Role of Phosphatidylinositol 3-Kinase in Platelet Aggregation

S.A. SAEED*, G. SHAMIM, Z. NAWAZ, A.H. GILANI AND B.H. SHAH
Department of Physiology and Pharmacology,
The Aga Khan University, Karachi-74800, Pakistan

Summary: Platelet aggregation plays an important role in haemostasis and vascular disorders. This mainly takes place by the action of endogenous agonists like ADP, platelet-activating factor (PAF), epinephrine, 5-hydroxytryptamine (5-HT) and arachidonic acid (AA). These agonists except AA interact with G-protein coupled receptors. Recent studies have shown that phosphatidylinositol 3-kinase (PI 3-kinase) and myosin light chain kinase (MLCK) play an important role in platelet aggregation. We have shown that low doses of the selective PI 3-kinase inhibitor, wortmannin, inhibits aggregation (IC_{50}; 20 nM) mediated by subthreshold doses of 5-hydroxytryptamine (5-HT; 5 μM) and epinephrine (1 μM). This study was undertaken to examine the effects of wortmannin in platelet aggregation induced by various platelet agonists. The results show that wortmannin inhibited aggregation mediated by various agonists with an IC_{50} for ADP (110 nM), AA (2 μM), collagen (280 nM) and PAF (520 nM). These studies suggest that wortmannin-mediated inhibition of aggregation induced by ADP, epinephrine, collagen and PAF may affect multiple enzymatic pathways and the most likely targets seem to be both PI 3-kinase and MLCK, as other kinases like cAMP- and calcium-calmodulin-dependent protein kinases are reported to be not affected by wortmannin.

Introduction

Platelet aggregation plays an important role in the haemostasis and vascular disorders. This mainly takes place by the action of endogenous agonists like ADP, platelet activating factor (PAF), epinephrine, 5-hydroxytryptamine (5-HT) and arachidonic acid (AA). Most platelet agonists bind to their specific receptors on platelets and activate guanine nucleotide binding proteins (G-proteins). Epinephrine and ADP interact with receptors coupled to Gi proteins on platelets and activation of these receptors suppresses the adenylyl cyclase activity [1-3]. These agents also increase Ca^{++}-influx. Collagen action is not mediated through G-proteins but it has been shown to decrease cAMP and increase Ca^{++} levels [4].

Receptors for PAF and thrombin in human platelets are coupled with Gq-phospholipase C (Gq/PLC) pathway, activation of this leads to generation of second messengers, inositol-1,4,5-triphosphate (IP_3) and diacylglyecrol (DAG) [1,5]. IP_3 causes release of calcium from internal stores and DAG activates protein kinase C (PKC). PKC acts in synergy with Ca^{++} mobilization for the activation of platelets [6]. However, inhibitory effects of PKC on receptor-mediated IP_3 production and Ca^{++} mobilization are wide-spread and also occur in platelets [7,8].

Recent studies have shown that phosphatidylinositol 3-kinase (PI 3-kinase) and myosin light chain kinase (MLCK) play important role in platelet aggregation [9,10]. Wortmannin, a compound derived from *Penicillium wortmannin* has been shown to be a selective and potent inhibitor of PI 3-kinase at very low doses (1-10 nM) and it also inhibits MLCK at higher doses (> 100 nM). We have recently shown that wortmannin in very low doses inhibits platelet aggregation induced by synergistic action of epinephrine and 5-HT, both of these act through activation of G-protein coupled receptors in platelets [11]. In continuation to our previous work, here we studied the role of PI 3-kinase and MLCK in response to aggregation induced by various platelet agonists.

Results and Discussion

The platelet agonists like epinephrine, ADP, AA, collagen and PAF increased the platelet aggregation in a dose dependent manner. However, 5-HT had no appreciable effect in a wide range of doses (not shown). The maximal effect produced by these agonists occurred at concentrations as following; epinephrine (20 μM), ADP (4.2 μM), collagen (638 nM), AA (1.7 mM) and PAF (0.8 μM).

*To whom all correspondence should be addressed.

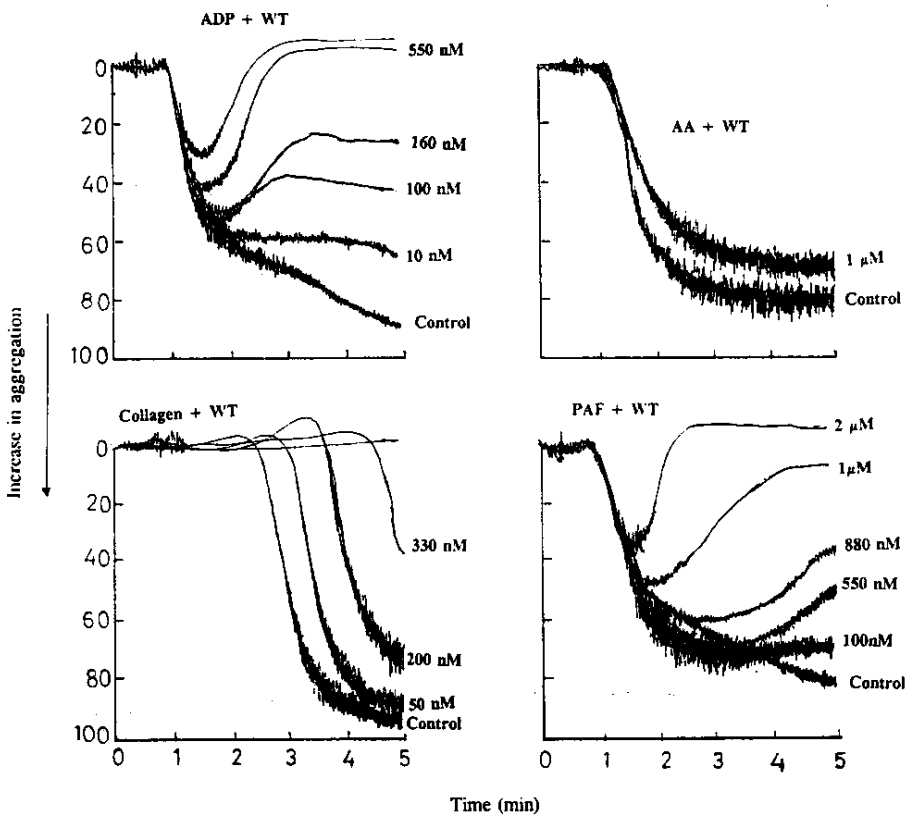

Fig. 1: Effect of phosphatidylinositol 3-kinase inhibi-tor, wortmannin, on platelet aggregation induced by ADP (4.2 μM), collagen (638 nM), arachidonic acid (0.75 μM) and platelet activating factor (0.8 μM) as described in methods. These full concentrations of agonists are indicated as Control.

Treatment of platelets in PRP with wortmannin alone up to doses of 4 μM had no effect on platelets. However, it inhibited the aggregation induced by maximal concentrations of these agonists with varying inhibitory concentrations (IC_{50}) as shown in Figure 1. The IC_{50} of wortmannin against ADP, collagen and PAF ranged between 45-520 nM, however much higher concentrations were needed to inhibit the aggregation caused by epinephrine and AA (Table 1). Platelet aggregation was substantially enhanced when subthreshold concentrations of epinephrine (1 μM) plus 5-HT (5 μM) were used (Fig. 2A). This synergistic effect was inhibited by low doses of wortmannin (IC_{50}; 20 nM) as depicted in Fig. 2B.

The activation of platelets with agonists leads to shape change and release of endogenous 5-HT and

Table-1:Inhibitory effects of wortmannin on platelet aggregation induced by various platelet agonists. The values are IC_{50} of wortmannin (n=4).

Agonists (concentration)		IC_{50} of wortmannin
ADP	(4.2 μM)	110 nM
Collagen	(638 nM)	280 nM
PAF	(0.8 nM)	520 nM
Epinephrine	(20 μM)	2.5 μM
Epinephrine	(1 μM)	
+ 5-HT	(5 μM)	45 nM

ADP from the dense tubular system. We tested if wortmannin has any effect on the release of 5-HT from platelets. It was found that 2 and 4 μM of wortmannin caused 10 and 62 % decrease in epinephrine-induced release of 5-HT in platelets (Fig. 3).

PI 3-kinase is a cytosolic enzyme and has been reported to play a trivial role in the platelet

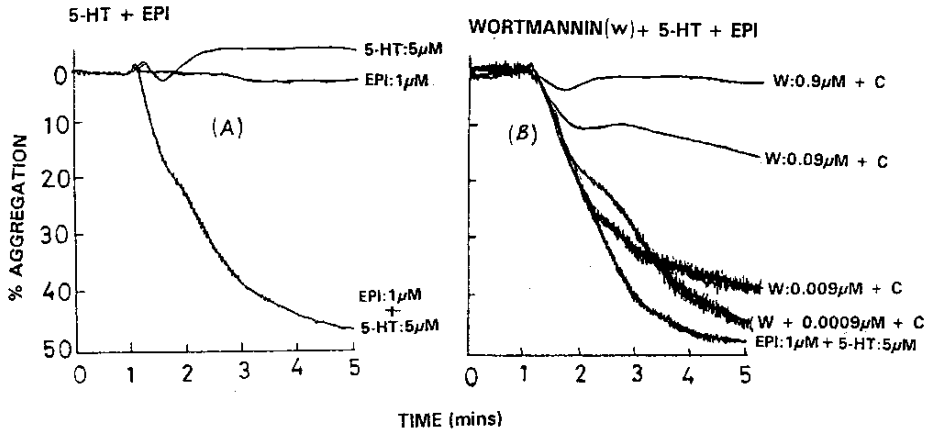

Fig. 2: (A) Effect of epinephrine and 5-HT on platelet aggregation. The subthreshold concentrations of epinephrine (1 μM) and 5-HT (5 μM) poten-tiate the aggregatory response. (B) Dose-response effect of wortmannin on platelet aggre-gation induced by subthreshold concentrations of 5-HT (5 μM) and epinephrine (1 μM).

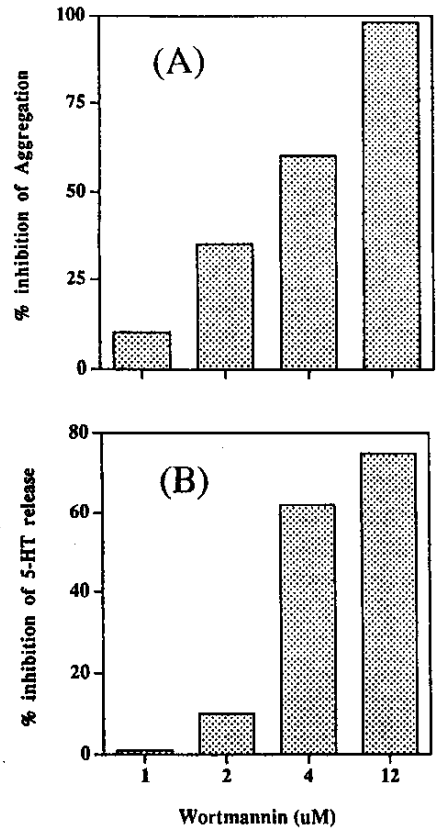

Fig. 3: Effect of wortmannin on the platelet aggregation and release of 5-HT from human platelets. Platelets were pre-labelled with ^{14}C[5-HT], aggregation and 5-HT release were measured as described in Methods.

aggregation through phosphorylation of substrate proteins located in the cytoskeleton of platelets [7,9]. We tested if PI 3-kinase is relocated from the cytosol to cytoskeleton in agonist-activated platelets. Our Western blot results in Fig. 4 show that platelet agonists caused shift of PI 3-kinase to the cytoskeleton of platelets.

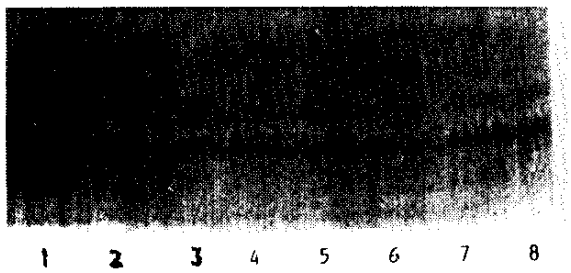

Fig. 4: Effect of various platelet agonists on the relocation of PI 3-kinase to cytoskeleton. PI 3-kinase was detected by Western blot as described in Methods. Lanes of bands on immunoblot indicate: Lane-1 (control); Lane-2 (Thrombin, 0.25 μ/ml); Lane-3 (epinephrine 0.4 μM); Lane-4 (Epinephrine, 0.4 μM + 5-HT, 5μM); Lane-5 (wortmannin, 10 μM); Lane-6 (thrombin + wortmannin); Lane-7 (epinephrine + wortmannin) and lane-8 (5-HT + epinephrine + wortmannin).

Wortmannin is a fungal metabolite which has been extensively used in studying signalling mediated by PI 3-kinase in platelets and other cells

[14]. It has proved to be a potent inhibitor of mammalian PI 3-kinase in virtually all the preparations tested so far [9, 14]. We show that wortmannin inhibits platelet aggregation induced by various platelet agonists with variable IC_{50}. Earlier studies have shown that platelet secretion and aggregation in response to thrombin, collagen or thromboxane A_2 (TXA_2) is inhibited by wortmannin without any effect on Ca^{++} release or Ca^{++}-influx. It was concluded that wortmannin acts on selective targets within the cell [9,14-15].

Both PI 3-kinase and MLCK are involved in the platelet aggregation process [9,16]. Wortmannin inhibits PI 3-kinase activity at very low concentrations (10 nM) but it inhibits MLCK activity at higher concentrations (> 1 μM) [9,15-17]. Based on this concentration-dependent differential inhibition of these enzymes, it is easy to characterize the mechanism and pathway of platelet aggregation induced by various platelet agonists. Our results in Table 1 indicate that IC_{50} of wortmannin aganist various platelet agonists ranges between 50-550 nM. Wortmannin is reported to have variable IC_{50} (ranging between 5-200 nM) against PI 3-kinase [17, 18]. However, much higher concentrations of wortmannin (3-6 μM) were required to inhibit the ADP responses on MLCK. Moreover, substantially higher amounts (about 8-10 μM) are required to cause inhibition of cAMP- and cGMP-dependent protein kinases and calmodulin-dependent protein kinase II [10].

The activation of PI 3-kinase occurs following activation of receptors coupled with tyrosine kinase and G proteins [5,11,19,20]. Activation of G protein causes dissociation of α-subunit from the βγ-subunits [1]. The activation of PI 3-kinase by these agonists seems to result as a consequence of interaction of ßγ-subunits of heterotrimeric G-proteins with PI 3-kinase [20] or through activation of a small GTP-binding protein rho [21]. In fact G protein βγ-subunit mediated activation of PI 3-kinase is more pronounced for agonists like epinephrine that interact with pertussis toxin sensitive G-proteins (Gi) in platelets [14].

Our immunoblot results show that PI 3-kinase is relocated from the cytosol to the cytoskeleton in platelets stimulated with agonists. Also increased in the cytoskeletal fraction are focal adhesion components such as vinculin, αIIbβ3, and talin, as well as $pp^{60\text{-}src}$ (a tyrosine kinase), PKC and rho protein [9, 21-24]. The PI 3-kinase in the soluble fraction of platelets is stimulated several fold by GTPγS and other agonists which activate G proteins. It seems as PI 3-kinase is activated directly or indirectly by rhoA. Thus rhoA, via PI 3-kinase, may exert effects on platelet cytoskeletal reorganization, perhaps at the platelet's αIIbβ3-containing "focal adhesion complex" [9, 25]. It is likely that relocation of PI 3-kinase to cytoskeleton and probably activation of MLCK plays some role in platelet shape change and in the process of platelet aggregation. The involvement of platelet integrin receptors (αIIbβ3) is well known during collagen induced platelet aggregation and PI 3-kinase stimulation is necessary for prolonged GPIIb-IIIa activation and irreversible platelet aggregation [26].

Human platelets react weakly to activation by 5-HT. Platelets show a shape change, a small reversible aggregation, the release of intragranular substances but without any irreversible aggregation [11,16]. However, subthreshold concentrations of 5-HT potentiate the aggregatory effect of epinephrine which can be blocked by low doses of wortmannin [11]. Our results suggest that wortmannin (> 4 μM) inhibits the release of 5-HT. Since the concentration required to elicit this response is much more compared to that needed to inhibit PI 3-kinase, it seems that the wortmannin-mediated inhibition of 5-HT release occurs due to inhibition of some other signalling pathway such as MLCK activity [16,26]. In conclusion, both PI 3-kinase and MLCK seem to play vital role in the platelet aggregation through phosphorylation cascade.

Experimental

Materials

Epinephrine, PAF, ADP, 5-HT, collagen and arachidonic acid and wortmannin were obtained from Sigma Chemical Co, St. Louis, USA. Chelerythrine was from Calbiochem-Novabiochem, UK. H-7 was from Seikagaku Corp., Japan. All other chemicals used were of analytical grade.

Preparation of human platelets

Blood was drawn by venepuncture from normal human volunteers 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°C to obtain platelet-rich plasma (PRP). Platelet count was made 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 x 10^8/ml of plasma.

Measurement of platelet aggregation

Aggregation was monitored using a Dual-channel Lumi aggregometer (Model 400 Chronolog Corporation, Chicago, USA) with 0.45 ml aliquots of PRP as described previously [11]. The final volume was made up to 0.5 ml with sodium chloride (0.9%, w/v) or test drug and incubated for 1 min before challenge with the aggregating agent. While studying the role of PI 3-kinase and MLCK, platelets were pre-incubated with varying doses of wortmannin for 1 min and then treated with optimal dose of the platelet agonist (ADP, PAF, collagen and epinephrine). Aggregation induced by various agents was recorded for 5 minutes. Statistical difference between control and drug-treated platelet preparations were determined by Student's t-test.

5-Hyroxytryptamine release

The PRP was prepared as described above and pre-labelled with ^{14}C-[5-HT], (CPM = 18 x 10^5, specific activity 55 mCi/mmol) and incubated for 1/2 hr. at 37°C in a shaking water bath. Aggregation was monitored using 450 µl aliquots of PRP. The PRP was preincubated with 10 µM imipramine and/or an appropriate amount of test drug for 1 minute before challenge with aggregating agent. Aggregation was induced by ADP (4.2 µM), PAF (0.8 µM) or AA (1.7 µM). Resulting aggregation was recorded and expressed as percent inhibition compared with control at 4 min after challenge. After aggregation, aliquots were transferred to microfuge tubes and were centrifuged. 100 µl aliquot of the supernatant was added to 5 ml of aquasol (liquid scintillation cocktail). Radioactivity was counted by Beckman LS6500 Multipurpose Scintillation Counter, to determine 5-HT release from the platelets.

Western Blotting of PI 3-kinase

PI 3-kinase plays important role in the platelet cytoskeleton reorganization during aggregation process. To examine any changes produced in the protein expression of PI 3-kinase, we did Western blotting using antisera raised against PI 3-kinase in rabbits. The platelets in PRP were treated by various platelet agonists as indicated in the Figure legend. The changes in PI 3-kinase were studied by pre-treating the platelets with wortmannin (10 µM). Platelets were homogenized in buffer-A containing Triton X-100 (2%), Tris (100 mM), EGTA (10 µM), leupeptin (50 µM) and PMSF (100 µM) at 4°C for 30 min. The homogenate was centrifuged at 12000 rpm for 15 min at 4 °C. The pellet was collected and washed with buffer-B (buffer-A diluted twice in normal saline). Pellet was dissolved in SDS-Polyacrylamide (SDS-PAGE) loading buffer. The samples were placed in boiling water bath for 5 min before loading on to 8% SDS-polyacrylamide gels as described.

Proteins were resolved by running gels overnight, transferred to nitrocellulose membranes (Schleicher and Schuell) and blocked for 3 h in 5% (w/v) gelatin in phosphate buffered saline (PBS), pH 7.5. Primary antisera against PI 3-kinase (p85) was raised in rabbit and was added (1:250) in 1% gelatin in PBS containing 0.2% Nonidet P-40 (NP-40) and incubated overnight. The primary antiserum was removed and blots washed extensively with PBS containing 0.2% NP-40. Secondary antiserum (anti-rabbit IgG coupled to horseradish peroxidase; HRP) in 1% gelatin/PBS/0.2% NP-40 was added and left for 3h. After removal of second antiserum, blots were washed extensively as above and developed with o-dianisidine hydrochloride as substrate for HRP as described [12,13]. The developed immunoblots were scanned and quantified as described [13].

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