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Collagen stimulates tyrosine phosphorylation of phospholipase C-γ2 but not phospholipase C-γ1 in human platelets

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Abstract Collagen is an important primary stimulus of platelets during the process of hemostasis. As with many other platelet stimuli, collagen signal transduction involves the hydrolysis of inositol phospholipids; however, the mechanism which underlies this event is not well understood. Neither the collagen receptor nor the isoform of phospholipase C that is activated have been identified. We report that collagen-activation of platelets induces tyrosine phosphorylation of phospholipase C-γ2 but not phospholipase C-γ1. We also show that the platelet low affinity Fc receptor (FcγRII), which mediates activation of platelets by immune complexes, and wheat germ agglutinin, which binds non-specifically to glycoprotein, stimulate phospholipase C-γ2 tyrosine phosphorylation. In contrast, we could not detect phospholipase C-γ2 tyrosine phosphorylation in platelets stimulated by either thrombin or a stable thromboxane A_2 analogue, U46619.

Key words: Collagen; Fcγ receptor; Wheat germ agglutinin; Phospholipase Cγ; Human platelet

1. Introduction

During the initial stages of hemostasis, platelets are activated by adhesion to collagen in the connective tissue surrounding the site of injury. The activation of platelets by collagen, as with the majority of platelet stimuli, involves activation of phosphoinositide-specific phospholipase C (PLC) [1]. The mechanism of PLC activation by collagen has not been characterised, nor has the collagen receptor been identified. Several platelet surface proteins with collagen binding properties are candidate receptors (reviewed in reference [2]). These include: gpIa/IIa (integrin $\alpha 2\beta 1$), gpIV (CD36), and two unidentified proteins, of 65 and 61 kDa. It is not yet certain which of these proteins is involved in collagen-stimulated activation of PLC.

Platelets contain several PLC isozymes, including PLC-γ1, PLC-γ2, PLC-β, PLC-δ and at least two unidentified cytosolic PLCs [3,4]. There is good evidence that PLC-β is coupled via G-proteins, to receptors of several platelet agonists, including thrombin and thromboxane A_2 (for review see [5]). Several platelet receptors however, do not have the seven transmembrane domains typical of G-protein-linked receptors, yet have been shown to stimulate PLC and platelet activation; e.g. the platelet low-affinity antibody receptor (FcγRII) [6] which mediates activation of platelets by immune complexes. Since none of the putative collagen receptors have been found to have seven transmembrane domains, it is possible that the collagen receptor uses a mechanism of PLC activation similar to that of FcγRII. Indeed, there is evidence, based on the effects of tyrosine kinase inhibitors, that both collagen and FcγRII stimulate PLC through a mechanism involving tyrosine phosphorylation [7,8]. These data suggest the involvement of PLCγ isozymes, which are regulated by tyrosine phosphorylation [9,10]. Indeed FcγRII induces tyrosine phosphorylation of PLCγ isoforms in two human monocytic cell lines, THP-1 [11] and U937 [12]. In contrast tyrosine kinase inhibitors have little or no effect on PLC activation by thrombin or U46619, a thromboxane A_2 (TxA_2) analogue [8]. Tyrosine phosphorylation of FcγRII during stimulation may be part of the mechanism of PLC activation, since it occurs upstream of PKC activation and Ca^{2+} mobilization [8]. Huang et al. [13] observed that the cytoplasmic tail of FcγRIIA, the form of FcγRII in platelets [14], contains a sequence motif closely related to a repeated tyrosine, leucine/isoleucine motif, known as an antigen recognition activation motif (ARAM), which is responsible for coupling the T-cell receptor to the tyrosine kinase ZAP-70 (reviewed in [15]). Phosphorylation of FcγRII on tyrosine may also permit direct association with PLCγ via SH2 interactions, as in the case of the receptors for, platelet-derived growth factor (PDGF-β) and epidermal growth factor [16].

The evidence we present here suggests that collagen activates platelets through a mechanism similar to that used by FcγRII cross-linking. Both stimulation by collagen and FcγRII cross-linking, but neither thrombin nor a stable TxA_2 analogue, stimulate tyrosine phosphorylation of PLC-γ2.

2. Materials and methods

2.1. Materials

Monoclonal antibody (mAb) IV.3, specific for FcγRII, was purchased from Madarex Inc., New Hampshire, USA. Anti-phosphotyrosine mAb PY20 was purchased from ICN Flow (High Wycombe, Bucks., UK). Rabbit antiserum raised against residues 461 to 481, and 1218 to 1239 of PLC-γ2 was prepared as described [17]. Rabbit antiserum (06-152) raised against PLC-γ1, mAb (05-163) against PLC-γ1 and anti-phosphotyrosine mAb 4G10 were purchased from UBI (New York, USA). Collagen (native collagen fibrils from equine tendons) was from Nycomed (Munich, Germany). Ro 31-8220 was a gift from Roche Products (Welwyn Garden City, Herts., UK). BAPTA-AM was purchased from Calbiochem-Novabiochem (Nottingham, UK). Thrombin, staurosporine, U46619, bovine serum albumin (fraction V) (BSA), Tween 20 and sheep $F(ab')_2$ raised against mouse IgG (M-1522) were purchased from Sigma (Poole, Dorset, UK). Nonidet P-40 was pur-

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Abbreviations: ARAM, antigen recognition activation motif; BAPTA-AM, acetomethoxy ester of 1,2-bis(2-aminophenoxyethane)-*N*,*N*,*N*′,*N*′-tetraacetic acid; mAb, monoclonal antibody; PLC, phosphoinositide-specific phospholipase C; FcγRII, the platelet low-affinity IgG receptor, Fcγ receptor II; SDS, sodium dodecyl sulfate; PIP2, phosphatidylinositol (4,5)-bisphosphate; PKC, protein kinase C; SH, src homology domain; TxA_2, thromboxane A_2; PMSF, phenylmethylsulfonyl fluoride; PDGF-β, platelet-derived growth factor-β.

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chased from BDH (Poole, UK). ST271 was a gift from the Wellcome Foundation (Beckenham, UK). Horseradish peroxidase conjugated sheep anti-mouse IgG (NA931), donkey anti-rabbit IgG (NA934), and ECL reagents were from Amersham International (Cardiff, UK). PVDF western blotting membrane was from Bio-Rad (Hertfordshire, UK). All other reagents were of analytical grade.

2.2. Platelet isolation and stimulation

Twice washed human platelets were prepared from drug free volunteers on the day of the experiment as previously described [7] and resuspended at a concentration of 8×10^8/ml in a modified Tyrode buffer consisting of NaCl (134 mM), KCl (2.9 mM) Na_2HPO_4 (0.34 mM), $NaHCO_3$ (12 mM), HEPES (20 mM) $MgCl_2$ (1 mM), indomethacin (10 μM) and EGTA (1 mM). All experiments were performed at 37°C with continuous stirring (1200 rpm). FcγRII was stimulated by cross-linking with mAb IV.3 (1 μg/ml) and $F(ab')_2$ anti-mouse IgG (30 μg/ml); mAb IV.3 was added 60 s before $F(ab')_2$, the latter time being taken as the start of stimulation. Collagen was used at a concentration of 100 μg/ml added from a stock of 1 mg/ml. Stocks of protein kinase inhibitors staurosporine (1 mM), Ro 31-8220 (1 mM) and ST271 (10 mM) were stored in Me_2SO. Staurosporine and Ro 31-8220 were added 60 s before agonist stimulation while ST271 was given 10 min before agonist addition. In experiments where platelets (8×10^8/ml) were incubated with the intracellular Ca^{2+} chelator BAPTA-AM (40 μM) the latter was added from a 10 mM stock in Me_2SO, 15 min prior to agonist stimulation. Appropriate Me_2SO controls were used.

2.3. Immunoprecipitation and immunoblotting

Following stimulation, platelets (8×10^8/ml) were lysed, either at 4°C for 30 min with an equal volume of ice-cold Nonidet P-40 extraction buffer [8] or radio-immunoprecipitation assay (RIPA) buffer, consisting of sodium deoxycholate (2% w/v), Triton X-100 (2% v/v), SDS (0.2%, w/v), NaCl (300 mM), Tris (20 mM), PMSF (1 mM), EDTA (10 mM), Na_3VO_4 (2 mM) at pH 7.3; or at 19°C with an equal volume of denaturing extraction buffer consisting of SDS (4% w/v), 2-mercaptoethanol (10% v/v), EDTA (10 mM), Tris (20 mM), PMSF (1 mM added from 100 mM in Me_2SO), pH 7.3. In the latter case, samples were heated to 100°C then diluted 25-fold into Tris-buffered saline, Tris (20 mM), NaCl (137 mM) pH 7.6, containing Tween 20 (0.1% v/v) (TBS-T), BSA (2 mg/ml), PMSF (1 mM), prior to immunoprecipitation. Insoluble cell debris was removed by centrifugation either at $3,000 \times g$ for 30 min, for the denaturing protocol, or at $13,000 \times g$ for 10 min, for the Nonidet P-40 and RIPA protocol. The supernatant was precleared with 20 μl of protein A-Sepharose CL-4B which had been hydrated in TBS-T containing 10% (w/v) BSA and sodium azide (0.05% w/v). The supernatant was incubated overnight with 5 μl of antiserum to either PLC-γ2, or PLC-γ1 and 20 μl of protein A-Sepharose CL-4B. The protein A-Sepharose CL-4B pellet was washed using at least 6 sequential 20-fold dilutions, in TBS-T (4°C, containing PMSF (1 mM) and EDTA (1 mM). Immunoprecipitated protein was resolved by SDS-PAGE (10%) and Western blotted. The Western blot was probed initially with horseradish conjugated secondary antibody and ECL detection was used to identify bands of direct secondary antibody binding. The blot was then probed for phosphotyrosine using either mAb PY20 or mAb 4G10, followed by reprobing with the secondary antibody and ECL detection. The blot was then stripped as described previously [7] and reprobed with either anti-PLC-γ1 monoclonal antibody and horseradish peroxidase conjugated sheep anti-mouse IgG, or antiserum to PLC-γ2 (1:200 dilution) and horseradish peroxidase conjugated donkey anti-rabbit IgG, followed by ECL detection. Each result was confirmed in at least three independent experiments.

3. Results

3.1. Phosphorylation of phospholipase C-γ2

Stimulation of platelets by both collagen, and by cross-linking FcγRII caused the tyrosine phosphorylation of PLC-γ2, as detected by immunoblotting of immunoprecipitated PLC-γ2 with two different antiphosphotyrosine monoclonal antibodies (Figs. 1A and 2). Fig. 1A shows the tyrosine phosphorylation of PLC-γ2 immunoprecipitated under non-denaturing conditions from Nonidet P-40 lysed platelets, as detected mAb PY20. Fig. 2 shows the tyrosine phosphorylation of PLC-γ2 immunoprecipitated from platelets lysed in 2% SDS, then diluted to permit immunoprecipitation, as detected using mAb 4G10. Equivalent amounts of PLC-γ2 were detectable in immunoprecipitates from both unstimulated and stimulated samples (Fig. 1A). We did not detect the presence of tyrosine phosphorylated protein specifically associated with immunoprecipitated PLC-γ2.

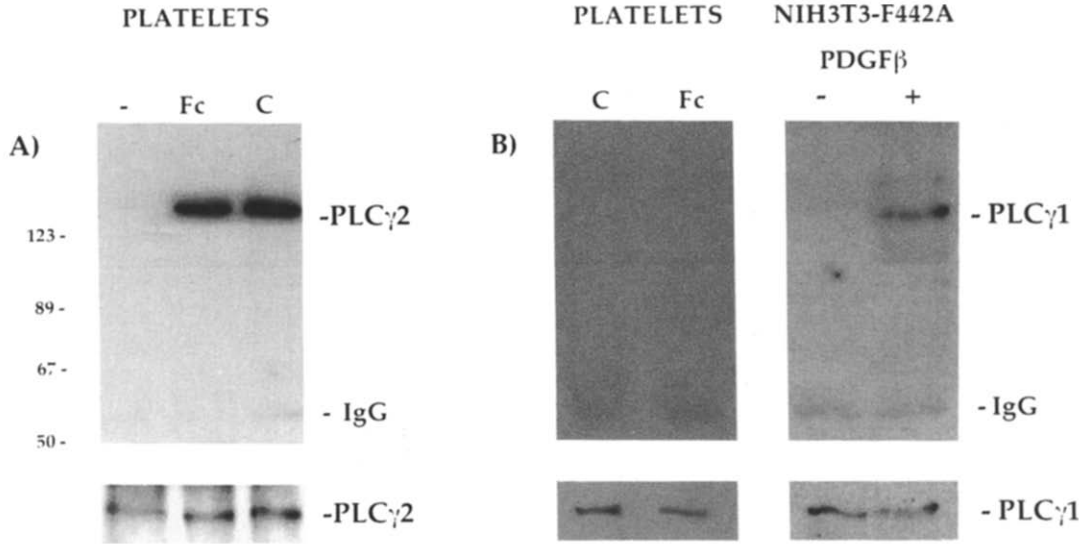

Fig. 1. Tyrosine phosphorylation of platelet PLCγ isozymes. (A) PLCγ2 was immunoprecipitated from Nonidet P-40 lysed unstimulated platelets (−), platelets stimulated for 120 s by cross-linking of FcγRII (Fc), as detailed in section 2, or platelets stimulated by collagen (100 μg/ml) for 120 s (C). The upper panel shows an anti-phosphotyrosine immunoblot using mAb PY20. The lower panel shows the appropriate section of the same blot after being stripped and reprobed for PLCγ2. Part B shows anti-phosphotyrosine (mAb PY20) immunoblots of PLCγ1 immunoprecipitated (using anti-PLCγ1 antiserum) from platelets (upper left hand panel) stimulated for 120 s with 100 μg/ml of collagen (C) or for 60 s by cross-linking of FcγRII (Fc); and right hand upper panel, NIH3T3-F442A cells grown to confluence in 25 cm^2 flasks unstimulated (−) or stimulated for 10 min with 2 nM PDGF-β (+), and lysed in ice-cold RIPA buffer. The lower two panels show the appropriate sections of the same blots after being stripped and reprobed for PLCγ1 using a mAb against PLCγ1. The position of the IgG heavy chain is marked on the upper panels.

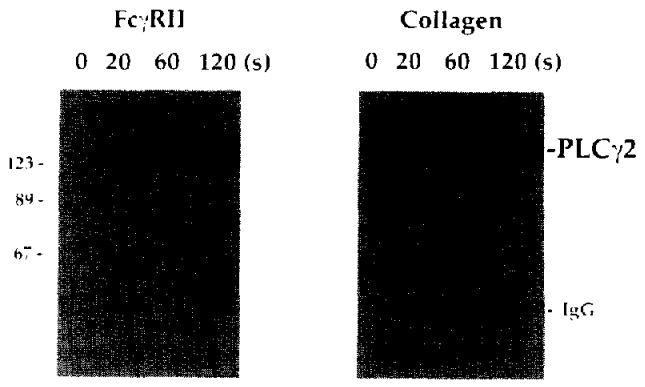

Fig. 2. Time course of PLCγ2 tyrosine phosphorylation. The two panels show anti-phosphotyrosine immunoblots (mAb 4G10) of PLCγ2 immunoprecipitated from platelets lysed and boiled in 2% SDS, as described in section 2. The left hand panel shows the time course of PLCγ2 tyrosine phosphorylation in response to FcγRII cross-linking. The right hand panel shows the time course of PLCγ2 tyrosine phosphorylation following the addition of 100 μg/ml collagen to the platelet suspension. The position of the IgG heavy chain is marked.

Collagen and FcγRII stimulated tyrosine phosphorylation of PLC-γ2 appears to occur on a time-scale similar to the onset of platelet functional responses, FcγRII stimulated tyrosine phosphorylation of PLC-γ2, detectable within 20 s and maximal by 60 s (Fig. 2), correlated with phosphorylation of pleckstrin, the major protein kinase C (PKC) substrate in platelets (not shown). Similarly, collagen stimulated phosphorylation of both PLC-γ2 and pleckstrin was first detectable 60 s after addition of collagen (Fig. 2 and not shown).

Wheat germ agglutinin, which shows binding specificity for glycoproteins containing sialic acid, *N*-acetylglucosamine, and *N*-acetylgalactosamine [18], including gpIb [19], also stimulated tyrosine phosphorylation of PLC-γ2 (Fig. 3). This is consistent with our unpublished observations that the potent non-selective protein kinase inhibitor, staurosporine, inhibits wheat germ agglutinin stimulated formation of inositol phosphates, and the report by Yatomi et al. [20] that staurosporine inhibits wheat germ agglutin stimulated rise in cytosolic Ca^{2+} concentration. The protein tyrosine phosphatase inhibitor, peroxovanadate, which stimulates inositol phosphate production in platelets [7], also stimulated strong tyrosine phosphorylation of PLC-γ2 (not shown).

We were unable to detect tyrosine phosphorylation of PLC-γ2 stimulated by a stable thromboxane A_2 analogue, U46619 (20 μM) or thrombin (1 unit/ml), over stimulation periods between 10 and 120 s (Fig. 3). This differs from a previous study [21] which reports transient thrombin-stimulated tyrosine phosphorylation of PLC-γ2, peaking at 60 s of stimulation. We tested whether the presence of indomethacin and EGTA in our platelet suspension inhibits this response, but were still unable to detect PLC-γ2 tyrosine phosphorylation with either mAb PY20 or mAb 4G10 (not shown).

3.2. *Phosphorylation of phospholipase C-γ1*

In contrast to the results obtained for PLC-γ2, and our previous study of peroxovanadate stimulated platelets [7], we have been unable to detect tyrosine phosphorylation of PLC-γ1 in platelets stimulated by collagen, FcγRII cross-linking (Fig. 1B) or thrombin (not shown). A positive control of PLC-γ1 tyrosine phosphorylation in PDGF-β stimulated NIH3T3-F442A cells is shown in Fig. 1C. Others have also been unable to detect thrombin stimulated tyrosine phosphorylation of PLC-γ1 in platelets [22,23]. In contrast, Guinebault et al. [24] detected PLC-γ1 in protein from thrombin stimulated, digitonin-lysed platelets, purified on agarose-conjugated antiphosphotyrosine antibody. However, this procedure does not distinguish between tyrosine phosphorylation of PLC-γ1 and its association with tyrosine phosphorylated protein. In platelets, PLC-γ1 associates with the GTPase activating protein of $p21^{ras}$ (rasGAP), which is phosphorylated on tyrosine in response to thrombin stimulation [23]. Similarly, PLC-γ1 associates with various growth factor receptors in fibroblast cell lines [16,25], as well as several uncharacterised tyrosine phosphorylated proteins (the most notable being pp35/36) in T- and B-lymphocytes and related cell-lines [26,27].

3.3. *Effect of intracellular Ca^{2+} chelation and protein kinase C inhibition on PLC-γ2 phosphorylation*

We have previously used the combination of the selective PKC inhibitor, Ro 31-8220, and the intra-cellular Ca^{2+} chelator, BAPTA-AM, to demonstrate that the tyrosine phosphorylation of FcγRII occurs independently of PKC activation and Ca^{2+} mobilization [8]. Similarly, PLC-γ2 is tyrosine phosphorylated in platelets pre-incubated with Ro 31-8220 and BAPTA-AM, prior to stimulation by collagen or FcγRII cross-

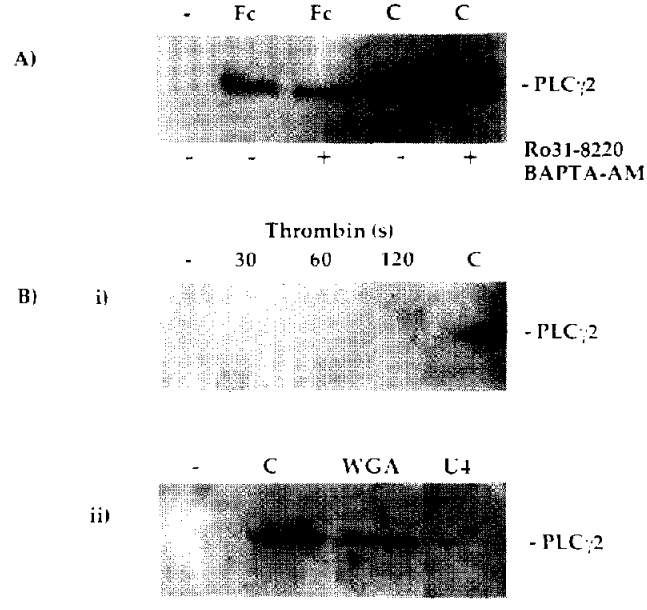

Fig. 3. Protein kinase C inhibition and intracellular Ca^{2+} chelation, and the effect of other platelet agonists on PLCγ2 tyrosine phosphorylation. All three panels show antiphosphotyrosine immunoblots (combined mAb PY20 and 4G10) of PLCγ2 immunoprecipitated from SDS lysed platelets. Part A shows the tyrosine phosphorylation of PLCγ2 from platelets which had been pre-incubated as indicated, with Ro 31-8220 and BAPTA-AM, as described in section 2, and stimulated for 120 s, either by cross-linking FcγRII (Fc) or by the addition of 100 μg/ml of collagen (C). Part B (i) shows the effect of thrombin (1 unit/ml) stimulation of platelets over 30, 60 and 120 s, on PLCγ2 tyrosine phosphorylation, as compared to unstimulated platelets (−) and collagen stimulated (120 s, 100 μg/ml) platelets (C). Part B (ii) shows the effect of wheat germ agglutinin (WGA; 60 s, 100 μg/ml) and U46619 (U4; 60 s, 20 μM) stimulation as compared to unstimulated platelets (−) and platelets stimulated for 120 s with 100 μg/ml of collagen (C).

linking (Fig. 3A). This is consistent with tyrosine phosphorylation of PLC-γ2 being upstream of second-messenger formation and responses such as aggregation which regulate tyrosine phosphorylation of several platelet proteins.

4. Discussion

Our studies show that stimulation of platelets by collagen, FcγRII cross-linking and wheat germ agglutinin, causes tyrosine phosphorylation of PLC-γ2 but not PLC-γ1. These observations offer a molecular explanation as to why these platelet stimuli are particularly sensitive to tyrosine kinase inhibitors [7,8,20]. It remains to be shown whether platelet PLC-γ2 is indeed activated by tyrosine phosphorylation, but there is sufficient precedent from the studies of PLC-γ1, and circumstantial evidence from the effect of tyrosine kinase inhibitors, to speculate that it is. Moreover, when Rat-2 cells overexpressing PLC-γ2 were treated with PDGF, an increase in both the tyrosine phosphorylation and the in vivo activity of PLC-γ2 was observed [28]. Also, the time course of anti-IgM stimulated PLC-γ2 tyrosine phosphorylation in WEHI-231 B-cells correlates well with the production of inositol phosphates [29].

There are contrasting reports with regard to the level of PLC-γ isoforms in platelets. Torti and Lapetina [23] suggest that PLC-γ1 accounts for approximately 62% of the total phosphatidylinositol (4,5)-bisphosphate (PIP2) hydrolysing activity in unstimulated platelets. This estimate contrasts with the results of Banno et al. [4], which show that the platelet Fast Q-Sepharose fraction containing both PLC-γ1 and PLC-γ2, accounts for no more than 6% of the total cytosolic PIP2 hydrolysing activity. Consistent with this, depletion of 40 to 50% of platelet PLC-γ2 by immunoprecipitation, does not cause significant reduction in PIP2 hydrolysing activity of platelet lysate [21]. Our results are consistent with tyrosine phosphorylation of PLC-γ2, but not PLC-γ1, playing a significant role in collagen and FcγRII-stimulation of platelets. This is analogous to anti-IgM stimulated B-cell lines in which both expression [30] and tyrosine phosphorylation [29] of PLC-γ2 is predominant over PLC-γ1.

Our findings prompt the question, which tyrosine kinase is responsible for the phosphorylation of PLC-γ2? This is not a trivial matter to resolve, especially in the case of collagen and wheat germ agglutinin stimulation because of the uncertainty of the receptor involved. More information, from studies in platelets and other-cell types, is available on FcγRII signal transduction. FcγRII co-immunoprecipitates with several src family tyrosine kinases: $pp59^{fyn}$ in human tonsil B-cells [31], $pp59^{hck}$ and $pp56^{lyn}$ in THP-1 cells [32] and $pp58^{fgr}$ in neutrophils [33]. Huang et al. [13] examined the role of the src-like kinases, in platelet FcγRII signal transduction. The only member of this family which had a detectable increase in autophosphorylating activity was $pp60^{src}$, 2 to 10 min after FcγRII stimulation, but the change was small. They were unable to detect association of any of the src-like kinases with FcγRII.

Studies in the human monocytic cell lines THP-1 [11] and HL60 [34] have implicated the ZAP-70-homologous tyrosine kinase $pp72^{syk}$ in FcγRII signalling. In HL60 cells, $pp72^{syk}$ is phosphorylated on tyrosine in response to FcγRII cross-linking. In THP-1 cells, $pp72^{syk}$ associates with FcγRII, is phosphorylated on tyrosine and has increased in vitro autophosphorylating activity following FcγRII stimulation. Similarly in platelets, FcγRII cross-linking induces tyrosine phosphorylation of a 70 kDa protein upstream of second-messengers [8]; this protein has been identified (Blake, Poole, Asselin, Schieven and Watson, unpublished results) as $pp72^{syk}$. We have also been able to detect an uncharacterised tyrosine kinase activity associated with FcγRII from platelets stimulated by FcγRII cross-linking or peroxovanadate, by subjecting FcγRII immunoprecipitates to in vitro kinase assay and observing the phosphorylation of FcγRII on tyrosine, as determined by phosphoaminoacid analysis (Blake, Poole, Asselin, Schieven and Watson, unpublished results). Interestingly, activation of porcine platelets by wheat germ agglutinin stimulates the activity of $pp72^{syk}$ [35], and both collagen and wheat germ agglutinin activation of human platelets stimulates tyrosine phosphorylation of $pp72^{syk}$ (Blake, Poole, Asselin, Schieven and Watson, unpublished results). These results lead us to speculate that $pp72^{syk}$ participates in the mechanism of PLC-γ2 tyrosine phosphorylation and activation in platelets stimulated by collagen, FcγRII cross-linking, and wheat germ agglutinin. This model would predict that the collagen receptor, like FcγRII, has an ARAM-like motif which, when phosphorylated, binds $pp72^{syk}$. However, $pp72^{syk}$ is also activated in porcine platelets stimulated by the thromboxane analogue U44069 [36], and by thrombin in a cell-free system [37]; yet these stimuli do not induce tyrosine phosphorylation of PLC-γ2. It is possible that phosphorylated $pp72^{syk}$ must have the appropriate sub-cellular localization in order to induce tyrosine phosphorylation of PLC-γ2.

We have failed to confirm previous observations [21] of thrombin-stimulated PLC-γ2 tyrosine phosphorylation. The reason for this discrepancy is not clear as we were careful to reproduce the conditions used in the original study. To our knowledge there has only been one other report of tyrosine phosphorylation of PLC-γ isoforms in response to activation of a G-protein coupled receptors. Gusovsky et al. [38] demonstrated that the m5 muscarinic receptor transfected into Chinese hamster ovary cells induced tyrosine phosphorylation of PLC-γ1, but in a manner dependent upon the entry of extracellular Ca^{2+}, suggesting that this is not a direct receptor-linked event; a result which is consistent with our findings.

In summary, we have demonstrated that a subset of platelet stimuli, which include adhesion to fibrilar collagen and cross-linking of FcγRII, induce the tyrosine phosphorylation of PLC-γ2, but not PLC-γ1. We suggest that this is part of a mechanism responsible for stimulating phosphoinositide hydrolysis and subsequent downstream responses, by non-G-protein-coupled receptors in platelets. Our comparison of the mechanisms of collagen and FcγRII stimulation lead us to speculate that the collagen receptor will prove to be a glycoprotein which is phosphorylated on tyrosine in response to collagen stimulation.

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