Biochemical and Biophysical Research Communications 389 (2009) 198–203

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Characterization of a novel focal adhesion kinase inhibitor in human platelets

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ARTICLE INFO

Article history:
Received 6 August 2009
Available online 28 August 2009

Keywords:
FAK
pp125FAK
Platelet
Lamellipodia
PF-573,228
$\alpha_{IIb}\beta_3$
Integrin
Focal adhesion kinase

ABSTRACT

Focal adhesion kinase (FAK) is activated in human platelets downstream of integrins, e.g. $\alpha_{IIb}\beta_3$, and other adhesion receptors e.g. GPVI. Mice in which platelets lack FAK have been shown to exhibit extended bleeding times and their platelets have been shown to display decreased spreading on fibrinogen-coated surfaces. Recently, a novel FAK inhibitor (PF-573,228) has become available, its selectivity for FAK shown *in vitro* and in cell lines. We determined the effect of this inhibitor on platelet function and signaling pathways. Like murine platelets lacking FAK, we found that PF-573,228 was effective at blocking human platelet spreading on fibrinogen-coated surfaces but did not affect the initial adhesion. We also found a reduced spreading on CRP-coated surfaces. Further analysis of the morphology of platelets adhered to these surfaces showed the defect in spreading occurred at the transition from filopodia to lamellipodia. Similar to that seen with murine neutrophils lacking FAK, we also observed an unexpected defect in intracellular calcium release in human platelets pre-treated with PF-573,228 which correlated with impaired dense granule secretion and aggregation. The aggregation defect could be partially rescued by addition of ADP, normally secreted from dense granules, suggesting that PF-573,228 has effects on FAK downstream of $\alpha_{IIb}\beta_3$ and elsewhere. Our data show that PF-573,228 is a useful tool for analysis of FAK function in cells and reveal that in human platelets FAK may regulate a rise in cell calcium and platelet spreading.

Introduction

Platelets are anucleate cells found in the bloodstream that assist in maintaining the integrity of the cardiovascular system by responding to damaged vessel walls and initiating a complex restorative process, haemostasis. Pathological activation of platelets, thrombosis, can occur at sites of ruptured or eroded atherosclerotic plaques which underlies coronary artery disease, stroke and peripheral artery disease [1]. The integrin $\alpha_{IIb}\beta_3$ plays an important role in clot formation by binding soluble ligands such as fibrinogen and vWF facilitating platelet–platelet interactions [2,3]. Diverse platelet agonists converge on this integrin, changing its conformation to allow higher affinity ligand binding [4,5]. Antagonists of this platelet integrin, e.g. abciximab and tirofiban, are sometimes used in treatment of acute coronary syndromes.

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that, in platelets, becomes tyrosine phosphorylated after ligand engagement of $\alpha_{IIb}\beta_3$ [6–8]. Recently, a megakaryocyte lineage-specific FAK null mouse has been generated which showed a key role for FAK in megakaryopoiesis, platelet spreading on fibrinogen and haemostasis [9]. A myeloid lineage-specific FAK null mouse has also been generated and revealed that FAK has important roles in adhesion signaling and adhesion-independent signaling in neutrophils [10]. Macrophages from this mouse exhibited altered spreading and migration [11]. Recently, a novel small molecule inhibitor of FAK, PF-573,228 (henceforth referred to as PF-228) has been described [12]. This molecule exhibits selectivity towards FAK, at least *in vitro*, and inhibits activation of FAK in various cell lines in the range 0.3–3 μM [12].

In this study, we describe the effect of this inhibitor on human platelet function and human platelet signaling pathways. We demonstrate defects in platelet spreading and aggregation, intracellular calcium mobilization and dense granule secretion when platelets are pre-treated with the novel FAK inhibitor PF-228 which is, in part, consistent with data obtained from the haematopoietic cell-type specific FAK knockouts.

Materials and methods

Reagents. Generally, laboratory chemicals were purchased from Sigma (Poole, UK) unless otherwise indicated. The focal adhesion kinase (FAK) inhibitor PF-573,228 (3,4-dihydro-6-[[4-[[[3-(methylsulfonyl)phenyl]methyl]amino]-5-(trifluoromethyl)-2-pyrimidinyl]amino]-2(1H)-quinolinone) was purchased from Tocris (Bristol, UK). $DiOC_6$ (3,3′-dihexyloxacarbocyanine iodide) was purchased from Alexis Biochemicals (Exeter, UK). Anti-Syk, clone 4D10.1; anti-phosphotyrosine, clone 4G10 were from Millipore (Watford,

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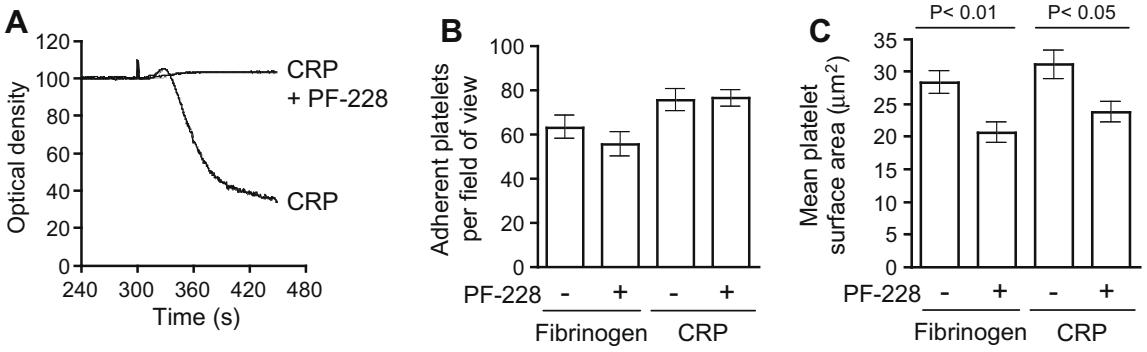
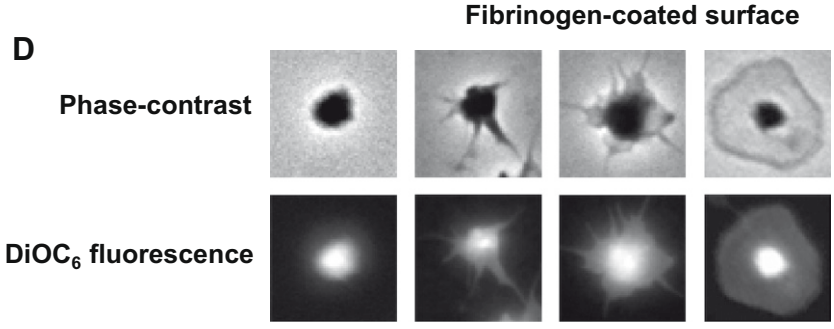
doi:10.1016/j.bbrc.2009.08.132

UK). Anti-FAK (clone 77) and anti-actin antibodies were purchased from BD Biosciences (Oxford, UK). All other primary antibodies were purchased from Cell Signaling Technology (NEB, Hitchin, UK). Anti-mouse and anti-rabbit horse-radish peroxidase (HRP) conjugated antibodies and enhanced chemiluminescent (ECL) reagent was purchased from GE Healthcare (Amersham, UK). Complete mini-protease inhibitor tablets were purchased from Roche (Burgess Hill, UK). Protein-G plus/protein-A agarose beads were purchased from Merck Chemicals (Nottingham, UK). Restore Plus Western blot Stripping Buffer was purchased from Pierce Biotechnology (Cramlington, UK).

Preparation and stimulation of human platelets. Human platelets were obtained from adult volunteers in accordance with the approved guidelines from the local Research Ethics Committee of the University of Bristol, UK; informed consent was obtained in accordance with the World Medical Association Declaration of Helsinki. Venous blood was drawn from volunteers with acid citrate dextrose as anticoagulant, used at a 1:7 (v/v) ratio. Platelet-rich plasma was obtained by centrifugation at 180*g* for 17 min. Platelets were isolated by centrifugation after treatment with prostaglandin E_1 (140 μM) and indomethacin (10 μM) at 550*g* for 10 min. The platelet pellet was resuspended in modified Tyrode's-Hepes buffer to a density of 2×10^8/ml.

Cell lysis and immunoprecipitation. For immunoblotting, platelets (400 μl) were lysed into 200 μl of SDS-sample buffer (62.5 mM Tris, pH6.8, 25%(v/v) glycerol, 2%(w/v) SDS and 340 mM DTT). For immunoprecipitation with anti-FAK antibody, platelets were lysed into an equal volume of 2× lysis buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 1%(v/v) NP-40 alternative, 1%(v/v) Triton X-100, 0.2%(w/v) SDS, complete protease inhibitors, 1 mM sodium orthovanadate and 20 mM 2-glycerophosphate). After incubation at 4 °C for 60 min, samples were centrifuged at 12,000*g* for 15 min and supernatants removed. Immunoprecipitations were performed using antibody precoupled to protein-G plus/protein-A agarose beads. The equivalent of 2 μg of antibody per sample was coupled to beads for 1 h at room temperature before extensive washing in 1× lysis buffer. Samples were then mixed with antibody–protein-G plus/protein-A agarose beads for 60 min at 4 °C. Beads were extensively washed with 1× lysis buffer. After washing, 40 μl of SDS-sample buffer was added followed by incubation at 96 °C for 3 min.





	Cell Type 1	Cell Type 2	Cell Type 3	Cell Type 4
Fibrinogen	35.06 (± 24.32)	31.79 (± 8.57)	17.98 (± 9.43)	15.17 (± 7.82)
Fibrinogen + PF-228	51.48 (± 16.97)	31.84 (± 8.86)	12.58 (±6.32)	4.11 (± 3.00)
CRP	14.52 (± 2.21)	30.39 (± 10.31)	32.32 (± 1.09)	22.77 (± 7.71)
CRP + PF-228	47.18 (± 13.30)	42.45 (± 8.52)	8.56 (± 3.67)	1.81 (± 1.81)

Fig. 1. Attenuation of platelet spreading by PF-228. Human platelets were either pre-treated with DMSO (−) or PF-228 (+) (1 μM) for 5 min in suspension before being allowed to settle on a either a fibrinogen or CRP-coated surface. Unbound platelets were washed off and bound platelets fixed. The numbers of platelets bound were counted with the assistance of Image J software and presented as the mean number bound per field of view (±SEM; 5 replicates, $n = 3$) (A). The platelet surface area was determined from the same samples using NIH Image J software and was recorded as the mean surface area (±SEM; 5 replicates, $n = 3$). Statistical significance was determined using a two-way ANOVA with Bonnferoni post-test (B). Bound platelets were subdivided into different classes: class 1, compact shape; class 2, filopodial extensions evident; class 3, filopodial extensions evident and some platelet spreading; class 4, fully spread with no filopodia evident. Platelets in each class were expressed as a mean percentage (±SEM; 5 replicates, $n = 3$) of the total number of bound platelets examined (C).