s. The homogenate was centri-

fuged at 1200×*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 3.7 MBq [1-^{14}C]-arachidonic acid in the presence and absence of the agonists. After 15 min of gentle shaking in air at 37 ºC, the reaction was stopped by adding 0.4 mL of citric acid (0.4 mol/L) and ethyl acetate (7.0 mL). After mixing and centrifuging at 600×*g* for 5 min at 4 ºC, 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, USA). 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 Berthold TLC. linear analyzer and chromatography data system (Model LKB 511, Berthold W Germany). Protein concentration was determined using human serum allumin as standard[16].

Data analysis IC_{50}=Concentration (μmol/L) producing 50 % inhibition of platelet aggregation (control response taken as 100 %). The 50 % inhibitory concentrations (IC_{50}) values were calculated as means±SD of 5-6 independent experiments.

Differences between control and test measurements were assessed by *t*-test.

RESULTS

Treatment of PRP with subthreshold concentrations of epinephrine (1 μmol/L) and AA (0.2 mmol/L) was synergistic with regard to aggregation (Fig 1). Synergism between epinephrine and AA was inhibited

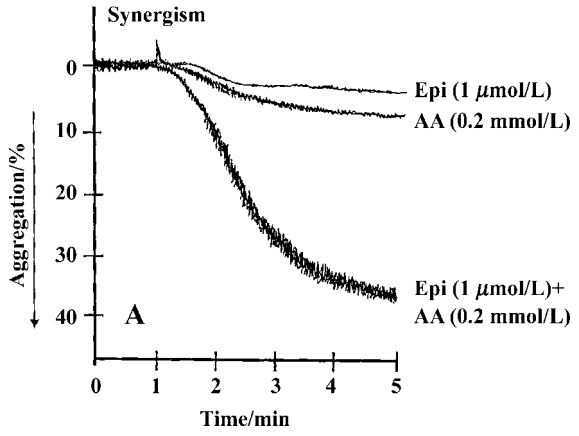

Fig 1. Tracings from a representative experiment showing synergism with epinephrine (1 μmol/L) and arachidonic acid (AA= 0.2 mmol/L) on platelet aggregation, *n*=7.

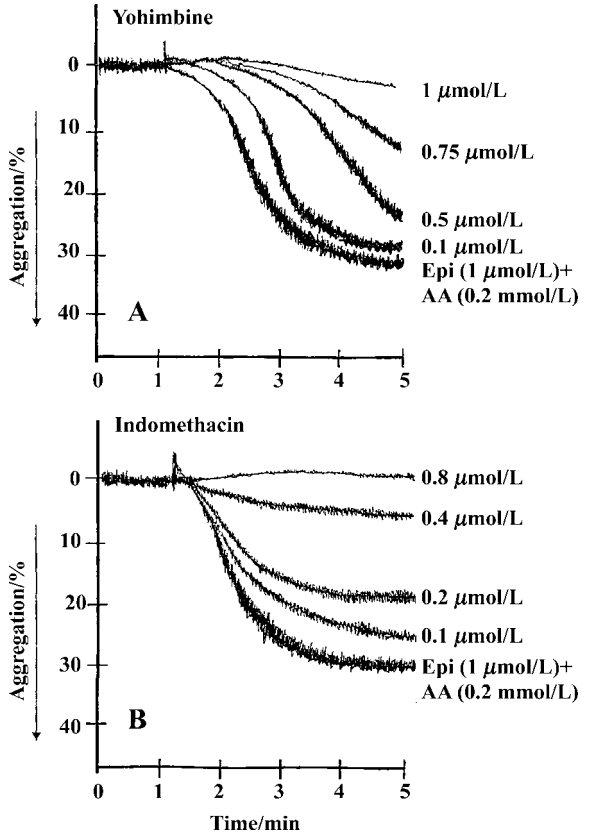

Fig 2. (A) Synergism between epinephrine and arachidonic acid is blocked by α_2-receptor antagonist, yohimbine; (B) Synergism blocked by AA-cyclooxygenase inhibitor, indomethacin. Control response is platelet aggregation induced by epinephrine (1 μmol/L) plus arachidonic acid (0.2 mmol/L) without the antagonist. *n*=5.

by pretreating PRP with α_2-adrenergic receptor blocker, yohimbine (IC_{50}=0.6 μmol/L) (Fig 2A), or AA-cyclooxygenase inhibitor, indomethacin (IC_{50}=0.25 μmol/L) (Fig 2B). We also determined the effect of these two agonists on COX activity by measuring TXA_2 formation in platelets exposed to epinephrine plus AA. Epinephrine (1 μmol/L) markedly potentiated the TXA_2 formation by low concentration of AA (0.2 mmol/L) (Fig 3).

We used a PLC inhibitor (U73122) to find out if the effect of epinephrine and AA involves activation of PLC. Results show that pretreatment of PRP with U73122 completely inhibited the synergistic effect of epinephrine and AA with an IC_{50} of 0.2 μmol/L (Fig 4). Therefore, we examined the effect of Ca^{2+} channel blockers (verapamil and diltiazem) on platelet aggregation. The synergistic effect of epinephrine and AA was inhibited by both diltiazem (Fig 5A) and verapamil (Fig 5B) and (IC_{50}=2.5 and 0.4 μmol/L

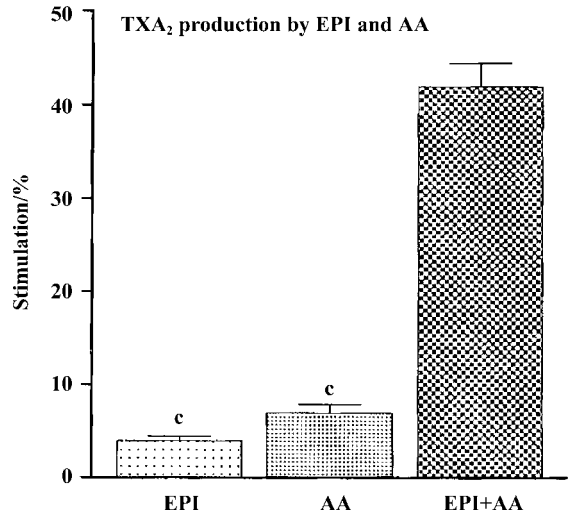

Fig 3. Effects of subthreshold concentrations of epinephrine and arachidonic acid on thromboxane A_2 (TXA_2) formation by human platelets. *n*=7.

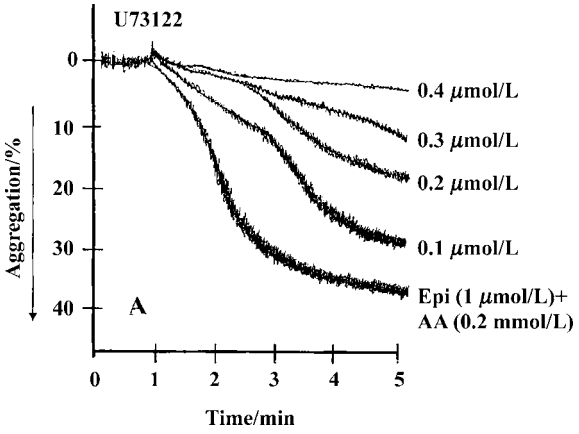

Fig 4. The effect of phospholipase C inhibitor, U73122 on the synergistic interaction of epinephrine (1 μmol/L) and AA (0.2 mmol/L). *n*=6.

respectively).

We used the selective MAP kinase inhibitor PD98059 in the epinephrine plus AA synergism model. Results show that pretreatment of PRP with PD98059 inhibited (IC_{50}=3.8 μmol/L) platelet aggregation usually produced by co-addition of subthreshold concentrations of epinephrine and AA (Tab 1 & Fig 6A).

The NO donor, SNAP, completely blocked platelet aggregation mediated by the synergistic interaction of epinephrine and AA (Tab 1). These data suggest the idea that NO negatively modulates human platelet aggregation. Herbimycin A, a specific inhibitor of tyrosine kinase, also inhibited epinephrine and AA induced aggregation with IC_{50} of 15 μmol/L, suggesting an involvement of tyrosine kinase in this cascade (Fig 6B).

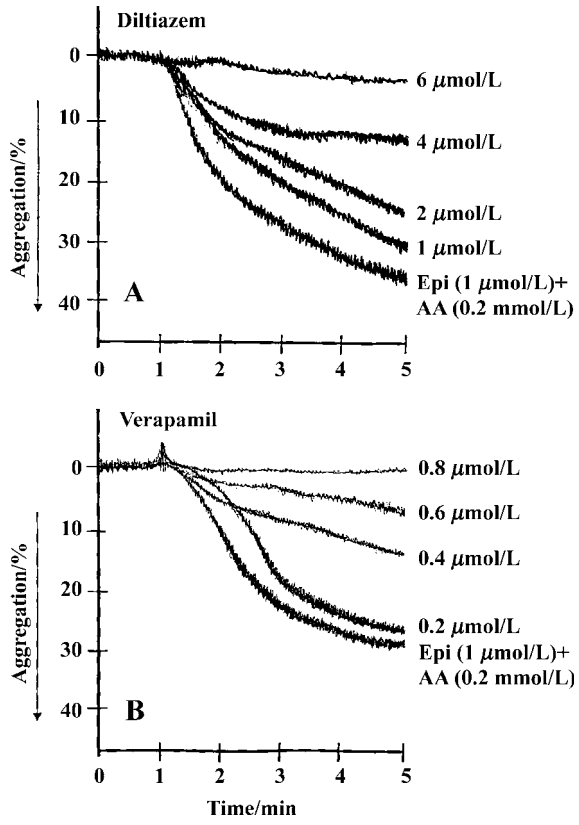

Fig 5. The effect of calcium channel blockers, (A) diltiazem and (B) verapamil on the synergistic effect of epinephrine (1 μmol/L) and AA (0.2 mmol/L). *n*=7.

Tab 1. Effect of various inhibitors on subthreshold concentration of epinephrine (1 μmol/L) and arachidonic acid (0.2 mmol/L)-induced platelet aggregation. *n*=5-7. Mean±SD.

Inhibitors	IC_{50}/μmol·L^{-1}	95 % Confidence limit
Yohimbine	0.60±0.09	0.49-0.71
Indomethacin	0.25±0.07	0.16-0.34
U73122	0.20±0.06	0.14-0.26
Diltiazem	2.5±0.7	1.85-3.15
Verapamil	0.40±0.09	0.32-0.48
SNAP	1.00±0.08	0.91-1.09
PD98059	3.8±0.5	3.18-4.42
Herbimycin A	15.0±2.5	11.90-18.10
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.

However, an inhibitor of PKC (chelerythrine; 20 μmol/L) had no effect.

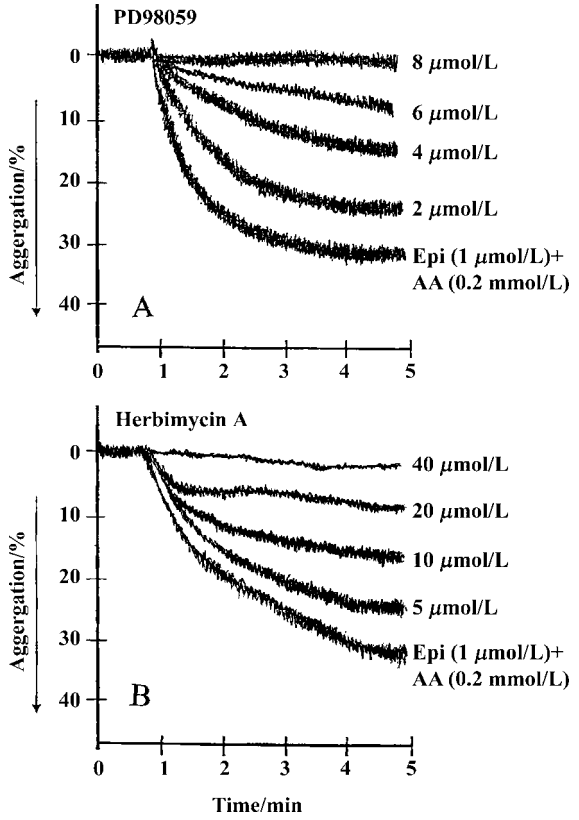

Fig 6. (A) The effect of MAP kinase inhibitor, PD98059 and (B) tyrosine kinase inhibitor, herbimycin A on the synergistic interaction of epinephrine (1 μmol/L) and AA (0.2 mmol/L), *n*=5.

DISCUSSION

Our study shows that epinephrine and AA, when added to PRP in subthreshold concentration, acted synergistically to produce platelet aggregation. This effect was dependent on transmembrane α_2-receptors. Synergism between epinephrine and AA was inhibited by calcium channel blockers, an α_2-receptor antagonist and inhibitors of PLC, MAP kinase, and COX pathways. Platelet α_2-receptors are linked to Gi proteins, which, in turn, activate PLC. Recent studies show that bg-subunits of activated Gi protein can activate PLC[17]. This sequence may explain why U73122, a selective inhibitor of PLC, inhibited platelet aggregation induced by co-activation of these agonists. Further, PLC activation leads to generation of IP_3, release of Ca^{2+} from internal stores and eventually store-depleted Ca^{2+} influx [13]. Ca^{2+} entry from the outside is inhibited by Ca^{2+} channel blockers, verapamil, and diltiazem. Moreover, an increase in cytosolic Ca^{2+} activates PLA_2 and COX-1 activity, thus stimulating TXA_2 synthesis[13]. Since the synergism was inhibited by indomethacin, a COX-1 inhibitor, it seems that agonist-mediated synergism follows activation of COX-1 distal to PLC/Ca^{2+} activation. Recent studies suggest an important role for nitric oxide (NO) in modulating platelet aggregation[18]. We tested the idea that increasing intracellular nitric oxide levels by using a NO donor, thus activating cGMP kinase, would inhibit platelet aggregation. Our results show that NO donor, SNAP, inhibited platelet aggregation (IC_{50}=1 mmol/L), suggesting that epinephrine/ AA synergism is sensitive to NO generation in platelets. However, a role for PKC seems unlikely, as PKC inhibition by chelerythrine chloride had no effect on the synergism.

Cyclic nucleotides, cAMP and cGMP, through activation of cGMP-dependent protein kinases, down-regulate Ca^{2+} responses and thus inhibit platelet aggregation[13]. In fact, platelets contain cAMP and cGMP dependent protein kinases that can inhibit PLC induced IP_3 production through inactivation of IP_3 and TXA_2 receptors[19]. Since stimulation of the G-protein/Ca^{2+} cascade leads to mitogen activated protein (MAP) kinase signaling[13], inhibition of epinephrine/AA synergism by MAP kinase inhibitor, PD98059 suggests the involvement of MAP kinase downstream from Gq/PLC[19]. Cytosolic PLA_2 is also a potential target for activation by an increase in cytosolic Ca^{2+}. Taken together, it appears that both Ca^{2+} signaling and MAP kinase play an important role in the synergy.

The selective MAPK inhibitor, PD98059 inhibits COX-1 and COX-2 activities[20]. Under our experimental conditions, PD98059 inhibited platelet aggregation with IC_{50} of 3.8 μmol/L. Therefore it is possible that inhibition of agonist-induced platelet aggregation by PD98059 may be due to blockade of COX activity.

Activation of platelets by some agonists increases the level of tyrosine phosphorylation resulting in the appearance of a new set of tyrosine-phosphorylated proteins[21]. To investigate the involvement of tyrosine kinase, we used herbimycin A, a known inhibitor of tyrosine kinase[22]. Herbimycin A blocked epinephrine/ AA-induced aggregation in a concentration-dependent manner (IC_{50}=15 μmol/L), showing that synergism may also involve tyrosine light chain kinase activation.

A common mechanism in synergism between various platelet agonists is thought to be due to activation of Ca^{2+} signaling cascade. A rise in intracellular Ca^{2+} concentration induced by the first agonist primes platelets for an enhanced functional response to the second

agonist[23]. Ca^{2+} plays a pivotal role in platelet aggregation. Synergism among various platelet agonists in the blood is of great clinical significance, as it could markedly potentiate platelet activation, producing cardiovascular pathology.

In conclusion, our studies show that subthreshold concentrations of epinephrine potentiate platelet aggregation mediated by AA. The synergism seems to derive from the PLC/Ca^{2+}, COX, and MAP kinase pathways and is negatively modulated by a nitric oxide donor. The results serve to reinforce the concept that aggregation synergisms are multifactorial interactions between products of G-proteins stimulation.

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