Fcγ receptor II stimulated formation of inositol phosphates in human platelets is blocked by tyrosine kinase inhibitors and associated with tyrosine phosphorylation of the receptor

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Abstract

We report that activation of phospholipase C (PLC) by cross-linking of the platelet low-affinity Fcγ receptor II (FcγRII) is inhibited by two structurally distinct tyrosine kinase inhibitors, staurosporine and ST271. This contrasts with PLC activation induced by thrombin and U46619, a thromboxane mimetic, whose receptors have seven transmembrane domains characteristic of G-protein coupled receptors. Several proteins undergo phosphorylation on tyrosine on FcγRII cross-linking upstream of protein kinase C (PKC), Ca²⁺ and aggregation, including the FcγRII itself. The role of FcγRII phosphorylation in the regulation of PLC is discussed.

Key words: Fcγ receptor; Phospholipase C; Tyrosine phosphorylation; Human platelet; Immune complex; Tyrosine kinase inhibitor

1. Introduction

As well as their major role in hemostasis, platelets participate in the immune response, forming microthrombi over the surface of immune complexes leading to local inflammation [1]. The interaction of platelets with immune complexes is mediated by a low-affinity Fc receptor, FcγRII (CD32), on the platelet surface [2]. Cross-linking of FcγRII using specific antibodies mimics the effect of the immune complex and leads to activation of PLC [3]. Cross-linking of FcγRII on the human monocytic cell line, U937, leads to a rapid transient phosphorylation of PLCγ-1 on tyrosine residues and the formation of inositol phosphates [4]. In the present study we have investigated whether the FcγRII in platelets regulates PLC through a tyrosine kinase-mediated pathway.

2. Materials and methods

2.1. Materials

Monoclonal antibody (mAb) IV.3 was purchased from Madarex Inc. (New Hampshire, USA). mAb PY20 was purchased from ICN Flow (High Wycombe, Bucks. UK). Ro 31-8220 was a gift from Roche Products (Welwyn Garden City, Herts., UK). Staurosporine, U46619,

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Abbreviations: FcγRII, Fcγ receptor II; mAb, monoclonal antibody; PKC, protein kinase C; PLC, phospholipase C; PMSF, phenylmethylsulfonyl fluoride.

bovine serum albumin (fraction V), Tween-20 and sheep F(ab')₂ raised against mouse IgG (M-1522) were purchased from Sigma (Poole, Dorset, UK). Nonidet P40 was purchased from BDH (Poole, UK). ST271 was kindly donated by the Wellcome Foundation. Horseradish peroxidase-conjugated sheep anti-mouse IgG, secondary antibody (NA931), ECL reagents and *myo*-[³H]inositol (sp.act. 18.2 Ci/mMol) were from Amersham International (Cardiff, UK). All other reagents were of Analytical grade.

2.2. Platelet isolation, stimulation and inositol phosphates

Human platelets were isolated from drug-free volunteers on the day of the experiment as previously described [5] and resuspended at a concentration of between 2–8 × 10⁸/ml. Platelets were pre-labelled with [³H]inositol for 3 h and inositol phosphates measured as previously described [5]. In some experiments, platelets (8 × 10⁸/ml) were incubated with the intracellular Ca²⁺ chelator BAPTA-AM (40 μM) for 15 min. Platelets were resuspended in a modified Tyrode buffer containing indomethacin (10 μM) and EGTA (1 mM) and all experimentation was performed at 37°C with continuous stirring [5].

The FcγRII was stimulated by cross-linking with mAb IV.3 (1 μg/ml) and F(ab')₂ anti-mouse IgG (30 μg/ml), the mAb IV.3 was added 60 s before F(ab')₂, the latter time being taken as the start of stimulation. The protein kinase inhibitors staurosporine and Ro 31-8220 were given 60 s before agonist stimulation while ST271 was given 10 min before agonist addition.

2.3. Immunoprecipitation and immunoblotting

Following stimulation, platelets (8 × 10⁸/1 ml) were lysed at 4°C for 30 min with an equal volume of ice-cold extraction buffer consisting of Nonidet P-40 (2%), NaCl (300 mM), Tris (20 mM), PMSF (1 mM), EDTA (10 mM), Na₃VO₄ (2 mM) at pH 7.3. Sufficient antibody was added to controls after lysis to make the antibody levels equivalent in all samples. Samples were diluted 1:20 in TBS-T (Tris, 20 mM; NaCl, 137 mM; Tween-20, 0.1% (v/v)) containing: bovine serum albumin (2 mg/ml), PMSF (1 mM) and EDTA (1 mM). Insoluble cell debris was removed by centrifugation at 3,000 × g for 30 min. mAb IV.3 (2 μg) was then added to the supernatant fraction, followed 30 min later by 40 μl of a suspension of protein A-sepharose CL 4B which had been hydrated in TBS-T containing 10% (w/v) bovine serum albumin and sodium azide (0.05%, w/v). After an overnight incubation, the protein A-sepharose CL 4B was pelleted by centrifugation at 3,000 × g for 10 min and

(A) Thrombin   (B) U46619   (C) FcγRII
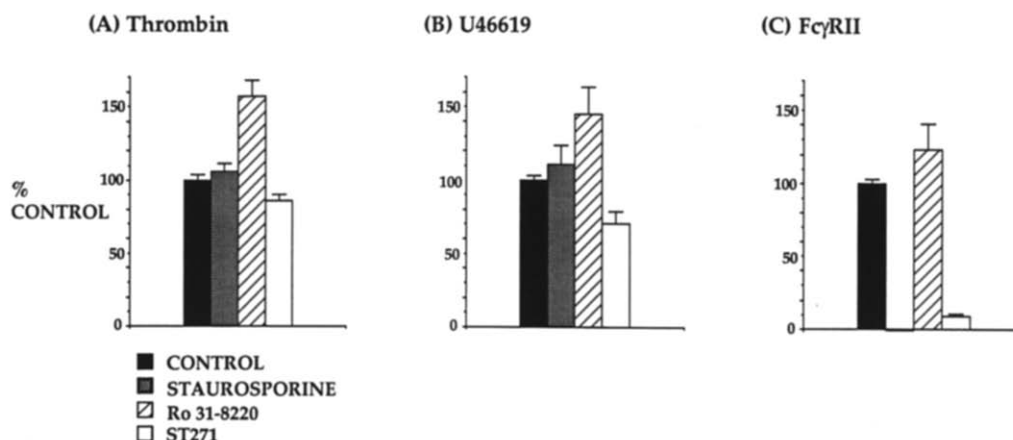

■ CONTROL
■ STAUROSPORINE
▨ Ro 31-8220
☐ ST271

Fig. 1. The effect of staurosporine (10 μM), Ro 31-8220 (10 μM) and ST271 (100 μM) on the formation of inositol phosphates stimulated by: (A) thrombin (1 unit/ml), (B) U46619 (20 μM), and (C) FcγRII cross-linking (as detailed in section 2). Platelets were incubated with kinase inhibitor then exposed to the above stimuli for 5 min. The data is expressed as mean ± S.E.M. of the percentage stimulation in the absence of the inhibitor. The response to each stimulus expressed as a percentage of basal ± S.E.M. were: thrombin, 1463 ± 55 (n = 7); U46619, 531 ± 15 (n = 11); and FcγRII cross-linking, 829 ± 67 (n = 6). The basal value was 123 ± 10 (n = 35) dpm.

then washed four times in 50 ml of TBS-T containing PMSF (1 mM) and EDTA (1 mM). Immunoprecipitated protein was resolved by SDS PAGE (12%) and Western blotted [6]. The Western blot was probed initially with HRP-conjugated secondary antibody and ECL detection was used to identify bands of direct secondary antibody binding. The blot was then probed for phosphotyrosine using mAb PY20 followed by reprobing with the secondary antibody and ECL detection.

2.4. Analysis of results
Results are expressed as mean ± S.E.M. from at least three experiments performed in quadruplicate. Statistical significance was indicated using Student's t-test.

3. Results

3.1. Measurement of inositol phosphates
Staurosporine (10 μM) had no significant effect on the

formation of inositol phosphates induced by thrombin or the thromboxane mimetic, U46619 (Fig. 1). In contrast, it completely blocked the response induced by FcγRII cross-linking (Fig. 1). The structural derivative of staurosporine, Ro 31-8220 (10 μM), which exhibits a high degree of selectivity for PKC over tyrosine kinases in platelets [6], did not reduce FcγRII stimulated inositol phosphate production and potentiated the response to thrombin and U46619 (Fig. 1). The tyrphostin, ST271 (100 μM), which is claimed to be a more selective inhibitor of tyrosine kinases, also caused complete inhibition of formation of inositol phosphates induced by FcγRII cross-linking, but also induced a small (< 30%) but significant inhibition of the response to thrombin and U46619 (Fig. 1).
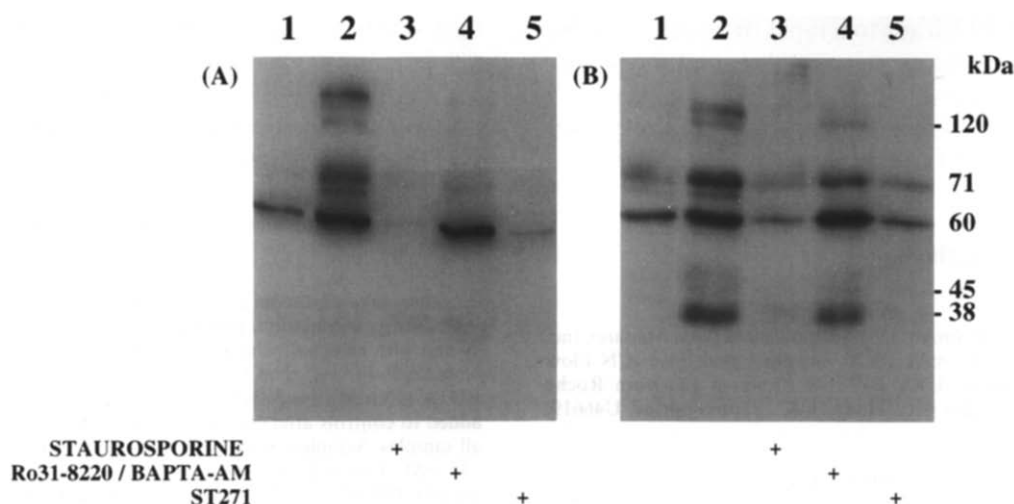

Fig. 2. The effect of kinase inhibitors and intracellular Ca^{2+} chelation on protein tyrosine phosphorylation stimulated by thrombin (1 unit/ml) in (A), lanes 2–5; and FcγRII cross-linking (as detailed in section 2) in (B), lanes 2–5. Lane 1 in each figure shows basal phosphorylation. Platelets resuspended at a density of 8 × 10⁸/ml were treated with: lane 3, staurosporine (10 μM); lane 4, Ro31-8220 and BAPTA-AM (as detailed in section 2); and lane 5, ST271 (300 μM). They were then exposed to the above stimuli for 2 min. SDS–12% PAGE, and anti-phosphotyrosine immunoblotting were as detailed in section 2. The results are representative of three similar experiments.

3.2. Measurement of protein tyrosine phosphorylation

Fig. 2 demonstrates the thrombin and FcγRII cross-linking induce tyrosine phosphorylation of several proteins throughout the lane of the gel. The increase in tyrosine phosphorylation is inhibited completely by staurosporine (10 μM) and ST271 (300 μM) (Fig. 2). In order to investigate whether the thrombin-induced increase in protein tyrosine phosphorylation was mediated downstream of PKC and Ca^{2+}, we used a combination of the PKC inhibitor, Ro 31-8220 (10 μM), and the intracellular Ca^{2+} chelator, BAPTA-AM (40 μM). In preliminary experiments, this concentration of BAPTA-AM inhibited the rise in intracellular Ca^{2+} induced by thrombin by greater than 90%. Under these conditions, thrombin did not stimulate detectable tyrosine phosphorylation (Fig. 2). In marked contrast, FcγRII cross-linking in the presence of Ro 31-8220 and BAPTA-AM resulted in a marked increase in tyrosine phosphorylation of two proteins of 38 and 71 kDa, and minor changes in several other proteins (Fig. 2) suggesting that these increases occur upstream of PKC and Ca^{2+}. Interestingly, thrombin induces significant phosphorylation of a 71 but not a 38 kDa protein in the absence of Ro 31-8220 and BAPTA-AM.

It has previously been reported that the FcγRII is phosphorylated on tyrosine residues following cross-linking of the receptor [7]. Fig. 3 confirms that the FcγRII is phosphorylated following receptor cross-linking and demonstrates that this is unaffected by pre-treatment with a combination of Ro 31-8220 and BAPTA-AM. The mobility of the 40 kDa phosphorylated FcγRII does not correspond with the phosphorylated 38 kDa band described in Fig. 2.

4. Discussion

4.1. Role of tyrosine phosphorylation in the generation of inositol phosphates

We have presented pharmacological evidence that stimulation of platelet PLC activity by FcγRII cross-linking is dependent on tyrosine phosphorylation. Two structurally dissimilar tyrosine kinase inhibitors, the indole carbazole-based staurosporine [8] and the styryl-based ST271 [9], inhibited completely the formation of inositol phosphates induced by FcγRII cross-linking. In contrast, staurosporine did not alter the formation of inositol phosphates induced by the G protein linked [10,11] receptor stimuli thrombin and U46619 while ST271 induced a small degree of inhibition. Staurosporine has also been shown to inhibit the increase in intracellular Ca^{2+} stimulated in platelets by wheat germ agglutinin [12], whose mechanism of activation involves the non-specific cross-linking of surface glycoprotein. These results are consistent with the reported tyrosine
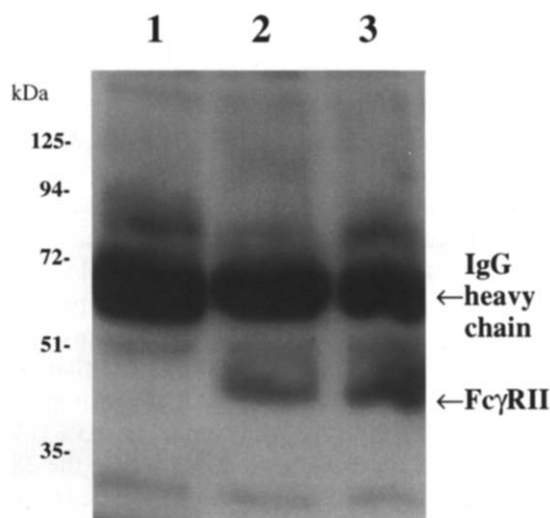

Fig. 3. Tyrosine phosphorylation of FcγRII in response to receptor cross linking is neither dependent on PKC activity nor on the elevation of the intra-cellular Ca^{2+} concentration. The figure shows an anti-phosphotyrosine immunoblot of FcγRII immunoprecipitated from: lane 1, untreated platelets; lane 2, platelets stimulated by cross-linking FcγRII (60 s); and lane 3, platelets pretreated with Ro31-8220 and BAPTA-AM, then stimulated as in lane 2. Stimulation and use of inhibitors, immunoprecipitation, SDS-PAGE, and anti-phosphotyrosine immunoblotting were as detailed in section 2.

phosphorylation of PLCγ-1 in U937 cells activated by FcγRII cross-linking [4].

The inhibitory effect of ST271 on the activation of PLC by thrombin and U46619 could represent a non-selective action resulting from the relatively high concentration of ST271 required to achieve full inhibition of tyrosine phosphorylation. Consistent with this, tyrphostins have been reported to inhibit oxidative phosphorylation in isolated fat cells [9]. Alternatively, however, the inhibitory effect of ST271 may reflect participation of PLCγ-1 in thrombin and U46619-induced formation of inositol phosphates, an effect that is likely to occur downstream of PKC and Ca^{2+}. This possibility provides an explanation for the paradoxical action of Ro 31-8220 on thrombin- and U46619-induced formation of inositol phosphates compared with the effect of staurosporine. Both Ro 31-8220 and staurosporine are potent inhibitors of PKC and yet only Ro 31-8220 induces potentiation of the formation of inositol phosphates by these two receptor stimuli, an effect that is likely to be mediated by inhibition of the negative feedback action of PKC on G protein receptor-induced activation of PLC [13]. The absence of a similar potentiation effect mediated by staurosporine may represent a cancelling out of the negative feedback action of PKC, which would lead to an increase in PLC activity, and an inhibition of tyrosine kinase activation of PLC. In contrast, ST271 has no effect on PKC activity and so only the inhibitory effect on tyrosine kinase activity is observed.

4.2. Mechanism of FcγRII signalling

In order to identify proteins that are uniquely tyrosine phosphorylated by FcγRII cross-linking relative to thrombin and U46619 receptors, we have employed a strategy to block increases in tyrosine phosphorylation that occur downstream of PKC and Ca^{2+} through the combined use of Ro 31-8220 and BAPTA-AM. This has led to identification of the prominent tyrosine phosphorylation of two proteins of 38 and 71 kDa, along with a number of minor proteins, which occur upstream of PKC and Ca^{2+}. In view of the fact that many tyrosine kinases undergo autophosphorylation, and that several tyrosine kinases are present in platelets in remarkably high levels (e.g. *src* and *syk* which make up 0.3% [14] and 0.15% [15] of platelet protein, respectively), the 38 and 71 kDa bands may represent tyrosine kinases. It is therefore provocative that *syk* is approximately 70 kDa in size and that FcγRII is reported to activate *syk* in the human monocytic cell line THP-1 [16].

One of the minor proteins which undergoes tyrosine phosphorylation has been identified as FcγRII. This provides the potential for direct receptor regulation of a tyrosine kinase or PLCγ-1 through interaction of the phosphotyrosine residue and an SH2 domain. Different SH2 domains have preference for different sequences neighbouring the phosphotyrosine. We have searched for a match between recently published SH2 domain sequence preferences [17] and the potential tyrosine phosphorylation sites on the cytoplasmic domain of FcγRIIA, the predominant subtype of FcγRII on platelets [18]. Human FcγRIIA has three tyrosine residues in its cytoplasmic domain [19] and the closest match found was in the sequence neighbouring tyrosine 310, YLTL, and the sequence preference of the N-terminal SH2 domain of PLCγ-1, YLDL. If tyrosine 310 undergoes phosphorylation, and allowing for the degeneracy of SH2 domain sequence preference, this may prove to be a binding site and 'membrane anchor' for PLCγ-1, bringing it in close proximity to its substrate in the membrane. Alternatively, we speculate that FcγRII phosphorylation enables the binding and activation of a separate tyrosine kinase, possibly *syk*, through an SH2 domain, which will then induce phosphorylation and activation of PLCγ-1.

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