Phenylarsine oxide inhibits tyrosine phosphorylation of phospholipase Cγ2 in human platelets and phospholipase Cγ1 in NIH-3T3 fibroblasts

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Abstract The sulphydryl reagent phenylarsine oxide (PAO) (1 μM) inhibited completely formation of inositol phosphates in human platelets induced by collagen or by cross-linking of the platelet low affinity Fc receptor, FcγRIIA, but did not alter the response to the G protein receptor agonist thrombin. PAO also inhibited completely tyrosine phosphorylation of PLCγ2 in collagen and FcγRIIA-stimulated cells, although tyrosine phosphorylation of other proteins including the tyrosine kinase syk was relatively unaffected. PAO (1 μM) also inhibited completely tyrosine phosphorylation of PLCγ1 induced by platelet derived growth factor (PDGF) in NIH-3T3 fibroblasts but only partially reduced phosphorylation of the PDGF receptor. These results provide further evidence that collagen and FcγRIIA cross-linking activate platelets through a pathway distinct from that used by thrombin and suggest that PAO may be a selective inhibitor of PLCγ relative to PLC β isozymes.

Key words: Collagen; Fcγ receptor; Phenylarsine oxide; Platelet-derived growth factor-β; Phospholipase Cγ; Human platelet

1. Introduction

Phenylarsine oxide (PAO) is a trivalent arsenical compound which induces a wide spectrum of effects in biologic systems through covalent modification of thiol groups [1]. In particular, PAO has been reported to inhibit a number of transmembrane signalling pathways that involve tyrosine phosphorylation including insulin-mediated activation of p21 ras in fibroblasts [2] and activation of T-lymphocytes through the T-cell antigen receptor complex [3,4]. PAO also inhibits tyrosine phosphorylation of PLCγ1 induced by cross-linking of the high affinity receptor for immunoglobulin E on mast cells [5] and by cross-linking of anti-CD3 monoclonal antibodies in T-lymphocytes [6].

There is considerable evidence for the role of tyrosine phosphorylation in the early events associated with platelet activation [7–12]. For example, we and others reported recently that collagen and cross-linking of the platelet low-affinity IgtG receptor FcγRIIA (CD32) stimulate PLC activity through a tyrosine kinase dependent pathway involving tyrosine phosphorylation of PLCγ2 [11,12]. In contrast, the G protein receptor stimuli thrombin and U46619, a thromboxane mimetic, induce little or no tyrosine phosphorylation of PLCγ2 [11,12]. The molecular events which underlie phosphorylation of PLCγ2 by collagen or by FcγRIIA-crosslinking are not established.

The aim of the present study was to investigate further whether FcγRIIA cross-linking and collagen induce platelet activation through a common mechanism. The effect of PAO on responses induced by FcγRIIA cross-linking and collagen has been compared in view of the recent report that the sulphydryl reagent inhibits platelet activation by collagen [13]. The results illustrate that PAO inhibits platelet activation induced by FcγRIIA cross-linking or by collagen through inhibition of tyrosine phosphorylation of PLCγ2. PAO also inhibits tyrosine phosphorylation of PLCγ1 in NIH-3T3 fibroblasts activated by PDGF suggesting that it may be a general inhibitor of PLCγ isoforms.

2. Materials and methods

2.1. Materials

Antiserum against PLCγ1 and the anti-phosphotyrosine monoclonal antibody (mAb), 4G10, were purchased from Upstate Biotechnology Inc. (TCS Biologicals Ltd., Bucks., UK). Rabbit antiserum raised against residues 461 to 481 and 1218 to 1239 of PLCγ2 was prepared as described [14]. mAb IV.3, specific for FcγRIIA, was purchased from Madarex Inc. (New Hampshire, USA). Collagen was from Nycomed (Munich, Germany). Indomethacin, Nonidet P-40 (NP-40), thrombin, Tween-20 and sheep $F(ab')_2$ raised against mouse IgG (M-1522) were purchased from Sigma (Poole, Dorset, UK). PAO was purchased from Aldrich. An anti-syk rabbit polyclonal antibody raised against a polyhistidine fusion protein containing amino acids 257–352 within the linker region of syk was purchased from Santa Cruz, California. Polyvinylidene difluoride (PVDF) western blotting membrane was from Bio-Rad (Herts., UK). Horseradish peroxidase-conjugated sheep anti-mouse Ig (NA931) and horseradish peroxidase-conjugated donkey anti-rabbit Ig (NA934), ECL reagents, and *myo*-[^{3}H]inositol (18.2 Ci/mmol) were from Amersham International (Cardiff, UK). NIH-3T3 cells were from the Dept. of Pathology, Oxford University. All other reagents were of analytical grade.

2.2. Preparation of human platelets

Human blood was taken from drug-free volunteers on the day of the experiment. Platelet-rich plasma was obtained by centrifugation at 200 × *g* for 20 min. Platelets were isolated from platelet-rich plasma by centrifugation at 1000 × *g* for 10 min in the presence of prostacyclin (0.1 μg/ml). Platelets were resuspended in 10 ml of a modified Tyrodes-HEPES buffer (134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 5 mM glucose, 1 mM $MgCl_2$, pH 7.3) in the presence of prostacyclin (0.1 μg/ml) and recentrifuged at 1000 × *g* for 10 min before resuspension at a concentration of 5×10^8 cells/ml in the above buffer. For experiments involving measurement of [^{3}H]inositol phosphates, platelets were initially resuspended in 1 ml of buffer following separation from plasma and labelled with 50 μCi [^{3}H]inositol for 3 h. At the end of the incubation, the volume of buffer was adjusted to 10 ml and platelets were centrifuged again at 1000 × *g* for 10 min and resuspended in the Tyrodes-HEPES buffer containing indomethacin (10 μM). Platelets were left 30 min and all experiments

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Abbreviations: FcγRIIA, the low-affinity IgG receptor, Fcγ receptor IIA; mAb, monoclonal antibody; PAO, phenylarsine oxide; PDGFβ, platelet-derived growth factor-β; PLC, phosphoinositide-specific phospholipase C; PVDF, polyvinylidene difluoride; SH, src homology domain.

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were carried out at 37°C in an aggregometer with continuous stirring (800 rpm).

2.3. Measurement of inositol phosphates

[^{3}H]Inositol-labelled platelets were resuspended in the above buffer to which 10 mM LiCl had been added in order to inhibit conversion of inositol phosphates to inositol. Platelet suspensions (200 μl) were stimulated for 5 min at 37°C under continuous stirring and reactions stopped by transfer to 1 ml of chloroform/methanol/HCl (50 : 100 : 1, by vol). [^{3}H]Inositol phosphates (i.e. inositol mono-, bis- and tris-phosphates) were separated by Dowex anion exchange chromatography as described previously [15].

2.4. Immunoblotting

An equal volume of Laemmli sample buffer was added and samples heated for 5 min at 95°C. Proteins were separated by 10% SDS-PAGE and transferred to PVDF blotting membranes using a semi-dry transfer system (120 min, 15V). Membranes were incubated for 30 min at room temperature with 10% BSA in TBS-T (20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.6), before probing for 1 h at room temperature with the primary antibodies 4G10 (anti-phosphotyrosine antibody), anti-PLCγ1 or anti-PLCγ2. Membranes were washed five times in TBS-T and bound primary antibody was detected by incubation for 1 h with horseradish-peroxidase-conjugated sheep anti-mouse Ig or donkey anti-rabbit Ig as secondary antibody. Membranes were washed in TBS-T and treated with ECL reagents before exposure to ECL-Hyperfilm. Where required, blots were stripped of bound antibody by washing in TBS-T containing 2% SDS at 80°C for 40 min. Membranes were washed three times with TBS-T and reprobed with secondary antibody to confirm removal of the primary antibody. Membranes were then probed with a different primary antibody.

2.5. Immunoprecipitation

Cells (1×10^9 cells/ml) were lysed on ice for 30 min with an equal volume of ice-cold lysis buffer (1% NP-40, 10 mM EDTA, 2 mM EGTA, 2 mM Na_3VO_4, 1 mM PMSF, 300 mM NaCl, 10 mM Tris, pH 7.3). Insoluble cell debris was removed by centrifugation at $11,600 \times g$ for 15 min and supernatant pre-cleared with 25 μl of protein A-Sepharose CL-4B which had been hydrated in TBS-T containing 10% BSA. Samples were divided and incubated with 3 μl of rabbit anti-PLCγ2 antiserum or 5 μg of anti-syk antiserum for 90 min before addition of 25 μl of protein A-Sepharose CL-4B. After a further incubation of 60 min, the protein A-Sepharose CL-4B was precipitated by centrifugation at $11,600 \times g$ for 30 s and washed five times in 1 ml of TBS-T containing 1 mM PMSF, 1 mM Na_3VO_4 and 1 mM EDTA. Immunoprecipitated proteins were subjected to SDS-PAGE, transferred to PVDF membrane and immunoblotted as described above. For the FcγRIIA immunoprecipitation, solubilized samples were incubated with 1 μg of mAb IV.3 without pre-clearing.

2.6. Preparation of NIH-3T3 fibroblasts

NIH-3T3 fibroblasts were grown in Dulbecco's modified eagle medium (Gibco) supplemented with 10% fetal bovine serum in 6-well plates. After cells reached semi-confluence, they were twice washed with fresh media before stimulation with 2 nM PGDFβ for 10 min at 37°C. The measurement of tyrosine phosphorylation of PLCγ1 was carried out as described above; immunoprecipitations were performed using 4 μg of anti-PLCγ1 antiserum.

3. Results

3.1. PAO inhibits collagen and FcγRIIA-induced activation of PLC

PAO inhibited formation of inositol phosphates induced by FcγRIIA cross-linking and collagen over a similar concentration range with half maximal and maximal inhibition observed at ~0.1 and 1 μM PAO, respectively (Fig. 1). This was accompanied by inhibition of aggregation and secretion of 5-hydroxytryptamine (not shown). In contrast, PAO (0.01–1 μM) had no apparent effect on formation of [^{3}H]inositol phosphates in thrombin-stimulated platelets, although potentiation was observed at a concentration of 10 μM (Fig. 1). These results provide further evidence that FcγRIIA cross-linking and collagen-induce platelet activation by a common pathway which is distinct from that utilised by thrombin.

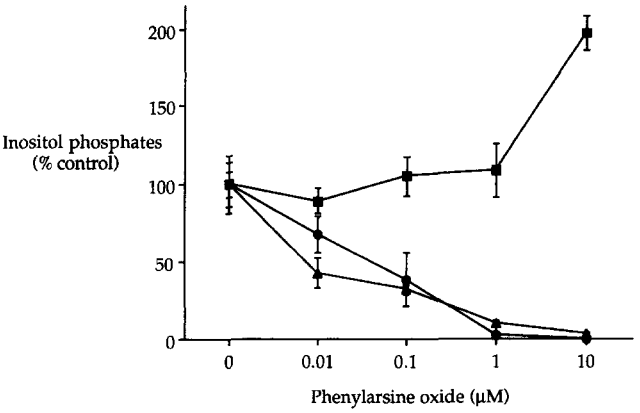

Fig. 1. Effect of phenylarsine oxide (PAO) on phospholipase C activity. [^{3}H]Inositol-labelled platelets were pretreated with or without various concentrations of PAO for 30 s and stimulated with thrombin (0.1 unit/ml), FcγRIIA cross-linking (IV.3 [1 μg/ml] and $F(ab')_2$ [30 μg/ml]), or collagen (100 μg/ml) for 5 min at 37°C. Formation of [^{3}H]inositol phosphates was measured as described in section 2. The results are the mean ± S.E.Mean of four experiments. Thrombin, ■; FcγRIIA cross-linking, ▲; collagen, ●; basal dpm was 348 ± 11 and each stimulus increased this value to 1472 ± 56, 772 ± 69 and 759 ± 73, respectively.

3.2. Effect of PAO on protein tyrosine phosphorylation

PAO (1 μM) had little effect on the general pattern of tyrosine phosphorylation induced by FcγRIIA cross-linking and collagen (not shown) despite the fact that this concentration produces complete inhibition of PLC activity. The effect of PAO on tyrosine phosphorylation of specific proteins was investigated in immunoprecipitation studies. Activation of platelets by cross-linking of FcγRIIA is associated with phosphorylation of FcγRIIA itself [16,17] a response that was increased slightly by 1 μM PAO in some but not all experiments (Fig. 2). This is consistent with the observation that tyrosine phosphorylation of FcγRIIA is independent of PLC [17]. Collagen and FcγRIIA cross-linking induce tyrosine phosphorylation of the tyrosine kinase syk [18,19] and this response this is maintained in the presence of 0.1–10 μM PAO (Fig. 3) demonstrating independence from PLC activity. In contrast, PAO inhibits completely tyrosine phosphorylation of PLCγ2 induced by cross-linking of FcγRIIA or by collagen over the same concentration range as that for inhibition of formation of inositol phosphates (Fig. 3).

3.3. PAO inhibits tyrosine phosphorylation of PLCγ1 in NIH-3T3 cells stimulated by PDGFβ

PAO has been reported to inhibit tyrosine phosphorylation of PLCγ1 in mast cells and T-cells following activation of the FcεRI receptor or the T-cell receptor complex. To our knowledge, however, there is no report of the effect of PAO on activation of PLCγ1 or PLCγ2 by receptors which contain intrinsic tyrosine kinase activity. We therefore examined the effect of PAO on PLCγ1 tyrosine phosphorylation in PDGFβ-stimulated NIH-3T3 fibroblasts. Immunoprecipitation of PLCγ1 with a specific antibody followed by western blotting with an antiphosphotyrosine antibody demonstrated that PAO

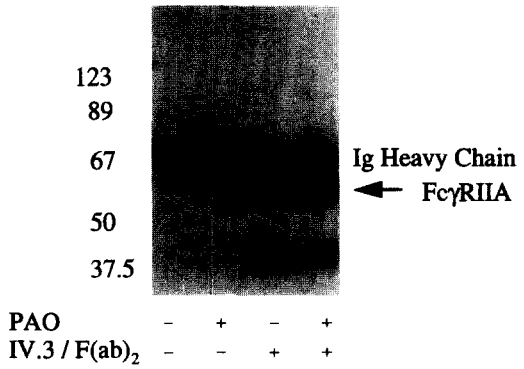

Fig. 2. Phenylarsine oxide (PAO) does not inhibit tyrosine phosphorylation of FcγRIIA. Platelets were pretreated with PAO (1 μM) or its solvent for 30 s and stimulated by FcγRIIA cross-linking (IV.3 [1 μg/ml] and $F(ab')_2$ [30 μg/ml]) for 60 s at 37°C. FcγRIIA was immunoprecipitated as described in section 2 and, following SDS-PAGE, immunoblotted for anti-phosphotyrosine. Molecular weight markers are shown on the left hand side. Results are representative of four experiments.

inhibits completely tyrosine phosphorylation of PLCγ1 by PDGFβ (Fig. 4). Anti-phosphotyrosine blots of whole cell lysates revealed that PDGFβ also induced marked tyrosine phosphorylation of a high mwt protein (~180 kDa), co-migrating with the PDGF receptor. Phosphorylation of this band was inhibited slightly by PAO (Fig. 4).

4. Discussion

In this report, we show that PAO causes complete inhibition of PLC activation in human platelets stimulated by collagen or FcγRIIA cross-linking at a concentration of 1 μM and that this is associated with inhibition of PLCγ2 tyrosine phosphorylation. However, at the same concentration PAO (1 μM) had no significant effect on the formation of inositol phosphates induced by the G protein receptor agonist thrombin. This observation is consistent with previous reports that thrombin induces little or no tyrosine phosphorylation of PLCγ2 in platelets [12,13] and confirms that tyrosine phosphorylation of PLCγ2 does not play a significant role in thrombin-induced formation of inositol phosphates. The mechanism underlying potentation of thrombin-induced formation of inositol phosphates by a higher concentration of PAO (10 μM) is not known; however, it is not due to increased phosphorylation of PLCγ2 (not shown).

The tyrosine kinase which underlies phosphorylation of PLCγ2 in human platelets is not established. The FcγRIIA has an immuno-receptor tyrosine activation motif (ITAM; also known as antigen receptor activation motif [ARAM]) in its cytoplasmic domain that is also present in a number of other immune receptors including the T-cell receptor, B-cell receptor and FcεRI receptor [20,21]. It is now established that cross-linking of immune receptors leads, in a manner that is poorly understood, to phosphorylation of the two tyrosine residues in the ITAM providing a site of interaction for the related tyrosine kinases syk and zap-70 via their two SH2 domains [11,22]. There is increasing evidence for a role of syk and zap-70 in tyrosine phosphorylation of PLCγ isoforms induced by immune receptors. For example, crosslinking of a chimeric receptor protein containing syk as its cytosolic tail is sufficient to stimulate tyrosine phosphorylation of PLCγ1 in rat basophilic

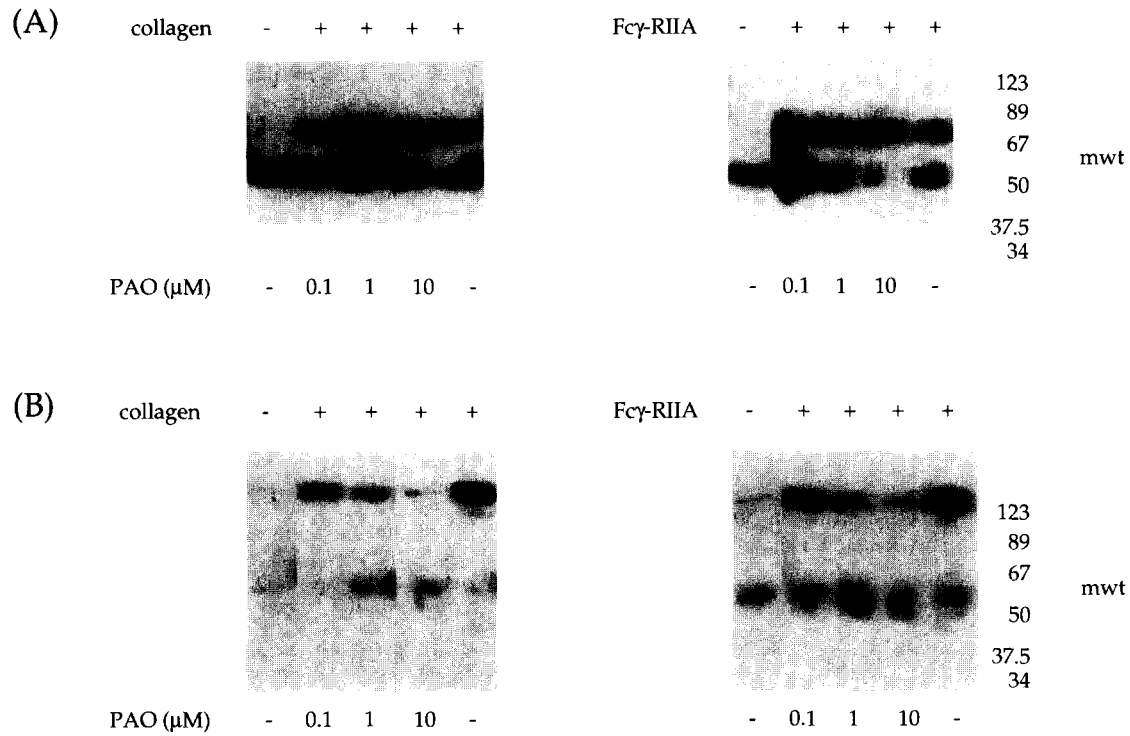

Fig. 3. Phenylarsine oxide (PAO) inhibits tyrosine phosphorylation of PLCγ2 but not syk. Platelets were pretreated with PAO (1 μM) or its solvent for 30 s and stimulated by FcγRIIA cross-linking (IV.3 [1 μg/ml] and $F(ab')_2$ [30 μg/ml]) or collagen (100 μg/ml) for 60 and 90 sec, respectively at 37°C. Immunoprecipitation of syk and PLCγ2, and anti-phosphotyrosine immunoblotting were performed as described in section 2: (A) syk-immunoprecipitation and (B) PLCγ2-immunoprecipitation. Molecular weight markers are shown on the right hand side. The results are from one experiment representative of three other similar experiments.

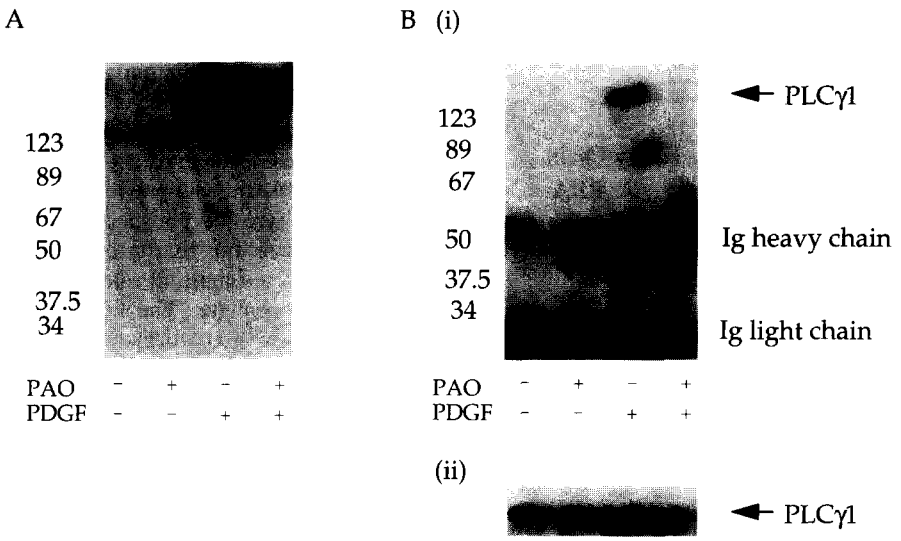

Fig. 4. Phenylarsine oxide (PAO) inhibits tyrosine phosphorylation of PLCγ1 in NIH-3T3 cells induced by platelet derived growth factor β (PDGFβ). NIH-3T3 cells were treated with PAO (1 μM) or its solvent for 30 s before stimulation with PDGFβ (2 nM) for 10 min. Part A shows whole cells lysates immunoblotted for phosphotyrosine as described in section 2. Part B shows PLCγ1 immunoprecipitates probed with (i) anti-phosphotyrosine antibody and, following stripping, (ii) anti-PLCγ1 antiserum. Molecular weight markers are shown on the left hand side. Results are representative of three experiments.

leukemia cells [23] while tyrosine phosphorylation of PLCγ2 and mobilisation of Ca^{2+} are absent in response to receptor cross-linking in syk-negative B-lymphocytes [24]. It is not established, however, whether syk and zap-70 directly phosphorylate PLCγ isoforms or whether intermediatory proteins, including other tyrosine kinases, are involved. The observation that tyrosine phosphorylation of syk induced by FcγRIIA cross-linking is maintained in the presence of PAO demonstrates that syk phosphorylation is independent of PLCγ2 activation. This result is consistent with a possible role of syk in the regulation of PLCγ2 phosphorylation. The demonstration that collagen-induced tyrosine phosphorylation of syk is also unaltered in the presence of PAO suggests that this kinase may also play a role in collagen-induced regulation of PLCγ2.

Unexpectedly, PAO also inhibited tyrosine phosphorylation of PLCγ1 in PDGF-stimulated NIH-3T3 cells, a response mediated by the tyrosine kinase activity intrinsic to the PDGF receptor [25–27]. Taken together with the results of the present study and earlier reports that PAO inhibits tyrosine phosphorylation of PLCγ1 induced by non-receptor tyrosine kinases in mast cells and T-cells [5,6], these observations suggest that PAO may be a general inhibitor of tyrosine phosphorylation of PLCγ isoforms. The mechanism which underlies this action of PAO is not known.

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