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Original Research

Synergism interaction between arachidonic acid by 5-hydroxytryptamine in human platelet aggregation is mediated through multiple signalling pathways[1]

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KEY WORDS serotonin; arachidonic acids; platelet aggregation; cyclooxygenase inhibitors; methysergide; cyproheptadine; thromboxane A_2; phospholipase C; calcium; drug synergism

ABSTRACT

AIM: To examine the signalling mechanisms involved in the synergistic interaction of 5-hydroxytryptamine (5-HT) and arachidonic acid (AA) in human platelet aggregation. **METHODS:** Blood was obtained from healthy human subjects, mixed with 3.8 % sodium citrate (9:1), and centrifuged to prepare platelet rich plasma (PRP). Aggregation was monitored using a Dual-channel Lumi-aggregometer. The agonist-induced influx of Ca^{2+} was measured using Fura-2 AM. TXA_2 formation was studied using radiochemical method. **RESULTS:** Subthreshold concentration of 5-HT (2 μmol/L) potentiated the effect of low dose of AA (0.2 mmol/L) in human platelets. This synergistic effect was blocked by 5-HT_2 receptor antagonist (methysergide IC_{50}=5.2 nmol/L; cyproheptadine IC_{50}=0.6 nmol/L), and thromboxane A_2 receptor antagonist (SQ 29 548; IC_{50}=30 nmol/L), showing that the effect is receptor-mediated. To examine the down-stream signalling pathways, we found that such an interaction was inhibited by calcium channel blockers (diltiazem; IC_{50}=3 μmol/L and verapamil; IC_{50}=5 μmol/L), phospholipase C (PLC) inhibitor (U73122; IC_{50}=4 μmol/L), cyclooxygenase inhibitor, (indomethacin; IC_{50}=0.2 μmol/L) and mitogen-activated protein (MAP) kinase inhibitor (PD98059; IC_{50}=3 μmol/L). The effect was also inhibited by a specific tyrosine light chain kinase (TLCK) inhibitor, herbimycin A with IC_{50} value of 5 μmol/L. Pretreatment of platelet with 5-HT and AA induced rise in intracellular calcium and this effect was blocked by verapamil. **CONCLUSION:** The synergism between 5-HT and AA in platelet aggregation involves activation of PLC/Ca^{2+}, COX, and MAP kinase pathways.

INTRODUCTION

A number of platelet agonists like 5-hydroxytryptamine (5-HT), arachidonic acid (AA), adenosine diphosphate (ADP), platelet-activating factor (PAF) and epinephrine when used together in subthreshold concentrations show synergism[1-4]. We have recently found that low concentration of platelet agonists such as 5-hydroxytryptamine potentiated the aggregating response of epinephrine[5]. Such a synergism between platelet agonists was found to be mediated through the activation of multiple signalling pathways. Moreover platelet agonists are also known to regulate AA metabolism by

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cyclooxygenase (COX) pathway[6,7]. These agonists act largely through the stimulation of G-protein-coupled receptors (GPCRs). The G-proteins mediate a variety of cellular processes by activating different effector molecules, including adenylate cyclase, phospholipase C, or ion channels[8,9].

It is known that both 5-HT and AA are pro-inflammatory mediators with potent cardiovascular and hematological actions. Platelets possess a well-defined serotonergic system that includes uptake and release of 5-HT after activation by most agonists. In platelets, stimulation of receptors coupled to Gq protein (eg, by 5-HT, PAF or thrombin) leads to the activation of PLC and thus generation of second messengers, 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[10]. Both Ca^{2+} and PKC stimulate platelet aggregation and also elicit synergism in platelet aggregation[11]. Consistent with the potential involvement of Gq/PLC pathway, the deficiency of Gq protein leads to impairment of agonist-induced platelet aggregation[12]. Stimulation of 5-HT_2 receptors evokes a shape change in human platelets and mediates many physiological functions that include increase in arterial constriction, modulation of perception, mood, feeding behaviour, and platelet aggregation[13,14]. Similarly 5-HT_{2A}-receptor density tend to increase in depression[15]. Like PAF, 5-HT also shows mitogenic effects in cardiovascular system and enhances the atherogenic and mitogenic effects of low-density lipoproteins (LDL) in aortic smooth muscles[16]. Combined thromboxane A_2 (TXA_2) and 5-HT_2 receptor blockade is proposed to prevent coronary artery thrombosis[17].

In platelet membranes, 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. TXA_2, produced by the action of cyclooxygenase (COX) on AA, is a potent vasoconstrictor and mediator of platelet aggregation[18]. It induces aggregation by binding to specific receptors on the platelet membrane. 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. It has been postulated that the main proaggregatory effects of TXA_2 are mediated by inhibition of an adenylate cyclase/cAMP complex[19].

This study was conducted to examine the interaction between 5-HT and AA to elucidate the possible intracellular signalling mechanism(s) involved in synergism.

MATERIALS AND METHODS

Materials 5-HT, arachidonic acid, methysergide, cyproheptadine, SQ 29548, indomethacin, diltiazem, verapamil, and chelerythrine were obtained from the Sigma Chemical Co (St Louis, Mo USA). U73122 was obtained from Alexis LC Labs (UK). PD 98059 and herbimycin A were 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 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 ºC to obtain platelet rich plasma (PRP). Platelet count was determined by phase contrast microscopy and all aggregation studies were carried out at 37 ºC with PRP having platelet counts between 2.5 and 3.0×10^9/L of plasma[20].

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). 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 of AA for aggregation was determined. To examine the synergistic effect of 5-HT and AA, we added subthreshold concentrations of these agonists (2 μmol/L and 0.2 mmol/L respectively), which induced a marked potentiation of aggregation. This response was taken as a control response against each inhibitor.

Anti-aggregatory effects of various inhibitors were studied by pretreating PRP with an inhibitor for 1 min, followed by the addition of sub-threshold concentrations of 5-HT and AA together (control response). Once the anti-platelet activity of various inhibitors against control was established, dose-response curves were constructed to calculate IC_{50} values of the inhibitors.

Thromboxane formation in platelets AA metabolism and TXA_2 formation were studied with the co-addition of 5-HT (2 μmol/L) and AA (0.2 mmol/L) using radiochemical methods[21]. 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 1200×*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 edetic 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 centrifuged at 1200×*g* for 20 min and 300 μL of the supernatant (containing 0.4 mg of protein) was incubated with 10 mg unlabelled AA and 3.7 kBq [1-^{14}C]AA in the presence and absence of the test compound. 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 mixture and centrifugation 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 mL ethanol and 20 mL solution was 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 use of a Berthold TLC. Linear analyzer and chromatography data system (Model LKB 511, Berthold, W Germany). Protein concentration was determined by the method of Lowry *et al* [22], using human serum albumin as standard.

Measurement of Ca^{2+} influx The agonist-induced influx of Ca^{2+} was measured using Fura-2 AM. Platelets (1×10^{11}/L) were suspended in Ca^{2+}-free standard medium (NaCl 145 mmol/L , KCl 5 mmol/L, $MgCl_2$ 1 mmol/L, HEPES 10 mmol/L, glucose 10 mmol/L, pH 7.4). Fura-2 AM dissolved in Me_2SO was added to the platelet suspension at 37 ºC for 45 min. The platelet suspension was centrifuged at 350×*g* for 15 min and the platelet pellet resuspended in fresh standard medium. Fura-2 AM fluorescence was monitored at 340 nm and 505 nm (excitation and emission) in platelets treated with the agonist AA and 5-HT.

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 mean±SEM of 5-6 independent experiments.

Differences between control and test measurements were assessed by Student's *t*-test.

RESULTS

Treatment of PRP with AA (0.1-1.73 mmol/L) showed a concentration-dependent increase in platelet aggregation. In contrast, 5-HT had no effect on platelet aggregation up to 200 μmol/L, it was found however, that low concentration of 5-HT (2 μmol/L) caused a marked potentiation of aggregation response mediated by low dose of AA (0.2 mmol/L) suggesting a synergism between the two agonists (Fig 1A). Such an effect was comparable to that obtained by higher concentrations of AA (1.73 mmol/L) alone.

Synergism between 5-HT and AA was inhibited by pretreating PRP with 5-HT_2 receptor antagonist, cyproheptadine; IC_{50}=0.6 nmol/L (Fig 1B) and methysergide; IC_{50}=5.2 nmol/L (Fig 2A). It is well known that TXA_2 is a potent AA metabolite and reported to be a vasoconstrictor and a potent mediator of platelet aggregation. We also tested SQ 29548, a specific TXA_2 receptor antagonist[23] against 5-HT- and AA-mediated platelet aggregation which inhibited the response at IC_{50} value of 30 nmol/L (Fig 2B) showing that the effect was receptor mediated.

Recent studies showed that activation of Gq protein by two different agonists at subthreshold concentrations was equally potent in eliciting the aggregation response by platelets. TXA_2 derived from 5-HT and AA caused stimulation of Gq protein followed by the activation of PLC. We used PLC inhibitor U73122 to examine whether the effects involved activation of PLC. Results showed that pretreatment of PRP with U73122 completely inhibited the synergistic effect of 5-HT and AA with IC_{50} of 4 μmol/L (Fig 3A).

Since activation of PLC leads 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[24], we examined the effect of Ca^{2+} channel blockers on platelet aggregation and found that the synergistic effect of 5-HT and AA was inhibited by both verapamil and diltiazem (IC_{50}=5 and 3 μmol/L respectively) as shown in Fig 3B (diltiazem) and Tab 1. Similar inhibitory effects of verapamil were also obtained using Fura-2 AM assay for the measurement of Ca^{2+} release as shown in Fig 4.

5-HT and AA are considered to be a potent activator of TXA_2 formation through activation of cyclooxygenase-1 (COX-1). To examine if these two agonists show synergism on COX-1 activity, we measured TXA_2 formation in agonist-treated platelets. Similar to its ef-

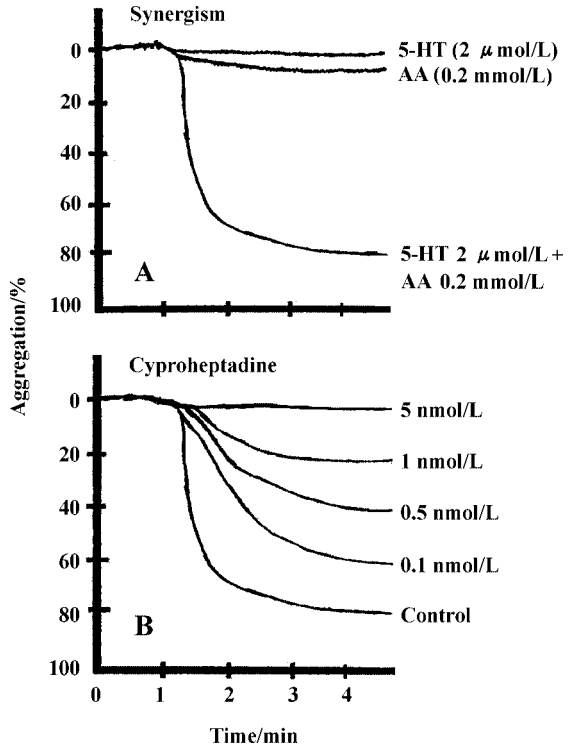

Fig 1. (A) Tracings from representative experiments showing synergism of 5-HT (2 μmol/L) and AA (0.2 mmol/L) on human platelet aggregation. (B) The synergistic interaction of 5-HT and AA is blocked by 5-HT$_2$ receptor antagonists, cyproheptadine. *n*=6.

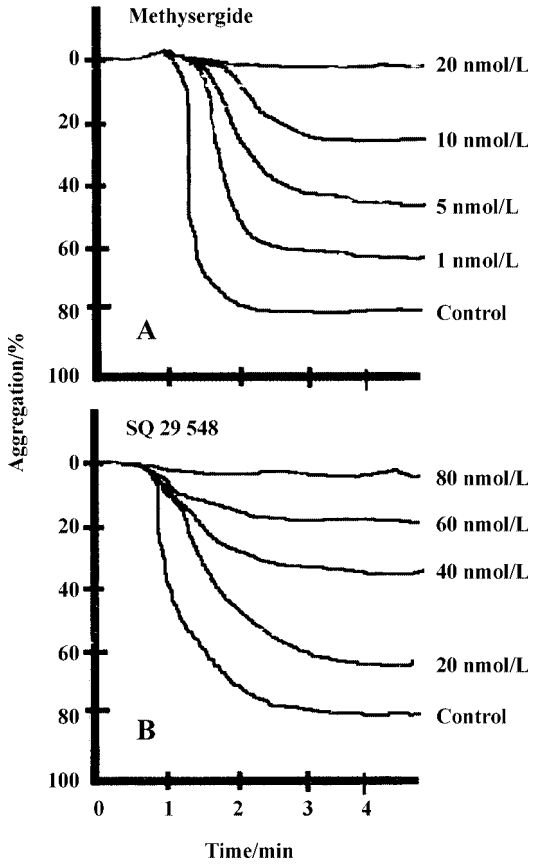

Fig 2. (A) Effect of 5-HT$_2$ receptor antagonist, methysergide. (B) TXA$_2$ receptor antagonist, SQ 29548 on synergistic interaction of 5-HT and AA in human platelet aggregation. *n*=6.

fect on platelet aggregation, AA markedly potentiated the TXA_2 formation by low concentrations of 5-HT (2 μmol/L) as shown in Fig 5. This effect was also blocked by AA-cyclooxygenase inhibitor, indomethacin (IC_{50}=0.25 μmol/L) indicating the involvement of COX pathway in the synergism.

As stimulation of the G-protein/Ca^{2+} cascade leads to mitogen-activated protein (MAP) kinase signalling, we used the selective MAP kinase inhibitor PD98059 in the 5-HT plus AA synergism. Results show that pretreatment of PRP with PD98059 inhibited (IC_{50}=3 μmol/L) platelet aggregation produced by co-addition of subthreshold concentrations of 5-HT and AA.

Herbimycin A, a specific inhibitor of tyrosine kinase also inhibited 5-HT and AA-induced aggregation with IC_{50} of 5 μmol/L indicating the involvement of tyrosine kinase in this cascade. The dose response effect of PD98059 and herbimycin A is shown in Fig 6. However, the inhibitor of protein kinase C (chelerythrine; 20 μmol/L) had no effect (Tab 1).

Tab 1. Effects of various inhibitors on subthreshold concentrations of 5-HT (2 μmol/L)- and arachidonic acid (0.2 mmol/L)-induced platelet aggregation. *n*=5-7. Mean±SEM.

Inhibitors	IC_{50}/μmol·L^{-1}	95 % CI
Methysergide	[1)]5.2±0.12	5.05-5.35
Cyproheptadine	[1)]0.60±0.03	0.56-0.64
SQ 29 548	[1)]30.000±0.001	30.0-30.0
Indomethacin	0.25±0.02	0.23-0.27
Verapamil	5.00±0.05	4.94-5.06
Diltiazem	3.0±0.4	2.50-3.50
U73122	4.00±0.01	3.99-4.01
PD 98059	3.0±0.1	2.88-3.12
Herbimycin A	5.0±1.5	4.38-5.62
Chelerythrine	no effect	-

1) Concentrations in nmol/L.

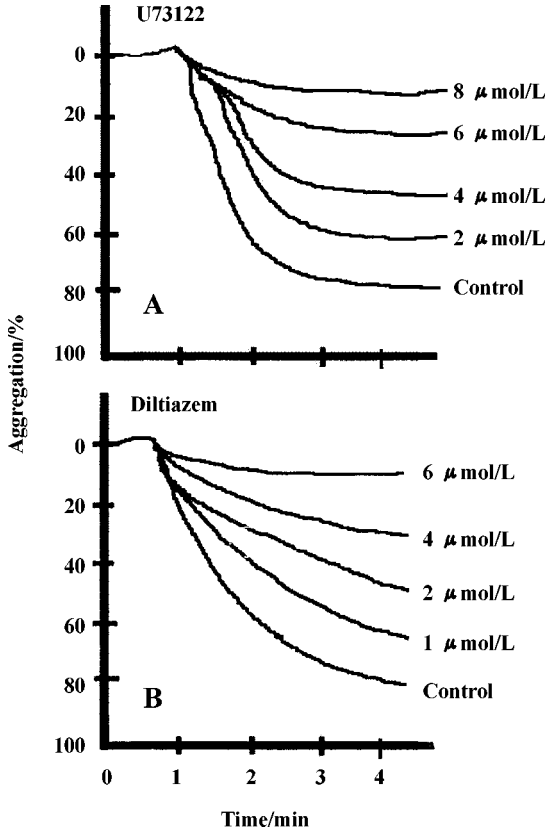

Fig 3. Synergistic effect of 5-HT and AA is blocked by (A) a PLC inhibitor, U73122 and (B) calcium channel blocker, diltiazem. *n*=6.

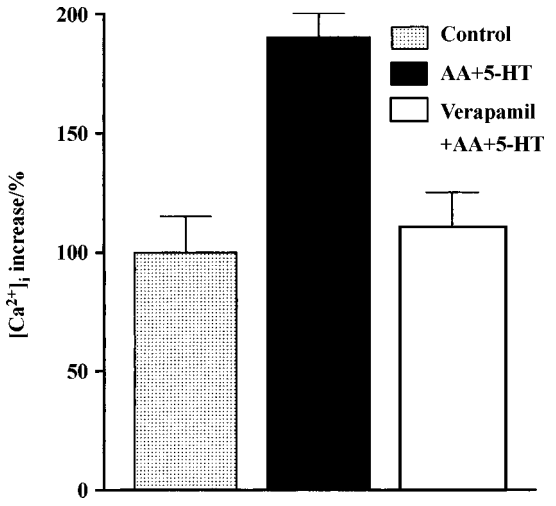

Fig 4. Effect of verapamil on 5-HT (2 μmol/L)- and AA (0.2 mmol/L)-induced rise in intracellular calcium $[Ca^{2+}]_i$. Platelets were loaded with Fura-2 AM and assays done as described in Methods. Control represents unstimulated platelets and is taken as 100 %. *n*=6. Mean±SEM.

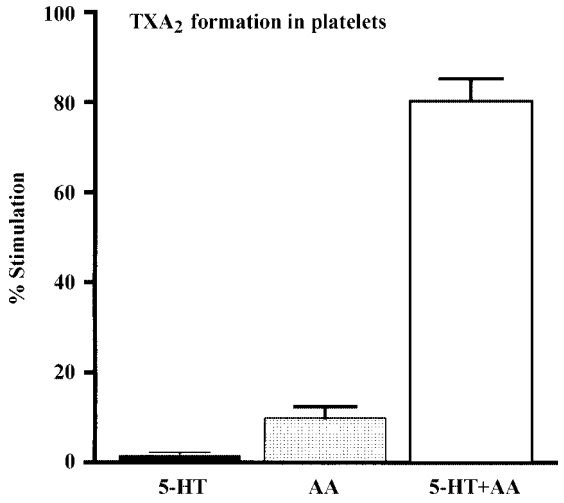

Fig 5. Effect of 5-HT and AA on thromboxane A_2 formation in human platelets. Low dose of 5-HT (2 μmol/L) potentiates the effect of AA (0.2 mmol/L) on TXA_2 formation in human platelets. *n*=6. Mean±SEM.

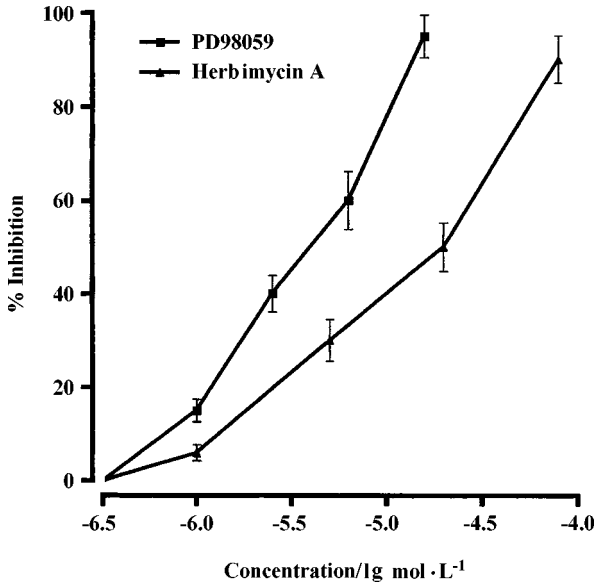

Fig 6. Dose-response curve of specific tyrosine kinase inhibitor, herbimycin A and MAP kinase inhibitor, PD98059 on co-addition of subthreshold concentrations of 5-HT and AA. *n*=6. Mean±SEM.

DISCUSSION

Our study shows that 5-HT and AA, when added to PRP in subthreshold concentrations acted synergistically to induce platelet aggregation. This effect was dependent on transmembrane 5-HT_2 and TXA_2 receptors. Synergism between 5-HT and AA was in-

hibited by 5-HT_2 receptor antagonists, calcium channel blockers, and inhibitors of PLC, MAP kinase, and COX pathways.

Platelet 5-HT_2 and TXA2-receptors are linked to Gq proteins which, in turn, activate PLC. This sequence may explain why U73122, a selective inhibitor of PLC, inhibited platelet aggregation induced by co-addition of 5-HT and AA. Further, PLC activation leads to the generation of IP_3, release of Ca^{2+} from internal stores and eventually store-depleted Ca^{2+} influx[24]. Moreover, an increase in cytosolic Ca^{2+} activates PLA_2 and COX-1 activity, thus stimulating TXA_2 synthesis[24]. Several studies[25,26] have reported that platelets lack L-type voltage-dependent calcium channels but contain receptor-operated calcium channels. The antiplatelet effects of calcium antagonists have been extensively studied *in vitro*, but such studies may involve high concentration of the drugs. Verapamil is well-documented calcium antagonist with regard to antiplatelet effects having the most varied possible mechanisms of action[27]. Our previous studies show that synergistic effect of various platelet agonists is blocked by calcium-channel blockers, verapamil and diltiazem in low concentration[4,5,20]. Similarly, the present findings also show that (5-HT and AA)-mediated platelet aggregation is also blocked by low concentration of verapamil and diltiazem. It is also supported by other studies that calcium channel blockers inhibit platelet activation induced by various agonists through different intracellular mechanisms[28].

Cyclic nucleotides, cAMP and cGMP, through activation of cGMP-dependent protein kinases, down-regulate Ca^{2+} responses and thus inhibit platelet aggregation[24]. 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[5]. Inhibition of 5-HT/ AA synergism by MAP kinase inhibitor, PD 98059, suggests the involvement of MAP kinase downstream from Gq/PLC[29,30]. 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+} signalling and MAP kinase play an important role in this synergism.

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

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. However; the role of PKC in the present study was excluded, as PKC inhibition had no effect on the synergism of 5-HT and AA in platelets.

The selective MAP kinase inhibitor, PD 98059, inhibits COX-1 and COX-2 activities[31]. Under our experimental conditions, PD 98059 inhibited platelet aggregation with IC_{50} of 3 μmol/L. Therefore, it is possible that inhibition of agonist-induced platelet aggregation by PD 98059 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. To investigate the involvement of tyrosine kinase, we used herbimycin A, a known inhibitor of tyrosine kinase[32,33]. It was found that herbimycin A blocked 5-HT/AA-induced aggregation in a concentration-dependent manner (IC_{50}=5 μmol/L), showing that synergism may also involve tyrosine light chain kinase (TLCK) activation.

Synergism among various platelet agonists in the blood is of great clinical significance, as it could markedly potentiate platelet activation, thus altering cardiovascular physiology. In conclusion, our studies show that subthreshold concentrations of 5-HT potentiate platelet aggregation mediated by AA. It seems to follow the activation of PLC/Ca^{2+}, COX, MAP kinase and TLCK pathways.

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