Phosphatidylinositol 3′-kinase and tyrosine-phosphatase activation positively modulate Convulxin-induced platelet activation. Comparison with collagen

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Abstract In this report we have studied the role of phosphatidylinositol 3′-kinase (PI3-K) and tyrosine phosphatase activation on platelet activation by Convulxin (Cvx). Wortmannin, a specific PI3-K inhibitor, and phenylarsine oxide (PAO), a sulfhydryl reagent that inhibits tyrosine phosphatase (PTPase), block Cvx-induced platelet aggregation, granule secretion, inositol phosphate production, and increase in $[Ca^{2+}]_i$. However, PAO does not inhibit Cvx-induced tyrosine phosphorylation of platelet proteins, including Syk and PLCγ2, but blocked collagen-induced platelet aggregation as well as tyrosine phosphorylation of PLCγ2. In contrast, Cvx-induced PLCγ2 tyrosyl phosphorylation was partially inhibited by wortmannin. We conclude that (i) although Cvx and collagen activate platelets by a similar mechanism, different regulatory processes are specific to each agonist; (ii) mechanisms other than tyrosine phosphorylation regulate PLCγ2 activity; and (iii) besides protein tyrosine kinases, PI3-K (and PTPase) positively modulate platelet activation by both Cvx and collagen, and this enzyme is required for effective transmission of GPVI-Fc receptor γ chain signal to result in full activation and tyrosine phosphorylation of PLCγ2 in Cvx-stimulated platelets.

Key words: Convulxin; Phospholipase Cγ; Protein tyrosine phosphorylation; Phenylarsine oxide; Phosphatidylinositol 3-kinase; Tyrosine phosphatase; Wortmannin; *Crotalus durissus terrificus*

1. Introduction

Convulxin is a 72 kDa glycoprotein, isolated from the venom of *Crotalus durissus terrificus* and *Crotalus durissus cascavella*, composed of 3α (13.9 kDa) and 3β (12.6 kDa) chains linked by disulfide bridges performing an α3β3 heterodimeric complex [1,2]. The cloning of both subunits of Cvx has recently been reported [3], and they present sequence homology with the carbohydrate recognition domain of the C-type lectin family; however, Cvx does not function as a lectin, as it does not induce agglutination of erythrocytes in a number of species [4]. Cvx is a potent inducer of platelet activation [5] and binds with high affinity to rabbit [4] and human [6] platelets. Cvx activates PLC [7] by a mechanism that is not blocked by the cyclooxygenase inhibitor aspirin, by the PAF antagonist WEB 2086, and/or by the ADP scavenger enzymatic system CP/CPK, indicating that PLC activation is independent of the secondary agonist produced by activated platelets [5,7]. Cvx-induced PLC activation is, however, blocked by inhibitors acting on the protein tyrosine kinases, genistein and staurosporine [8]. Actually, Cvx stimulates a rapid tyrosine phosphorylation of several platelet proteins, including PLCγ2, by a mechanism independent of fibrinogen interaction with integrin αIIbβ3 and insensitive to cAMP [8,9]. This early event is followed by the dephosphorylation of several proteins in a platelet aggregation-dependent manner [8,9]. It has been shown that Cvx binds to the putative collagen receptor GPVI [6,10] and that Cvx-mediated platelet adhesion, platelet aggregation, and $[Ca^{2+}]_i$ mobilization are blocked by the Fab fragments of a polyclonal anti-GPVI antibody [6]. More recently, it has also been shown that GPVI physically associates with the Fc receptor γ chain [11,12] and that this complex associates physically and functionally with the Src family kinases Fyn and Lyn [13]. A model has been proposed to explain the early steps of signaling by both Cvx [9] and collagen [14]. Accordingly, GPVI ligation by both molecules induces Src kinase activation, leading to tyrosyl phosphorylation of the ITAM motif of the Fc receptor γ chain and subsequent tyrosyl phosphorylation of Syk and PLCγ2 [8–17]. This model clearly shows that a cascade of kinase activation is essential for PLCγ2 tyrosyl phosphorylation by collagen and Cvx; however, PLC activation is modulated in a number of cells by additional mechanisms, e.g. the identification of the tyrosine phosphatases (PTPases) CD45 and SHP1 as positive and negative regulators, respectively, of lymphocyte signaling [17]. More recently, the identification of PI3-K, a lipid kinase, as another enzyme involved in the regulation of phosphoinositide metabolism and PLCγ activation upon stimulation by growth factor receptors, indicates that phosphoinositide metabolism is under the control of different enzymes [18,19]. In fact, phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phosphatidylinositol 3′-kinase (PI3-K) leads to the production of phosphatidylinositol 3,4,5-trisphosphate (PIP_3). PIP_3 serves as a docking site for the PH domain of both Bruton's tyrosine kinase, a Src-related tyrosine kinase

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Abbreviations: Cvx, convulxin; PAO, phenylarsine oxide (PTPase inhibitor); PH domain, pleckstrin homology domain; PI3-K, phosphatidylinositol 3′-kinase; PTPase, tyrosine phosphatase; PY20, antiphosphotyrosine monoclonal antibody

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that is involved in B cell antigen receptor-mediated activation of PLCγ2 [20], and PLCγ itself.

The aim of our study was to investigate further the mechanism of PLC activation by Cvx, studying the effects of two structurally and functionally unrelated reagents on platelet responses to this toxin, including Syk and PLCγ2 tyrosine phosphorylation. The first inhibitor, phenylarsine oxide (PAO), is a sulfhydryl reagent that inhibits PTPase. It is a suitable inhibitor to be tested because PTPase inhibitors have been shown to block collagen-induced platelet activation [21,22], including PLCγ2 tyrosyl phosphorylation [22]; PAO also inhibits FcRI-induced myeloid oxidant burst signaling in U937IF cells [23]. The second inhibitor, PI3-kinase inhibitor wortmannin, was used in an attempt to detect the contributions of PI3-K activation to signaling triggered by Cvx. Our findings suggest that both PTPase and PI3-K activation function as positive modulators of platelet activation by Cvx.

2. Materials and methods

2.1. Materials

Cvx was purified from the venom of *Crotalus durissus terrificus* from FUNED (Fundação Ezequiel Dias, Minas Gerais, Brazil) as previously described [4]. Antiphosphotyrosine monoclonal antibody (PY20), anti-PLCγ2 and anti-Syk polyclonal antibodies, and protein A/G-Sepharose were from Santa Cruz Biotechnology (Santa Cruz, CA). The peroxidase-coupled donkey anti-rabbit and sheep anti-mouse IgGs, the chemiluminescent reagent ECL, [^{14}C]5-hydroxytryptamine and *myo*[2-^{3}H]inositol were from Amersham (Les Ulis, France). Dowex 1-X8 AG anion exchange resin (format form) was from Bio-Rad (Richmond, CA). Collagen from equine tendons was from Horm (Hormon-Chemie, Munich, Germany). Bovine serum albumin fraction V (BSA), prostaglandin E1, apyrase fraction V, gelatin, phenylarsine oxide, vanadate, fura 2-acetoxymethylester (fura 2-AM), and Nonidet P40 were from Sigma (St. Louis, MO), and polyvinylfluoride membranes (PVDF) from Millipore (Bedford, MA). The PI3-kinase inhibitors wortmannin and LY 294002 were from Calbiochem (San Diego, CA).

2.2. Platelet preparation

Blood from healthy volunteers not on medication was collected on acid-citrate-dextrose anticoagulant. Platelet-rich plasma was obtained by centrifugation at $110\times g$ for 15 min. In some cases, platelets were labeled by incubating PRP with 0.6 μM [^{14}C]5-hydroxytryptamine for 30 min at 37°C. After acidification of the PRP to pH 6.5 with 1/10 vol of acid-citrate-dextrose anticoagulant and the addition of 100 nM PGE1 and 25 μg/ml apyrase, platelets were sedimented by centrifugation at $1100\times g$ for 15 min and washed twice by centrifugation in the washing buffer (103 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5 mM glucose, 36 mM citric acid, pH 6.5, containing 3.5 mg/ml BSA, 100 nM PGE1, and 25 μg/ml apyrase). Washed platelets were resuspended at the concentrations indicated in the text in the reaction buffer consisting of 5 mM HEPES, 137 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 5.5 mM glucose, pH 7.4, containing 1.5 mg/ml BSA or 2.5 mg/ml gelatin.

2.3. Platelet aggregation and secretion

Washed platelets were preincubated with PAO or wortmannin for 2 and 10 min, respectively at 37°C, before aggregation was initiated by the addition of Cvx or collagen. Control experiments were performed with platelets preincubated with a buffer containing the same concentration of DMSO as that contained in the inhibitors. Experiments were performed with stirring at 37°C in a Chrono-Log aggregometer (Chrono-Log Corp., Haverton, PA). Release of [^{14}C]5-hydroxytryptamine was measured as described previously [24]. Alternatively, platelets were lysed for analysis of protein tyrosine phosphorylation or for immunoprecipitation.

2.4. Measurement of intracellular Ca^{2+} concentration

Intracellular free calcium ($[Ca^{2+}]_i$) transients were monitored by fura-2 fluorescence as previously described. Briefly, platelets loaded with 2 μM fura 2-AM were washed twice and resuspended in a reaction buffer. Platelets (2×10^8/ml) were then preincubated with 2 mM $CaCl_2$ or 2 mM EGTA in the cuvette prior to the addition of Cvx or collagen. Fluorescence was measured at 37°C using two excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm on an Hitachi H-2000 spectrofluorometer (Sciencetec, Les Ulis, France). Ca^{2+} concentrations were calculated using a K_d of 224 nM for the interaction between fura 2 and Ca^{2+} [24].

2.5. Measurement of inositol-phosphates production

After the first washing step, platelets (2×10^9/ml) were incubated at 37°C for 3 h, with *myo*[2-^{3}H]inositol (25 μCi/ml) in a reaction buffer (without $NaHCO_3$) at pH 7.4, in which 2 mM EDTA, 100 nM PGE1 and 25 μg/ml apyrase were added to prevent platelet activation. After washing, platelets were resuspended at 5×10^8/ml in a reaction buffer containing 10 mM $LiCl_2$. Platelets were incubated with 1 μM PAO, 0.1 μM wortmannin or DMSO as above. Platelets were activated by 2 nM Cvx or 2 μg/ml collagen for 4 min in the aggregometer. Activation was stopped by ice-cold 0.1 M EDTA and centrifugation. Platelets were lysed by 10 mM formic acid and two freezing and thaw cycles and samples were neutralized with 10 mM ammoniac. Total inositol phosphates were separated on a Dowex 1-X8 AG anion exchange resin (format form). Following elution of [^{3}H]inositol and [^{3}H]glycerophosphoinositol with 40 mM ammonium formate, total inositol phosphates were eluted with 2 M ammonium formate. The radioactivity present in all fractions as determined by scintillation counting (Beckman Instruments).

2.6. Analysis of protein tyrosine phosphorylations

Platelets (200 μl, 5×10^8/ml) were lysed by the addition of 20 μl of a 10×SDS lysis buffer composed of 0.625 M Tris pH 6.8, containing 50% (v/v) 2-mercaptoethanol, 30% SDS, 100 mM vanadate, and 10 mM PAO. Platelet lysates were heated for 5 min at 100°C. Samples were adjusted for their platelet content before analysis.

2.7. Immunoblotting studies

Proteins from whole platelet lysates or immunoprecipitated proteins were separated by SDS-PAGE and transferred to PVDF membranes in 50 mM Tris, 95 mM glycine, 20% methanol, containing 0.1% (w/v) SDS and 100 μM Na_2VO_4. The membranes were blocked with 15 mg/ml BSA in 20 mM Tris, 150 mM NaCl, pH 8 (TBS) and were incubated with 1 μg/ml PY20 in TBS containing 0.05% (v/v) Tween 20 and 0.1% BSA for 2 h at room temperature. Membranes were washed in TBS-Tween and incubated with anti-mouse peroxidase-coupled secondary antibody diluted 1:7500. Following washing immunoblots were developed using enhanced chemiluminescence detection. Equivalent protein loading for immunoprecipitated proteins was verified by reprobing with a relevant antibody.

2.8. Immunoprecipitation

Platelets (500 μl, 5×10^8/ml) were lysed by the addition of 200 μl of 3.5×ice-cold immunoprecipitation buffer (10 mM Tris, 300 mM NaCl, 12 mM EDTA, pH 8, containing 1% Nonidet P40, 5 mM Na_2VO_4, and 2 mM PMSF). After 30 min on ice, samples were precleared by incubation with protein A-Sepharose for 15 min at 4°C and centrifugation to limit non-specific precipitation. Cleared lysates adjusted for their platelet content were then incubated with anti-PLCγ2, anti-Syk polyclonal antibody, or PY20 (1 μg/ml) for 1 h at 4°C. After the addition of protein A/G-Sepharose, incubation was continued overnight at 4°C. Samples were centrifuged at $4500\times g$ for 2 min. The supernatant was kept for further precipitation, and the protein A/G pellet was washed twice in the immunoprecipitation buffer. Immunoprecipitated proteins were solubilized by 2% (w/v) SDS reduced with 5% (v/v) 2-mercaptoethanol. In some experiments, quantification of tyrosine phosphorylation of immunoprecipitated proteins was performed in a Onescanner MacIntosh, and the analysis was made with an NIH Image. Results are expressed as % of tyrosine phosphorylation of at least two independent experiments; 100% was settled as phosphorylation in the presence of inhibitor vehicle (DMSO, 0.5–1%, v/v).

3. Results and discussion

3.1. PAO inhibits Cvx- and collagen-induced platelet aggregation, secretion, inositol phosphate production and $[Ca^{2+}]_i$ increase

Aggregation of washed platelets induced by an optimum concentration of Cvx (2 nM) was inhibited by pretreatment of platelets with PAO (Fig. 1A). The effect of PAO is dose dependent, with an IC_{50} of 0.4 μM. PAO also inhibited collagen-induced platelet aggregation (Fig. 1A), while thrombin-induced platelet aggregation was not significantly reduced by PAO at concentrations up to 2.5 μM, in agreement with previous reports [21,22]. As platelet aggregation, dense-granule release induced by Cvx or by collagen was inhibited by PAO we have thus investigated the effect of PAO on Cvx-induced IPs production. Inhibition of Cvx- and collagen-induced platelet aggregation by PAO was accompanied by more than 65% and 45% inhibition of IP production, respectively (Fig. 1B). Next, the effects of PAO on the Cvx-induced increase in Ca^{2+} were studied. Convulxin induces Ca^{2+} increase in the presence and absence of external Ca^{2+} (Fig. 1C) indicating that Cvx induces intracellular Ca^{2+} mobilization as well as Ca^{2+} entry, in agreement with previous results [6,8]. In the presence of 0.1 μM PAO, Cvx-induced increase in $[Ca^{2+}]_i$ was decreased by about 25%, while it was completely blocked by 1 μM PAO. These results indicate that an early step in the signal transduction pathway induced by Cvx is susceptible to PAO. Furthermore, at this point of our study, the observation that PAO similarly inhibited platelet responses triggered by Cvx or collagen favored the hypothesis that Cvx and collagen induce platelet activation by a common pathway.

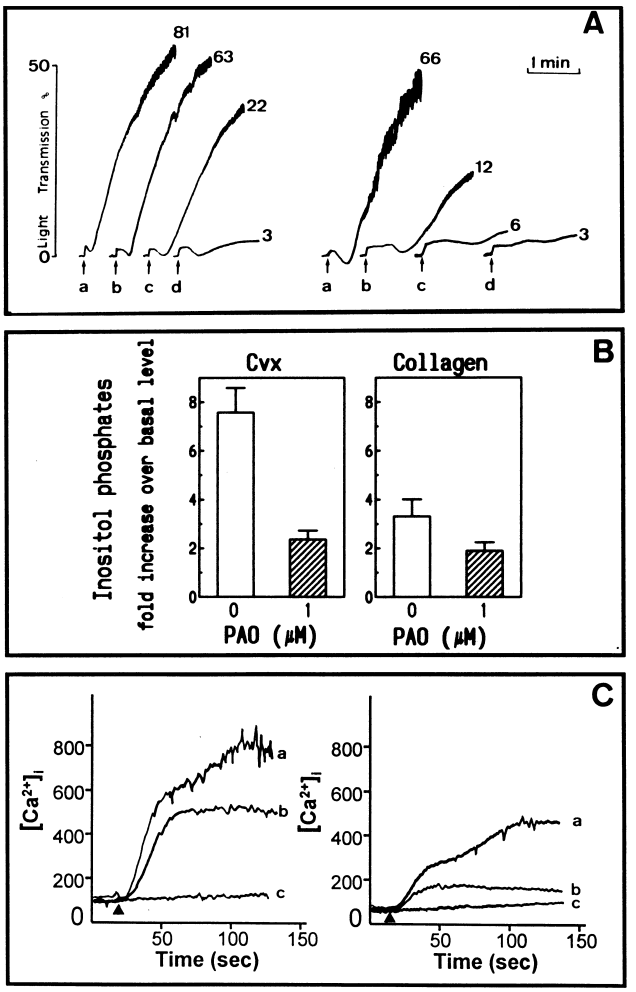

Fig. 1. Effect of tyrosine phosphatase inhibitor, phenylarsine oxide (PAO) on Cvx-induced platelet aggregation, secretion, inositol-phosphates production, and $[Ca^{2+}]_i$ increase. Comparison with collagen. A: Washed platelets were incubated with buffer (a) or in the presence of 0.2 (b), 0.5 (c), or 1 μM PAO (d). Aggregation was initiated (arrow) by 2 nM Cvx (left panel) or 1 μg/ml collagen (right panel). The percentage of serotonin released in indicated beside the curves. B: Effect of PAO on Cvx- and collagen-induced inositol-phosphates production. Washed platelets loaded with *myo*[2−^{3}H]inositol were stimulated by 2 nM Cvx (left panel) or 1 μg/ml collagen (right panel) in the absence of presence of PAO (1 μM). Separation of inositol phosphates was performed as described in Section 2. Results are expressed as the increase in inositol phosphates over the basal level (non-stimulated platelets) in the absence or presence of PAO. C: Effect of PAO on Cvx-induced calcium mobilization fura 2-loaded platelets were preincubated with buffer (a), or PAO 0.1 μM (b) or 1 μM (c). Calcium increase was induced by 2 nM Cvx in the presence of 2 mM $CaCl_2$ (left panel) or 2 mM EDTA (right panel).

3.2. PAO does not inhibit Cvx-induced protein tyrosine phosphorylation

The inhibition by PAO of Cvx-induced $[Ca^{2+}]_i$ increase indicates that an early step of the signaling cascade triggered by Cvx was blocked. Indeed, the observation that inositol-phosphates production was impaired by PAO, suggested that this compound might inhibit PLC activation or activity. In fact, PAO has previously been reported to inhibit tyrosine phosphorylation of PLCγ2 in collagen- and FcγRIIA-stimulated platelets [22]. Since Cvx induces tyrosine phosphorylation of several proteins including PLCγ2 and Syk, we have studied the effect of PAO on these responses. Non-stimulated platelets contain constitutively tyrosine phosphorylated proteins, in particular proteins of 55–60 kDa. Platelet pretreatment with 1 μM PAO alone slightly increased the intensity of some proteins (Fig. 2A,B), probably by inhibiting constitutively activated PTPases. Fig. 2A shows that whole platelet protein tyrosyl phosphorylation triggered by Cvx was increased by pretreatment with PAO at concentrations that also attenuated platelet aggregation, suggesting that the inhibitory effect of PAO is associated with inhibition of PTPases. In contrast, the intensity of the protein tyrosine phosphorylation triggered by collagen was not modified in the presence of 1 μM PAO (Fig. 2B), confirming previous reports and providing evidence to suggest that a different site from PTPase, yet to be identified, may be blocked by PAO in collagen-stimulated platelets [22].

The effects of PAO on the intensity and number of tyrosine-phosphorylated proteins in Western blots of Cvx-stimulated platelets may be explained by a change in the equilibrium between the phosphorylation/dephosphorylation reactions toward the phosphorylation reaction. Although our results do not reveal the PAO inhibitory mechanism of Cvx-induced platelet activation, they do support the contention that PTPase activation has a positive modulatory function on platelet activation [25–28]. Indeed, dephosphorylation of Src-like kinases that are involved in signaling by Cvx and collagen [13,14], results in the catalytic activation of Src-related kinases [29]. More recently, it has also been shown that PAO blocks FcγRI-induced myeloid oxidant signaling together with an increase of the intensity and number of tyrosyl-phospho-

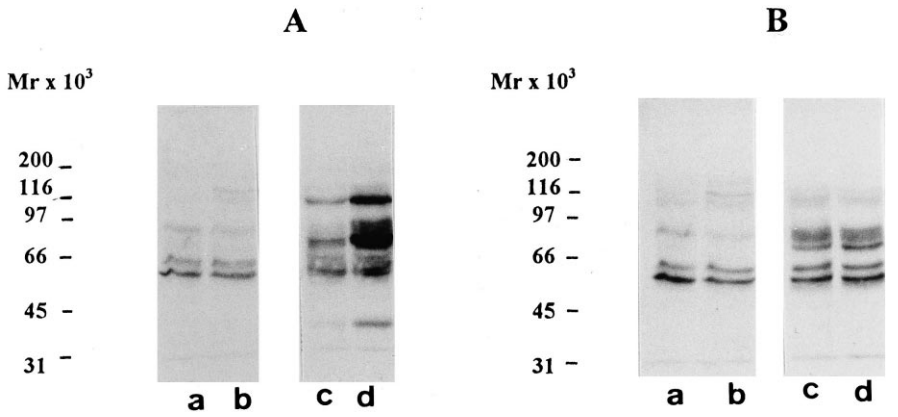

Fig. 2. Effect of PAO on platelet protein tyrosine phosphorylation induced by Convulxin or collagen. A: Washed platelets were incubated with buffer (a, c) or PAO 1 μM (b, d) before activation by 2 nM Cvx for 20 s (lanes c, d). B: Washed platelets were incubated with buffer (a, c) or PAO 1 μM (b, d) before activation by 1 μg/ml collagen for 40 s (lanes c, d). In A and B platelets were lysed and proteins analyzed by immunoblotting with PY20. The same number of platelets (10^7) was present in all the wells.

rylated proteins in U937IF cells [23]. It is of interest that this event is accompanied by an augmentation of the interaction of the adaptor protein Cbl with other phosphoproteins, among them SHC and CRKL. Since Cvx also phosphorylates Cbl in addition to syk, PLCγ2, the Fc receptor γ chain, and the 36–38 kDa adaptor proteins [8–10], it is plausible to suggest that some of these proteins bind to each other and that this association should be modulated by the level of tyrosyl phosphorylation and hence, by PTPases. In other words, tyrosine dephosphorylation is required for effective transmission of GPVI signaling to result in full platelet activation by Cvx and at least in part, by collagen.

In an attempt to determine the site of inhibition of Cvx- and collagen-induced platelet activation by PAO, we investigated the effects of this inhibitor on tyrosine phosphorylation of Syk and PLCγ2 by immunoprecipitation assays (Fig. 3A,B). Although GPVI ligation by both collagen [22] and Cvx induces a PAO-insensitive tyrosyl phosphorylation of the tyrosine kinase Syk (Fig. 3B), PLCγ2 tyrosyl phosphorylation induced by collagen [22], but not by Cvx, is inhibited by PAO (Fig. 3A). Differences in signals triggered by Cvx and collagen have been previously reported. In fact, we have shown that inhibition of Cvx-, but not collagen-induced platelet responses by PAO is accompanied by an increase in PTP. In addition, PLCγ2 tyrosyl phosphorylation triggered by Cvx occurs as early as 10 s after the Cvx addition to platelets [8,9], whereas PLCγ2 is tyrosyl phosphorylated by collagen at later time points [30,31]. Moreover, piceatannol, a Syk inhibitor, blocks platelet aggregation and protein tyrosine phosphorylation triggered by collagen, whereas it produced only a partial inhibition of Cvx-induced platelet aggregation [10]. Taking into consideration that collagen may interact with several receptors at the platelet surface (GPIV, integrin α2β1, p65) [32–35] while Cvx-induced platelet activation appears to occur mainly through GPVI ligation [6,10], it is not surprising that some differences exist at the level of the signaling pathways and their regulation. In addition, our results indicate that tyrosine phosphorylation of PLCγ2 and PLC activity

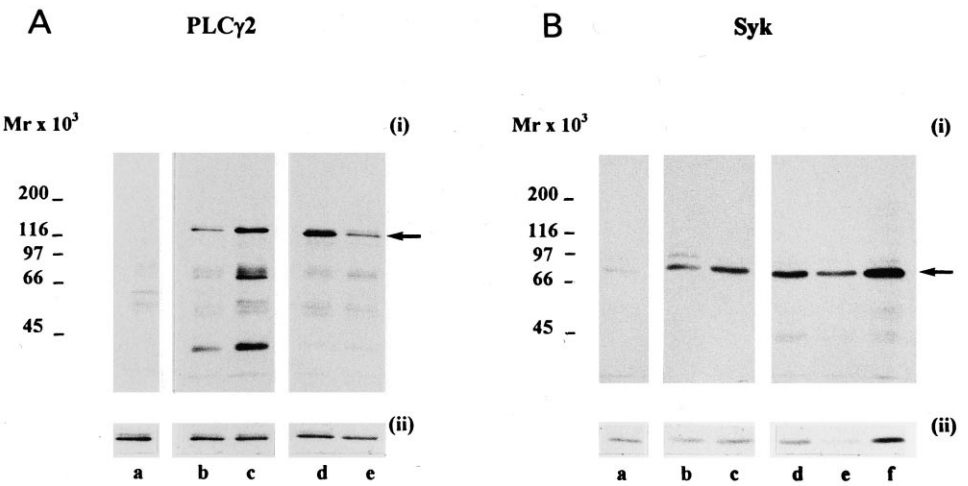

Fig. 3. Effect of PAO on PLCγ2 and Syk phosphorylation triggered by Convulxin or collagen. A: PLCγ2 was immunoprecipitated from platelets incubated with buffer (a, b, d) or PAO 1 μM (c, e) and activated by 2 nM Cvx (b, c) or 1 μg/ml collagen (d, e). Blots were incubated with anti-PY20 (i) and anti PLCγ2 (ii). B: Syk was immunoprecipitated from platelets incubated with buffer (a, b, d) or 1 μM PAO (c, e, f) before activation with Cvx (b, c) or collagen (d–f). Blots were incubated with anti-PY20 (i) and anti-Syk (ii). The intensity of the phosphorylated band (i) in d–f is proportional to the amount of protein (Syk) in the gel (ii). As a control, in f, the content of platelet protein loaded is 2.5 times higher than in d.

may uncouple, PLCγ2 being strongly phosphorylated in response to Cvx in the presence of PAO but without producing $[Ca^{2+}]_i$ increase and bringing inositol phosphates and platelet aggregation to near basal levels. Uncoupling of tyrosine phosphorylation of PLCγ2 and production of inositol phosphates has also been shown in platelets stimulated by Cvx, in the presence of the cAMP increasing agent, forskolin [9]. Thus, additional regulatory mechanisms may be involved in the expression of PLC activity by Cvx-induced tyrosine-phosphorylated PLCγ2.

3.3. PI3-kinase is involved in Cvx- and collagen-induced platelet activation

It has been shown that the binding of growth factors to appropriate cells is accompanied by tyrosyl phosphorylation of the receptor with its subsequent interaction with PLCγ1, leading to its tyrosyl phosphorylation; however, PLCγ1 express PLC activity once a lipid kinase, PI3-kinase, is also activated by a receptor-mediated mechanism [18,19,36–39]. Indeed, PI3-K is essential for PLCγ activity because the phosphorylation of the 3′ position of the inositol ring of PtdIns(4,5)P_2 produces PtdIns(3,4,5)P_3, a phospholipid that serves as a docking site for the pleckstrin homology (PH) domain of tyrosyl-phosphorylated PLCγ, targeting the enzyme to the vicinity of its membrane substrate, PtdIns(4,5)P_2 [18,19]. To determine whether PI3-kinase was involved in Cvx-induced platelet activation, experiments were performed in the presence of the PI3-kinase-specific inhibitor, wortmannin. Cvx-, as well as collagen-induced platelet aggregation and secretion, was largely inhibited by wortmannin, suggesting that an early step of platelet activation was blocked (Fig. 4A). Another PI3-K inhibitor, LY294002, inhibited Cvx-induced platelet aggregation and secretion as efficiently as wortmannin but was less potent in inhibiting collagen-induced platelet aggregation and secretion (not shown). Wortmannin at concentrations that brought platelet aggregation to basal levels also caused 80% inhibition of IP production triggered by Cvx (Fig. 4B) and 50% triggered by collagen (Fig. 4B). The finding that total inhibition of platelet aggregation by wortmannin was accompanied by partial inhibition of IP production by Cvx and collagen suggest that (i) wortmannin (and PAO) may also affect the platelet aggregation itself (e.g. modulation of integrin αIIbβ3 function) and/or (ii) additional mechanism, PI3-kinase-independent, may trigger IP production in Cvx- and collagen-stimulated platelets such as PLA2-dependent, TXA2-mediated IP production, or via tyrosine-kinase activation (see below). Fig. 4C shows that wortmannin lowered the Cvx-induced increase in $[Ca^{2+}]_i$ in the presence and in the absence of external Ca^{2+} (Fig. 4C). The effects of PI3-kinase inhibition on PLCγ2 phosphorylation were thus tested (Fig. 4D). At concentrations that completely blocked platelet aggregation, wortmannin caused 55% and 48% inhibition of tyrosine phosphorylation of PLCγ2 when the agonist was Cvx or collagen, respectively.

Taken together, our results suggest that inhibition by wortmannin of Cvx and collagen-induced PLCγ2 activation may

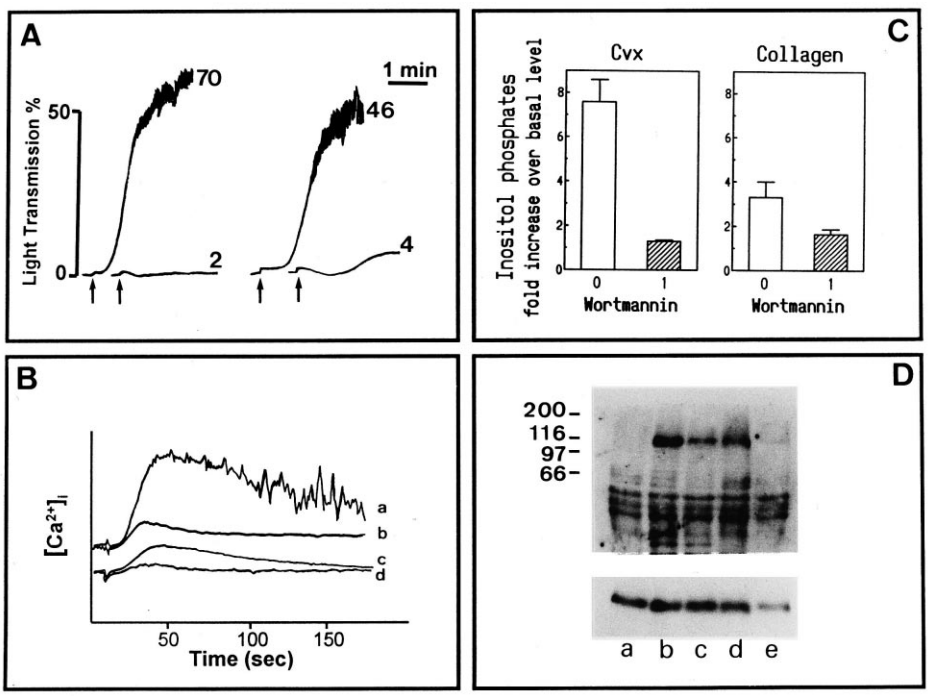

Fig. 4. Effect of PI3-kinase inhibition on Cvx-induced platelet aggregation, secretion, inositol-phosphates production, $[Ca^{2+}]_i$ increase, and PLCγ2 phosphorylation. Comparison with collagen. A: Washed platelets were incubated with DMSO (a) or 100 nM wortmannin (b) before activation with 2 nM Cvx (left) or 1 μg/ml collagen (right). The percentage of serotonin released is indicated beside the curves. B: Platelets loaded with *myo*[2−^{3}H]inositol were stimulated by 2 nM Cvx (left panel) or collagen (right panel) in the absence or presence of wortmannin (0.1 μM). Separation of inositol phosphates was perfomed as described in Section 2. Results are expressed as the increase in inositol phosphates over the basal level (non-stimulated platelets) in the absence or presence of wortmannin. C: Fura-2-loaded platelets were incubated with buffer (a, c) or 100 nM wortmannin (b, d) before activation with 200 pM Cvx in the presence of 2 mM $CaCl_2$ (a, b) or 2 mM EDTA (c, d). D: Tyrosine phosphorylation of PLCγ2 was carried out as in Fig. 3. (a) non-activated platelets; (b) Cvx 75 pM for 20 s; (c) wortmannin 100 nM for 5 min followed by Cvx as in b; (d) collagen, 1 μg/ml for 40 s; (e) wortmannin 100 nM followed by collagen as in d. Blots were incubated with anti-PY20 (i) and anti-PLCγ2 (ii).

proceed by at least two mechanisms. First, the inhibition of $PI(3,4,5)P_3$ production may impair PLCγ2 docking at the membrane in the proximity of its substrate $PI(4,5)P_2$ [18,19]. Indeed, a number of papers have shown that the biological effects mediated by PI3-K activation occur independently of tyrosine phosphorylation; (i) activation of phospholipase C-γ by $PI(3,4,5)P_3$ occurs independently of tyrosine phosphorylation [40–42], (ii) $PI(3,4,5,)P_3$ triggers Ca^{2+} influx in rabbit platelets, leading to platelet aggregation [43], (iii) wortmannin treatment did not influence the ability of PDGF to induce tyrosine phosphorylation of PLCγl in COS-1 cells but largely decreased $Ins(1,4,5)P_3$ production [19], (iv) FcγRIIA mediated-platelet secretion and aggregation, as well as phospholipase C (PLC) activation, but not PLCγ2 tyrosyl phosphorylation, is abolished by wortmannin or LY294002 [40].

Second, since we observed partial but reproducible inhibition of PLCγ2 tyrosyl phosphorylation by wortmannin in Cvx- and collagen-stimulated platelets, PI3-K may be also involved in an alternative pathway leading to PLCγ2 tyrosyl phosphorylation other than, or complementary to, Fc receptor γ chain-Syk-mediated tyrosyl phosphorylation. This pathway may be the activation of Bruton's tyrosine kinase, a PH domain containing Src-related PTK related to PLCγ2 activation in the B cell [20] and in collagen-activated platelets [44]. Additional tyrosine kinases and adaptor molecules may also be involved in this event. Finally, the finding that the p85 subunit of PI3-K associates with the Fc receptor γ chain in platelets stimulated with collagen and Cvx, provides an additional support to the contention that PI-3K participates in the GPVI-Fc receptor γ chain collagen receptor [45].

In conclusion, our results show that the unrelated inhibitors PAO and wortmannin similarly affect platelet function in Cvx-stimulated platelets by a mechanism that uncouples tyrosyl phosphorylation of PLCγ2 and PLC activity. Our results provide evidence that phosphoinositide metabolism in Cvx-stimulated platelets is a highly regulated and redundant event that may involve cross-talk between tyrosine kinases, lipid kinases, adaptor proteins, tyrosine and lipid phosphatase. The molecular mechanism involved in this cascade of signaling events awaits further investigation.

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