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Research Article

The molecular mechanisms involved in lectin-induced human platelet aggregation

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Abstract

We have compared the effect of three legume lectins wheat germ agglutinin (WGA), *Phaseolus vulgaris* agglutinin (PHA) and *Lens culinaris* agglutinin (LCA) on function of human platelets. We have found that WGA is more active than PHA in stimulating platelet activation/aggregation, while LCA has no effect. Studies on the mechanisms involved put in evidence that WGA and PHA induce phosphorylation/activation of PLCγ2 and increase $[Ca^{2+}]_i$. For the first time it has been shown that Src/Syk pathway, the adapter protein SLP-76 and the exchange protein VAV participate in the PLCγ2 activation by these lectins. Moreover WGA and PHA stimulate PI3K/AKT pathway. PI3K, through its product phosphatidylinositol-3,4,5-trisphosphate activates Bruton's tyrosine kinase (BTK) and contributes to PLCγ2 activation. In conclusion, our findings suggest that PLCγ2 activation induced by WGA and PHA is regulated by Src/Syk and by PI3K/BTK pathways through their concerted action.

Keywords: human platelets; *Phaseolus vulgaris* agglutinin; PI3K/AKT pathway; PLCγ2 pathway activation; wheat germ agglutinin.

Introduction

Human platelets are small subcellular fragments of megakariocytes which play a crucial role in hemostasis and thrombosis. Upon activation that can be induced by soluble molecules including thrombin, thromboxane A2 (TXA2), adenosine diphosphate (ADP), serotonin or by adhesive extracellular matrix protein such as vonWillebrand factor and collagen, platelets change their shape from small discs to spherocytic cells with filopodia resulting in significant remodelling of the cytoskeleton. These changes are associated with important functions of platelets such as adhesion to endothelium and aggregation. In most cases platelet activation and aggregation is mediated by mobilized glycoprotein receptors. Their mobilization can be probed by specific lectins, carbohydrate-binding proteins, that can recognize specific sugars of cell surface glycocalyx. Lectin binding may also induce transmembrane signalling and produce cell aggregation similar to physiological agonists of platelets (Higashihara *et al.*, 1985; Torti *et al.*, 1999; Ohmori *et al.*, 2001). The best characterized lectins are derived from plants. However recently the animal lectins galectins have been found in a wide variety of tissues. Galectins through a link with betagalactoside (Barondes *et al.*, 1994) bind to cell-surface and extracellular matrix glycans and affect a variety of cellular processes such as cell adhesion, proliferation, differentiation and apoptosis (Schattner, 2014). There is increasing evidence that galectins have a role in different physiological and pathological processes including inflammatory responses, tumor development and progression, neuronal degeneration and cardiovascular diseases (Yang *et al.*, 2008). Recently it has been shown that galectins can trigger many platelet responses including adhesion, P-selectin expression, aggregation and granule release (Romaniuk *et al.*, 2010, 2012; Etulain *et al.*, 2014). Like most traditional platelet agonists, galectins induce intracellular Ca^{2+} elevation and promote morphological changes in the cytoskeleton such as spreading and F–actin polymerization (Yang *et al.*, 2008). Legume lectins, despite their strong similarity at the level of amino acid sequences and tertiary structures, greatly vary in their carbohydrate specificities and quaternary structures. They display a wide variety of chemical and biological functions leading to their use in hematology or immunology or as specific markers for membrane glycoprotein structures. Thus the mechanisms of platelet activation and aggregation caused by lectins may greatly differ. For example wheat germ agglutinin (WGA), a strong platelet agonist, induces a rapid intracellular Ca^{2+} increase that is sensitive to the non-specific protein kinases inhibitor staurosporine but resistant to inhibition by cAMP (Yatomi *et al.*, 1993). On

the contrary *Sambucus nigra* agglutinin (SNA) does not lead to intracellular Ca^{2+} mobilization (Samal *et al.*, 1998).

In the present study we have compared the effect of three legume lectins with different determinants (Greenberg and Jamieson, 1974) on platelet aggregation. In particular we have studied WGA, *Phaseolus vulgaris* agglutinin (PHA) and *Lens culinaris* agglutinin (LCA). WGA is a plant lectin that exhibits specificity for two types of N-acetylated sugars, N-acetyl-D-glucosamine (Kumar *et al.*, 2012) and N-acetyl-D-neuraminic acid and interacts with sialylated-cell-surface receptors (Bhavanandan and Katlic, 1979; Lovrien and Anderson, 1980). The molecular structure of WGA has been extensively characterized (Wright, 1977, 1992; Wright *et al.*, 1984) and the WGA biosynthesis *in vivo* was demonstrated (Raikhel and Wilkins, 1987; Smith and Raikhel, 1989). Moreover WGA has been used as a tool for studying platelet activation (Ganguly and Fossett, 1979, 1980), glucose transport and lypolisis (Cuatrecasas, 1973), nerve growth factor stimulation (Grob and Bothwell, 1983), cell growth inhibition (Stanley and Carver, 1977) and morphological states of erythrocytes (Lovrien and Anderson, 1980). PHA is a tetrameric protein with a molecular weight of 126 kDa, each subunit of 31 kDa (Loris *et al.*, 1998). The lectin recognises and binds specifically to terminal galactose, N-acetylglucosamine and mannose residue of complex glycans on mammalian glycoprotein (Liener, 1986). PHA exists in different isoform (D,E,L) with different structure and properties. For instance PHA-E has been characterized as potential allergen for patients allergic to edible legume seeds (Rougé *et al.*, 2010) and PHA-L is the leukocyte-agglutinating form of this lectin that accumulates in the vacuoles of storage parenchyma cells (Mirkov and Chrispeels, 1993). LCA is a legume lectin that binds specifically to mannose (Qian *et al.*, 1994; Yamamoto *et al.*, 2000; Arce *et al.*, 2003) and fucose (Matsumura *et al.*, 2007). Its primary structure has been defined and its secondary structure predicted (Foriers *et al.*, 1981). LCA has been used to put in evidence N-glycan profiling changes in human gastric cancer cells (Zhao *et al.*, 2014), and can be useful probe for the detection in serum of a core fucosylated α-fetoprotein (AF8-L3) fraction, a well known marker for the diagnosis of the hepatocellular carcinoma (Tateno *et al.*, 2009). In this study we have tested WGA, PHA and LCA as agonists of human platelets. We have found that WGA and, at a lesser extent, PHA are able to stimulate platelet aggregation in a dose- and time-dependent manner, whereas LCA, in the range 1-50 μg/ml, has no effect. In addition, we have investigated the molecular mechanisms involved. We have found that the signalling pathways Src/Syk and PI3K/AKT/Bruton's tyrosine kinase (BTK) are greatly activated by WGA, contributing to the

activation of PLCγ2. Also PHA activates these pathways but with minor potency. Likely WGA and PHA behave as true agonists of human platelets being able to activate concerted mechanisms.

Results

Platelet aggregation and P-selectin exposure induced by lectins

The WGA, PHA or LCA effect on platelet aggregation was tested. WGA (Figure 1A) and PHA (Figure 2A) induced platelet aggregation in a dose- and time-dependent manner. WGA was more effective than PHA since at five min 50 μg/ml WGA produced the maximal aggregation (about 60 %), whereas PHA caused about 30 % of aggregation. However WGA is less active than collagen (Figure 1B). On the other hand LCA, in the range 1-50 μg/ml was unable to stimulate platelet aggregation, as previously demonstrated (Altankov and Setchenska, 1988) (Figure 3A). In our experimental conditions no spontaneous platelet aggregation was observed (Figure 1B). Moreover platelet function expressed through P-selectin (CD62P) surface exposure in platelets challenged by WGA, PHA or LCA was measured. Data obtained by these experiments are in agreement with aggregation data above reported. In fact it was shown that WGA (Figure 1C) was more effective than PHA (Figure 2B), whereas LCA was uneffective (Figure 3B) at all concentrations of lectins tested.

Intracellular Ca^{2+} mobilization induced by lectins.

The WGA effect on intracellular Ca^{2+} elevation was dose-dependent. In human platelets activated without stirring, WGA at 50 μg/ml elicited a prompt and sharp increase of intracellular Ca^{2+} concentration that was more 3 times the basal level (Figure 4A). The response in the presence of 10 or 5 μg/ml WGA was shifted and the peak of Ca^{2+} concentration was smaller. In addition 1 μg/ml WGA was ineffective. Different was the effect of PHA on Ca^{2+} mobilization. PHA at 50 μg/ml produced a Ca^{2+} elevation of about 100 nM that was unchanged during 5 min (Figure 4B). As expected LCA did not change basal Ca^{2+} level (Figure 4C). We tested the effect of some inhibitors on WGA-induced Ca^{2+} elevation (Figure 4D). All compounds tested (PP2, inhibitor of src kinases; piceatannol, inhibitor of p72syk; staurosporine, a non specific inhibitor of protein kinases including tyrosine kinases; U73122, inhibitor PLCγ2 pathway; LY294002 and MK2206, inhibitors of PI3K and AKT

respectively) reduced significantly Ca^{2+} elevation induced by WGA. Among these compounds staurosporine produced the maximal inhibiting effect (Figure 4D) as it was demonstrated for collagen (Tamaoki, 1991).

Activation of PLCγ by WGA and PHA

Protein tyrosine phosphorylation plays an important role in mechanisms induced by collagen. In particular collagen activates Src family tyrosine kinases, leading to Syk and PLCγ2 activation (Watson, 1999). Thus we examined the tyrosine phosphorylation on specific activation residues of Src, Syk and PLCγ2 in platelets stimulated by WGA and we compared the WGA effect on these enzymes with the PHA effect. Obviously we did not subjected LCA to further investigations. WGA induced the phosphorylation of Src on Tyr527 residue in a dose- (Figure 5A) and time- (Figure 6A) dependent manner. Also PHA stimulated Src Tyr527 phosphorylation but in a slower and weaker manner (Figures 5A, 6A). In a like manner WGA and PHA induced Syk phosphorylation on the residues Tyr525-526, confirming that WGA is more effective than PHA in the stimulation of some important enzymes leading to PLCγ2 phosphorylation/activation. In addition WGA and, to a lesser extent, PHA induced PLCγ2 phosphorylation on Tyr753 residue. The effect of both lectins was dose- and time-dependent (Figures 5A, 6A). Several proteins play important roles in the activation of PLCγ2 downstream of Syk in human platelets. These included the adapter protein SLP-76 and the exchange protein VAV implicated in tyrosine phosphorylation/activation of PLCγ2. In addition, the p85-110kDa dimeric form of PI3K and the BTK are required for regulation of PLCγ2 activity but not its tyrosine phosphorylation. Thus we tested the phosphorylation status of SLP-76 in platelets stimulated with lectins. We found that WGA and, with minor potency, PHA stimulate SLP-76 phosphorylation on Tyr128 residue (Figures 5A, 6A). The exchange protein VAV was also phosphorylated in platelets stimulated with WGA and PHA (Figures 5A,6A) by probable binding to SLP-76 and Syk to mediate PLCγ2 activation. Moreover WGA and PHA stimulated BTK phosphorylation at residue Tyr223 through PI3K activation, producing a dose- and time-dependent effect. WGA was more rapid and more effective than PHA (Figures 5A, 6A). The activation of PI3K and the consequent phosphatidylinositol-3,4,5-trisphosphate (PIP3) formation produced phosphorylation/activation of AKT in platelets treated with WGA and PHA (Figures 5A, 6A). Data indicative of the phosphorylation status of each enzyme are shown in tables of Figures 5,6 (panels B) and have been expressed as the "ratio of ratios" that was indicative of the real increase of the phosphorylation. Finally we

tested some specific inhibitors on PLCγ2 phosphorylation (Figure 7A,B). As expected PP2, pan inhibitor of Src kinase family, and piceatannol, a specific Syk inhibitor, abolished PLCγ2 phosphorylation induced by WGA or PHA. In addition LY294002 and MK2206 inhibitors of PI3K and AKT activities respectively, and CNX-774, a specific BTK inhibitor, greatly reduced PLCγ2 phosphorylation induced by WGA or PHA. On the contrary the PLCγ2 pathway inhibitor U73122 and the PKC specific inhibitor GF109203X had no effect. In addition we tested some inhibitors of PLCγ2 and PI3K/AKT pathways on AKT phosphorylation. We found that AKT phosphorylation was abolished by PP2 and piceatannol and by the pan PI3K inhibitor LY294002 (Figure 7C, D).

Discussion

Several studies concerning the WGA effect in cells have been performed, whereas only few recent data on PHA are reported. Previously it was shown that WGA induces NADPH-oxidase activity in neutrophyls (Karlsson, 1999), monocyte-mediated tumor cell killing (Ogawara *et al.*, 1985), Syk activation in porcine splenocytes (Yamada *et al.*, 1991), and IL2 production and IL2 receptor expression in lymphocytes (Reed *et al.*, 1985). PHA was shown to induce apoptosis in carcinoma cells (Kochubei *et al.*, 2015, 2016), can have anti-cancer therapeutic properties through the induction of transcription of iNOS gene (Fu *et al.*, 2011), stimulates production of NO in macrophages (Kesherwani and Sodhi, 2007) and inhibits electrogenic Na^+ absorption in both colon and trachea (Kunzelmann *et al.*, 2004). Many years ago it was shown that WGA stimulates platelet aggregation and release reaction (Greenberg and Jamieson, 1974; Ganguly and Fossett, 1979, 1980; Ganguly *et al.*, 1979; Higashihara *et al.*, 1985;), inducing PLCγ2 activation, Ca^{2+} mobilization and signalling (Higashihara *et al.*, 1985; Yatomi *et al.*, 1993, 1995). Moreover, seven bands phosphorylated in tyrosine with molecular masses ranging from 140 to 35 kDa have been identified by immunoblot in human platelets stimulated with WGA (Inazu *et al.*, 1991). Among these proteins, the protein tyrosine kinase p72Syk was identified (Ohta *et al.*, 1992). Concerning the effect of PHA in platelets only very old studies on platelet aggregation induced by PHA are reported (Majerus and Brodie, 1972; Altankov and Setchenska, 1988) but the action mechanism of this effect was never clarified. In the present study we have investigated some novel molecular mechanisms involved in platelet activation induced by WGA and PHA (Figure 8). We have confirmed that the tyrosine kinases Src and Syk lead to PLCγ2 activation. Likely the cross

linking of the receptor (probably a glycoprotein) with lectin induces the tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) through the Src kinases. This leads to binding of the protein kinase Syk to the phosphorylated ITAM, resulting in autophosphorylation and activation. Thus in human platelets stimulated with WGA or PHA Syk plays a pivotal role in the regulation of PLCγ2 as shown for collagen (Melford *et al.*, 1997; Briddon and Watson, 1999). For the first time we have demonstrated that some proteins downstream of Syk have a role in the activation of PLCγ2 by lectins. This includes the adaptor protein SLP-76 and the exchange factor VAV which are required for the phosphorylation and activation of the PLCγ2 (Gross *et al.*, 1999). Activated PLCγ2 generates secondary messengers, IP_3 and DAG which releases Ca^{2+} from the intracellular stores and activates PKC, respectively. These mechanisms lead to a common platelet activation cascade, resulting in platelet aggregation and dense granule secretion. Moreover we have also demonstrated that the PI3K/AKT pathway is activated in platelets challenged with WGA or PHA as occurs in platelets stimulated by agonists (Pasquet *et al.*, 1999; Kroner *et al.*, 2000; Li *et al.*, 2003; Signorello *et al.*, 2011; Signorello and Leoncini, 2014). Since in our experimental conditions AKT phosphorylation is blocked in platelets pretreated with the pan PI3K inhibitor LY294002 (Figure 7C,D), AKT phosphorylation was considered as a read out of PI3K activity (Kim *et al.*, 2009). PI3K is the p85/110kDa heterodimeric form of the enzyme that catalyzes the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2) to PIP3. PI3K interact with PLCγ2 and supports the enzyme recruitment to the membrane (Gratacap *et al.*, 1998). PIP3 is required for the activation of BTK that is also involved in the regulation of PLCγ2, as the specific BTK inhibitor CNX-774 abolishes PLCγ2 phosphorylation (Figure 7A,B). In addition BTK, activated by WGA and PHA, could contribute to the regulation of Ca^{2+} entry (Pasquet *et al.*, 2000). Comparing the WGA and PHA effect, we have found that both stimulate PLCγ2 phosphorylation/activation and Ca^{2+} elevation, being PHA less potent than WGA. Thus WGA and, with smaller power, PHA stimulate mechanisms leading to platelet activation/aggregation, largely similar to those produced by collagen upon GPVI activation in platelets. However at the moment it has not been clarified which receptor could be involved in WGA and PHA effect even if we can exclude a G-protein coupled receptor.

Materials and methods

Materials

Apyrase, Colorburst™ electrophoresis markers, digitonin, GF109203X, β-mercaptoethanol, piceatannol, PP2 inhibitor, prostaglandin E1 (PGE1), staurosporine, U73122 and all chemicals were from Sigma-Aldrich, St. Louis, MO, USA. Lectins (LCA, PHA and WGA) and FURA 2/AM were purchased from Merck Millipore, Billerica, MA, USA. LY294002 was from Tocris Bioscience, Bristol, UK. MK2206 and CNX-774 were from Selleck Chem, Houston, TX, USA. Inhibitors were diluted in saline from a stock DMSO solution immediately before each experiment. Anti-Src (Y527) and anti-AKT (S473) were from Cell Signalling Technology, Danvers, MA, USA. Anti-BTK (Y223) was from Bioss, Woburn, MA, USA and anti-SLP-76 (Y128) from Assay Biotech, Sunnyvale, CA, USA. Anti-CD62P-FITC, anti-PLCγ2 (Y753), anti-Syk (Y525-526), anti-VAV (Y174), horseradish peroxidase-conjugated secondary antibodies, anti-β-actin (loading control) and the antibodies recognising the total enzyme were purchased from Santa Cruz Biotechnology, Dallas, TX, USA. Nitrocellulose membranes (pore size 0.45 μm) were from Bio-Rad Laboratories, Berkeley, CA, USA.

Blood collection and preparative procedures

Freshly drawn venous blood from healthy volunteers of the "Centro Trasfusionale, Ospedale San Martino" in Genoa was collected into 130 mM aqueous trisodium citrate anticoagulant solution (9:1). The donors claimed to have not taken drugs known to interfere with platelet function during two weeks prior to blood collection, and gave their informed consent. Washed platelets were prepared centrifuging whole blood at 100 *g* for 25 min. To the obtained platelet-rich plasma (PRP) 4 mU/ml apyrase and 4 μM PGE1 were added. PRP was then centrifuged at 1100 *g* for 15 min. Pellet was washed once with pH 5.2 ACD solution (75 mM trisodium citrate, 42 mM citric acid and 136 mM glucose), centrifuged at 1100 *g* for 15 min and then resuspended in calcium-free 10 mM HEPES buffer containing 145 mM NaCl, 5 mM KCl, 1 mM MgSO4, 10 mM glucose (pH 7.4).

Platelet aggregation

Platelet aggregation was followed in a Bio-Data Aggregometer according to Born's method (Born, 1962) and quantified by the light transmission reached within 6 min. Washed platelets

(3.0×10^8 platelets/ml) were preincubated with saline for 3 min at 37°C before platelet stimulation with lectins or collagen.

Flow cytometric analysis of CD62P

Washed platelets (1.0×10^9 platelets/ml) were preincubated at 37°C with saline and then stimulated with lectins at 37°C for 120 s. At the end of incubation suitable aliquots of samples were fixed in 2% paraformaldehyde in PBS for 30 min at 4°C. Then anti-CD62P-FITC was added and each sample was analysed by flow cytometry.

Intracellular Ca^{2+} measurement

Washed platelets (3.0×10^8 platelets/ml) were incubated with 1 μg/ml FURA 2/AM for 45 min at 37°C. Two μM PGE1 and 1 mM EGTA were added before centrifuging loaded platelets for 15 min at 1100 *g*. The pellet, resuspended at 2.0×10^8 platelets/ml in Ca^{2+}-free HEPES buffer (pH 7.4), was preincubated at 37°C with saline or inhibitors and then lectins added. Fluorescence of FURA 2/AM-loaded platelets was monitored at 37°C under unstirring conditions for 3 min in a Perkin-Elmer fluorescence spectrometer model LS50B, with excitation at 340 nm and 380 nm and emission at 509 nm. The fluorescence of fully saturated FURA 2/AM (Fmax) was obtained by lysing platelets with 50 μM digitonin, in the presence of 2 mM Ca^{2+}, while Fmin was determined by exposing the lysed platelets to 1 M EGTA. In order to calculate the autofluorescence value, the fluorescence was fully quenched with 5 mM Mn^{2+}. A software combined with the fluorescence spectrometer converted data into cytosolic Ca^{2+} concentration, yielding a K_d value for FURA 2/AM and Ca^{2+} of 135 nM.

Immunoblotting analysis of phospho-proteins

Washed platelets (1.0×10^9 platelets/ml), preincubated for 10 min at 37°C with saline, were stimulated with lectins. In the experiments in which the dose effect of lectins was evaluated, platelets were stimulated with increasing concentrations of WGA or PHA for 120 s. The time-dependence was assessed in platelets challenged with 10 μg/ml lectins. In other experiments platelets (1.0×10^9 platelets/ml), preincubated for 10 min at 37°C with varying compounds able to inhibit specific steps of several signalling pathways, were stimulated with 10 μg/ml WGA or PHA for 120 s. Incubation was stopped by adding 2×Laemmli-SDS reducing sample buffer. Samples, heated for 5 min at 100°C, were separated by 5-10 % SDS-PAGE, and transferred to nitrocellulose membranes. Running was performed in the

presence of Colorburst™ Electrophoresis weight markers. Blots were blocked in 5 % BSA dissolved in TBST (Tris buffer saline, pH 7.6, containing 10 mM Tris, 150 mM NaCl, and 0.1 % Tween 20) at 37°C for 30 min, and then incubated overnight at 4°C with anti-Src (Y527), anti-Syk (Y525-526), anti-PLCγ2 (Y753), anti-SLP-76 (Y128), anti-VAV (Y174) anti-BTK (Y223), or anti-AKT (S473) (1:1000 dilutions) antibodies. Membranes were extensively washed and incubated for 60 min at room temperature with horseradish peroxidase-conjugated secondary antibody. After further washings, blots were developed using the ECL® system and revealed by the Bio-Rad Chemi-Doc software package. Nitrocellulose membranes were then stripped by incubation with 62.5 mM Tris/HCl (pH 6.7), 2% SDS, 100 μM β-mercaptoethanol for 30 min at 50°C, reprobed with anti-β-actin. Immunoblotting with respective anti-total antibodies was performed on a different gel.

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Figures

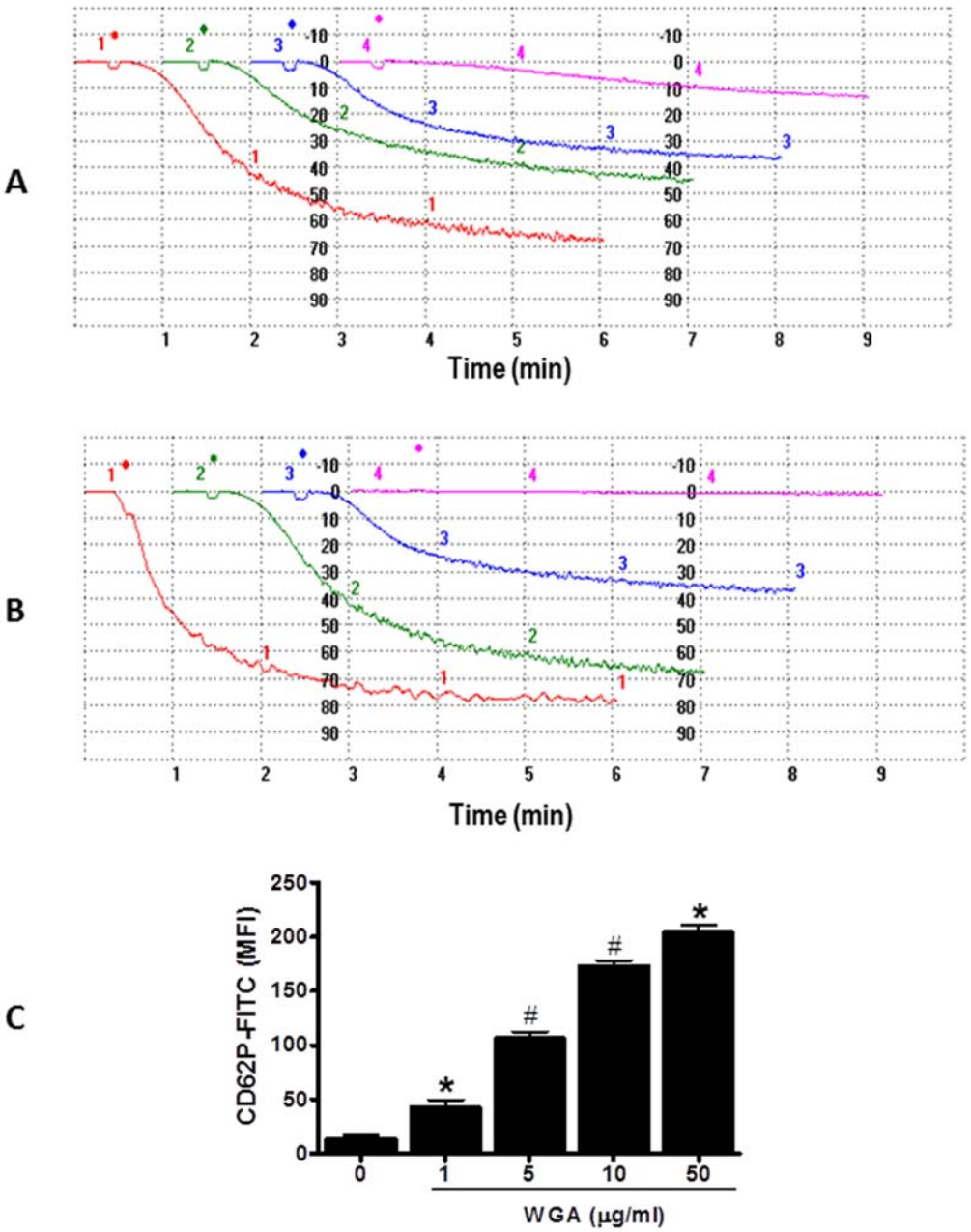


Figure 1 WGA-induced platelet aggregation and P-selectin exposure.

Panel (A): washed platelets (3.0×10^8 platelets/ml) preincubated at 37°C in the presence of saline were challenged with WGA as indicated: 50 µg/ml (line 1), 10 µg/ml (line 2), 5 µg/ml (line 3) and 1 µg/ml (line 4). Panel (B): lines 1, 2, 3: washed platelets (3.0×10^8 platelets/ml) were preincubated with saline at 37 °C and then challenged with 5 µg/ml collagen (line 1), 50 µg/ml WGA (line 2) or PHA (line 3); line 4 indicates spontaneous platelet aggregation (SPA) monitored in the presence of saline. Tracings are representative of four independent

experiments. Panel (**C**): washed platelets (1.0×10^9 platelets/ml) were preincubated with saline at 37°C and then stimulated for 120 s with WGA as indicated. Each bar represents the mean ± SD of four independent experiments. One way ANOVA-Bonferroni's *post hoc* test: *P<0.05; §P<0.01; $^{\#}$P<0.001.

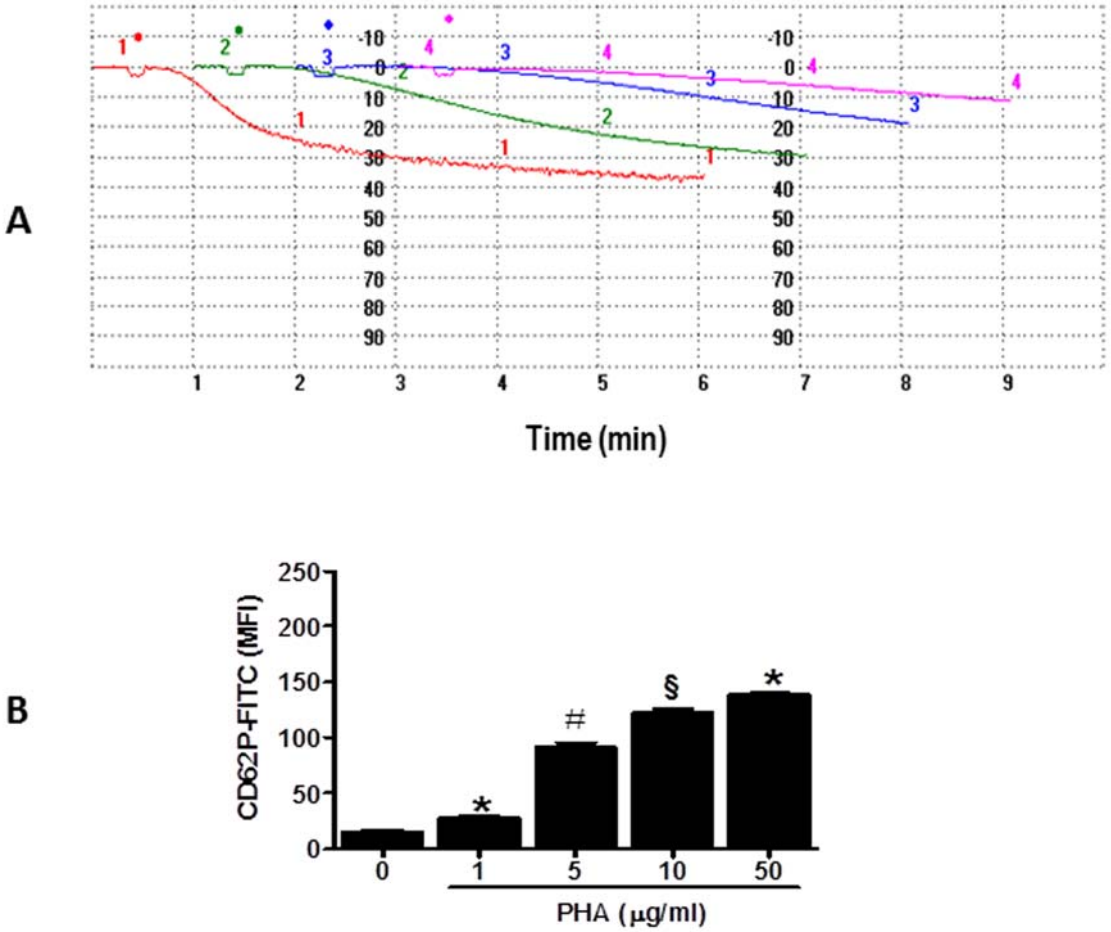


Figure 2 PHA-induced platelet aggregation and P-selectin exposure.

Panel (A): washed platelets (3.0×10^8 platelets/ml) preincubated at 37°C in the presence of saline were challenged with PHA as indicated: 50 µg/ml (line 1), 10 µg/ml (line 2), 5 µg/ml (line 3) and 1 µg/ml (line 4). Tracings are representative of four independent experiments. Panel (B): washed platelets (1.0×10^9 platelets/ml) were preincubated with saline at 37°C and then stimulated for 120 s with PHA as indicated. Each bar represents the mean ± SD of four independent experiments. One way ANOVA-Bonferroni's post hoc test: *P<0.05; §P<0.01; $^{\#}$P<0.001.

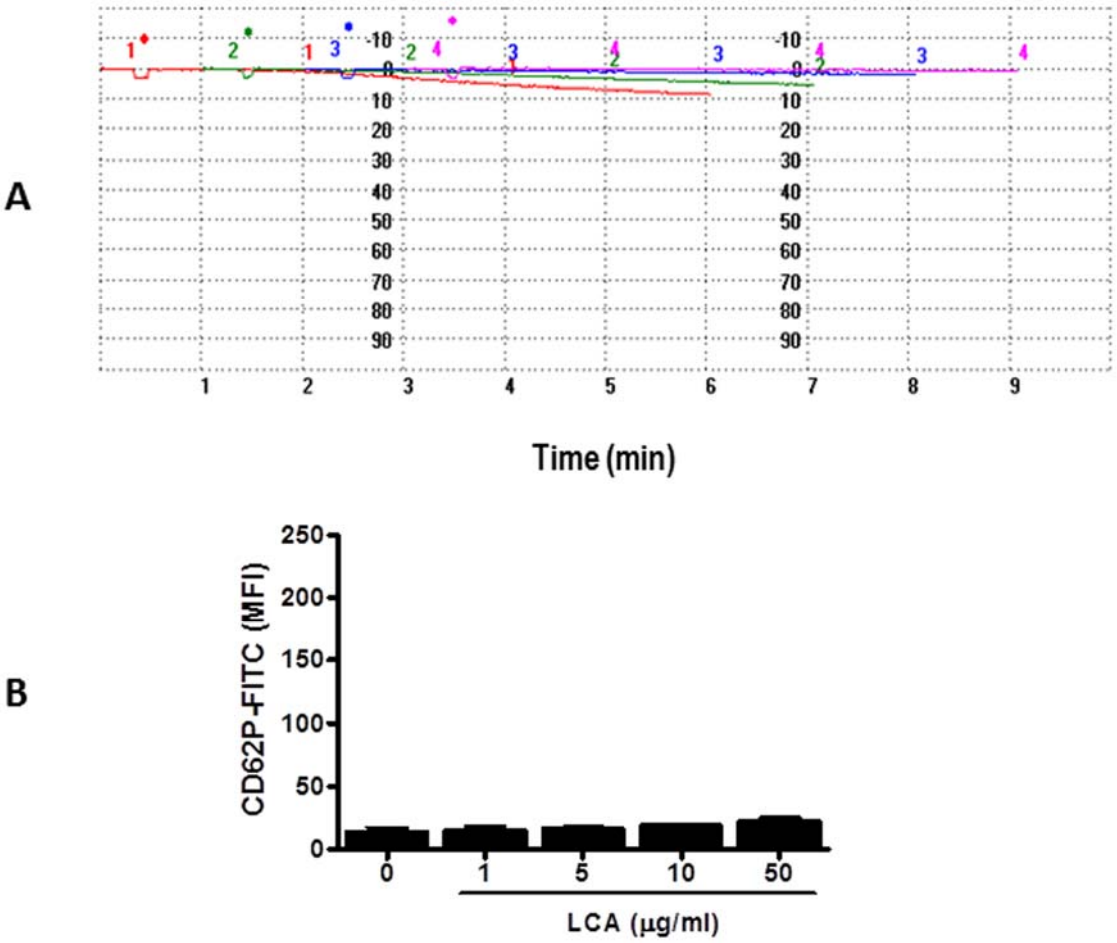


Figure 3 LCA-induced platelet aggregation and P-selectin exposure.

Panel (A): washed platelets (3.0×10^8 platelets/ml) preincubated at 37°C in the presence of saline were challenged with LCA as indicated: 50 μg/ml (line 1), 10 μg/ml (line 2), 5 μg/ml (line 3) and 1 μg/ml (line 4). Tracings are representative of four independent experiments. Panel (B): washed platelets (1.0×10^9 platelets/ml) were preincubated with saline at 37°C and then stimulated for 120 s with LCA as indicated. Each bar represents the mean ± SD of four independent experiments.

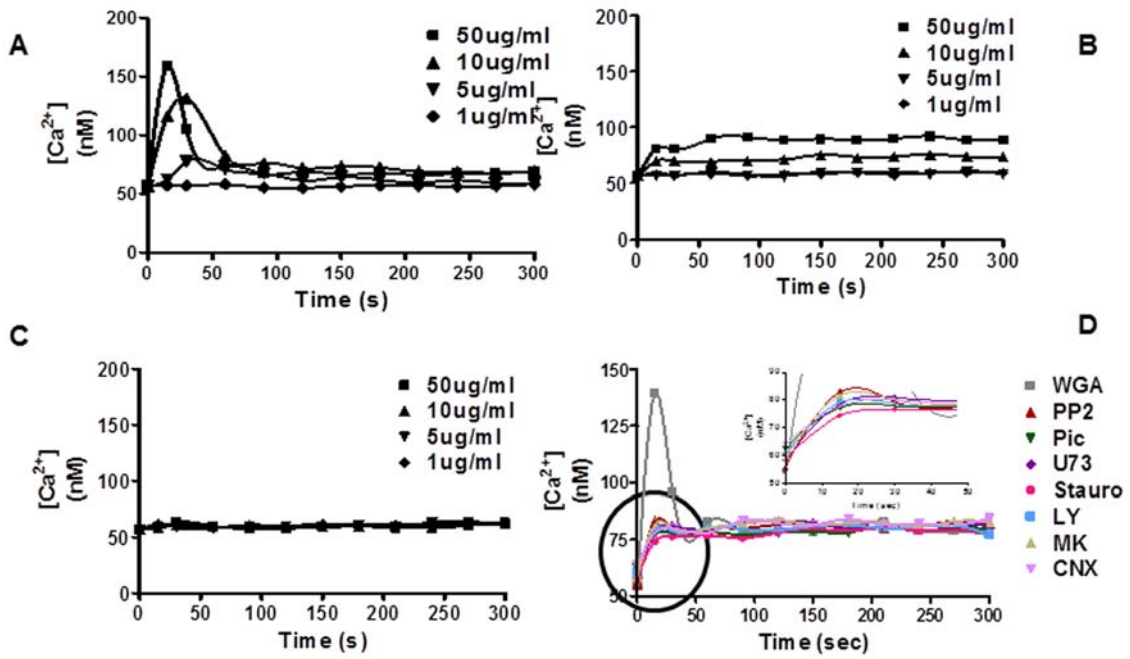
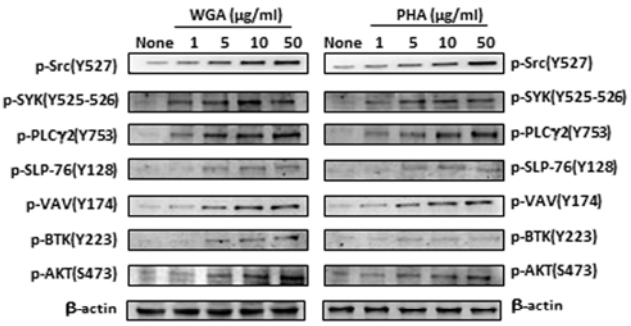


Figure 4 Lectin-induced intracellular Ca^{2+} elevation.

Intracellular Ca^{2+} elevation was quantified in FURA 2-loaded platelets (2.0×10^8 platelets/ml), preincubated with saline at 37°C before the addition of lectin as detailed in the materials and methods section. In panel (D), FURA 2-loaded platelets (2.0×10^8 platelets/ml) were preincubated with 10 µM PP2, 30 µM piceatannol (Pic), 5 µM staurosporine (Stauro), 10 µM U73122 (U73), 20 µM LY294002 (LY), 10 µM MK2206 (MK), or 1 µM CNX-774 (CNX) and then challenged with 10 µg/ml WGA. Ca^{2+} concentration was monitored at 37°C in a fluorescence spectrophotometer, as detailed in the materials and methods section. Tracings are representative of four independent experiments.

A



B

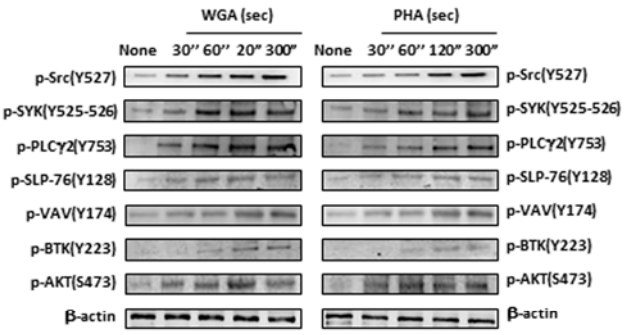
	WGA (μg/mL)				
	0	1	5	10	50
Src(Y527)	0.59	0.92	1.52	1.69	1.82
Syk(Y525-526)	0.66	0.75	0.77	0.85	0.79
PLCγ2(Y753)	0.59	0.71	0.99	1.11	1.14
SLP-70(Y128)	0.61	0.61	0.65	0.68	0.71
VAV(Y174)	0.64	0.65	0.78	0.82	0.85
BTK(Y223)	0.67	0.68	0.85	0.93	0.99
AKT(S473)	0.72	0.74	0.80	0.86	1.04

	PHA (μg/mL)				
	0	1	5	10	50
Src(Y527)	0.52	0.77	0.81	0.92	1.34
Syk(Y525-526)	0.69	0.71	0.76	0.78	0.79
PLCγ2(Y753)	0.60	0.68	0.79	0.93	1.08
SLP-70(Y128)	0.65	0.65	0.68	0.71	0.71
VAV(Y174)	0.67	0.69	0.77	0.82	0.84
BTK(Y223)	0.63	0.64	0.69	0.70	0.72
AKT(S473)	0.71	0.72	0.74	0.74	0.80

Figure 5 The dose-dependent effect on phosphorylation of several proteins of PLCγ2 pathway stimulated by WGA or PHA.

Panel (A): washed platelets (1.0×10^9 platelets/ml), prewarmed at 37°C, were incubated for 120 s with varying concentrations of lectins. At the end of incubation suitable aliquots were immunoblotted with specific primary antibodies as detailed in the materials and methods section. Blots are representative of four independent experiments. In panel (B) the "ratio of ratios", that indicates the real measure of the phosphorylation increase of each enzyme, is reported. For each enzyme the ratio of the measured phosphorylation form and the loading β-actin (ratio 1) and then the ratio between the total enzyme and the loading β-actin (ratio 2) were calculated. The "ratio of the ratios" was obtained by the ratio between ratio 1 and ratio 2.

A



B

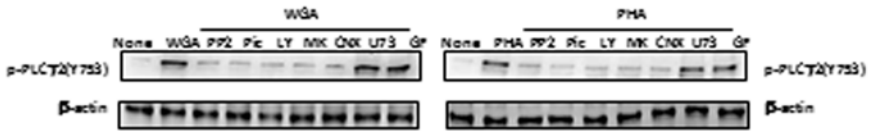
	WGA (sec)				
	0	30	60	120	300
Src(Y527)	0.77	0.95	1.26	1.43	1.61
Syk(Y525-526)	0.79	0.91	1.21	1.35	1.33
PLCγ2(Y753)	0.61	0.77	0.91	1.26	1.44
SLP-70(Y128)	0.65	0.68	0.72	0.78	0.77
VAV(Y174)	0.73	0.77	0.79	0.88	0.97
BTK(Y223)	0.72	0.73	0.81	0.96	0.96
AKT(S473)	0.75	0.80	0.91	1.00	1.03

	PHA (sec)				
	0	30	60	120	300
Src(Y527)	0.75	0.85	0.99	1.26	1.42
Syk(Y525-526)	0.73	0.82	0.97	0.98	1.05
PLCγ2(Y753)	0.61	0.72	0.85	1.01	1.17
SLP-70(Y128)	0.62	0.64	0.66	0.68	0.68
VAV(Y174)	0.67	0.72	0.80	0.87	0.96
BTK(Y223)	0.61	0.61	0.68	0.71	0.71
AKT(S473)	0.64	0.69	0.78	0.76	0.72

Figure 6 The time-dependent effect on phosphorylation of several proteins of PLCγ2 pathway activated by WGA and PHA.

Panel (A): washed platelets (1.0×10^9 platelets/ml), prewarmed at 37°C, were stimulated with 10 μg/ml lectins. At the times indicated suitable aliquots of incubation mixture were withdrawn and immunoblotted with specific primary antibodies as detailed in the materials and methods section. Blots are representative of four independent experiments. In panel (B) the "ratio of ratios", that indicates the real measure of the phosphorylation increase of each enzyme, is reported. For each enzyme the ratio of the measured phosphorylation form and the loading β-actin (ratio 1) and then the ratio between the total enzyme and the loading β-actin (ratio 2) were calculated. The "ratio of the ratios" was obtained by the ratio between ratio 1 and ratio 2.

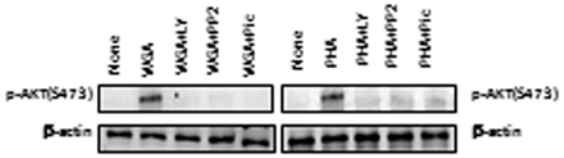
A



B

	PLCγ2(Y753)								
	None	Lectin	PP2	Pic	LY	MK	CNX	U73	GF
WGA	0.61	1.21	0.75	0.77	0.75	0.76	0.79	1.08	1.17
PHA	0.63	0.95	0.77	0.73	0.75	0.75	0.76	0.89	0.91

C



D

	AKT (S473)				
	None	Lectin	LY	PP2	Pic
WGA	0.59	0.84	0.61	0.63	0.61
PHA	0.50	0.80	0.65	0.64	0.67

Figure 7 Effect of varying inhibitors on the phosphorylation of PLCγ2 and AKT induced by WGA and PHA.

Washed platelets (1.0×10^9 platelets/ml) were preincubated at 37°C for 10 min with saline or 10 μM PP2, 30 μM Piceatannol (Pic), 20 μM LY294002 (LY), 10 μM MK2206 (MK), 1 μM CNX-774 (CNX), 10 μM U73122 (U73) or 10 μM GF109203X (GF), and then stimulated for 120 s with 10 μg/ml lectins. At the end of incubation suitable aliquots were immunoblotted with anti-PLCγ2 (Y753) (panel A) or anti-AKT (S473) (panel C) as detailed in the materials and methods section. Blots are representative of four independent experiments. In panels (B) and (D), for each enzyme the ratio of ratios is reported. The calculation of the "ratio of ratios" is detailed in the legends of Figures 5 and 6.

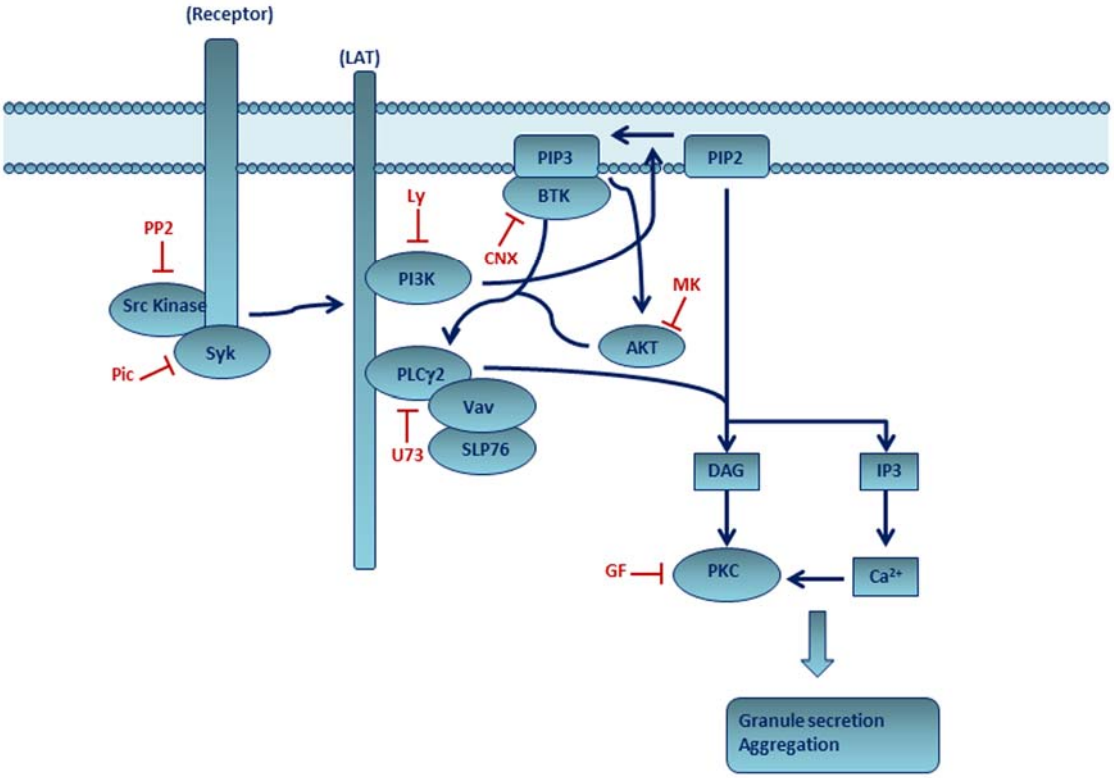


Figure 8 Signalling pathways involved in platelet activation by WGA or PHA. Activation of unknown receptor induces the recruitment of Syk mediated by Src kinases. Activated Syk promotes activation of signalling cascade that leads to the activation of PLCγ2 and PI3K. PI3K phosphorylates PIP2 to PIP3 that participates to the activation of BTK and AKT. These enzymes, together with SLP-76 and VAV, are involved in the full activation of PLCγ2, leading to intracellular Ca^{2+} increase, granule secretion and platelet aggregation.