FEBS Letters 573 (2004) 26–30 FEBS 28701

Phospholipase Cγ2 contributes to stable thrombus formation on VWF ☆

Vipul Rathore[a], Demin Wang[a,b], Debra K. Newman[a,b], Peter J. Newman[a,c,d,e,*]

[a]*Blood Research Institute The Blood Center of Southeastern Wisconsin, P.O. Box 2178, 638 N. 18th St., Milwaukee, WI 53201, USA*
[b]*Department of Microbiology, Medical College of Wisconsin, Milwaukee, WI 53226, USA*
[c]*Department of Pharmacology, Medical College of Wisconsin, Milwaukee, WI 53226, USA*
[d]*Department of Cellular Biology, Medical College of Wisconsin, Milwaukee, WI 53226, USA*
[e]*Department of the Cardiovascular Center, Medical College of Wisconsin Milwaukee, WI 53226, USA*

Received 12 March 2004; accepted 19 July 2004

Available online 8 August 2004

Edited by Veli-Pekka Lehto

Abstract Though phospholipase C PLCγ2 is known to play an important role in platelet activation by collagen and fibrinogen, its importance in GPIb-mediated platelet activation is less well understood. To better understand the role of PLCγ2 in GPIb-mediated adhesion and thrombus formation, we examined the ability of wild-type and PLCγ2- deficient murine platelets to spread on immobilized von Willebrand factor (VWF) under static conditions, and to attach to and form thrombi on VWF under conditions of arterial shear. While absence of PLCγ2 had only a minimal effect on platelet adhesion to immobilized VWF, its absence impaired spreading and profoundly affected thrombus growth and stability on VWF.

Keywords: Phospholipase C; Thrombosis; GPIb; von Willebrand factor; Signaling

1. Introduction

Platelet activation at sites of vascular injury triggers the rapid hydrolysis of the minor membrane-anchored phospholipid, phosphatidylinositol 4,5 bisphosphate (PIP_2) – a reaction that is carried out by members of the phospholipase C (PLC) family of lipid-hydrolyzing enzymes. PLC activity results in the generation of the second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) [1]. DAG promotes the activation of certain isoforms of protein kinase C (PKC), while IP_3 binds to and controls the permeability of specific calcium channels present on intracellular membranes of the endoplasmic reticulum and the dense tubular system, which sequester most of the intracellular calcium in resting cells.

Mammalian cells contain eleven genes that encode phospholipase C (PLC) isozymes, including four members each of the PLC-β and PLC-δ subfamilies, the newly discovered PLCε, and two isoforms of PLCγ [1]. While the β isozymes figure prominently in signal transduction by $G_q\alpha$ protein-coupled receptors, the PLCγ isoforms, γ1 and γ2, are regulated by tyrosine phosphorylation [2], and function downstream of a wide variety of receptor tyrosine kinases and immunoreceptor tyrosine-based activation motif (ITAM)-linked immunoglobulin superfamily receptors, including T and B cell receptors and Fc receptors [3]. Whereas PLCγ1 is widely distributed, the expression of PLCγ2 is limited to cells of the hematopoietic lineage. Consistent with their expression patterns, PLCγ1 deficiency results in embryonic lethality [4], while mice genetically deficient in PLCγ2 exhibit defects in B cell development and function, natural killer cell activation, and mast cell FcεR signaling [5]. In addition, PLCγ2-deficient mice have platelet defects, suffer from spontaneous subcutaneous hemorrhaging during embryogenesis, and gastrointestinal and intraperitoneal bleeding during adulthood [5].

In order for platelets to control bleeding, they must undergo a sequential progression of adhesive and signaling events that are initiated, at least in part, by interaction of the platelet plasma membrane glycoprotein GPIb/V/IX complex with von Willebrand factor (VWF) immobilized on exposed collagen surfaces [6]. Shear forces applied to the GPIb complex, which is linked to the membrane skeleton on the inner face of the plasma membrane [7], result in the activation of one or more Src family kinases [8] followed by transient, low magnitude calcium oscillations that allow initially adherent platelets to undergo cytoskeletal rearrangements, spread, and stably arrest [9]. Sustained calcium mobilization serves to amplify these initial responses by promoting integrin activation, granule secretion, and recruitment of additional platelets to the site of vascular injury [10,11].

Though PLCγ2 is known to play an important role in platelet activation by collagen [5,12,13] and fibrinogen [14,15] (via GPVI and the GPIIb-IIIa complex, respectively), the importance of PLC-dependent phosphoinositide turnover in GPIb-mediated platelet activation is just now beginning to be unraveled. Mangin et al. [16] recently reported that the small amount of IP_3 that is generated downstream of GPIb-mediated platelet adhesion to immobilized VWF under static conditions, as well as the low-level calcium transients and cytoskeletal changes that follow, can be completely inhibited by the pan-PLC pharmacological antagonist, U73122. Interestingly, these early activation responses do not appear to involve any of the PLCβ isoforms, and were significantly reduced, but not completely abolished, in PLCγ2-deficient platelets, suggesting that both PLCγ2 and at least one other isoform of PLC are involved in the early stages of GPIb-mediated platelet activation, at least under static conditions.

☆ Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2004.07.048.

* Corresponding author. Fax: +1-414-937-6284.
E-mail address: pjnewman@bcsew.edu (P.J. Newman).

0014-5793/$22.00
doi:10.1016/j.febslet.2004.07.048

Thrombus formation on VWF requires concerted actions of GPIb and GPIIb-IIIa [10,17]. It is not known which (or if both) receptor requires PLCγ2 to transduce VWF-dependent signals or whether the PLCγ2 requirement extends to thrombus formation on VWF under shear conditions. To address these issues, we compared the abilities of wild-type and PLCγ2-deficient platelets to adhere to and either spread or form thrombi on VWF under static vs. shear conditions, respectively. Under static conditions, PLCγ2 was more important for spreading than for adherence of platelets to immobilized VWF. Under shear conditions, PLCγ2 contributed profoundly to growth and stability of platelet thrombi on VWF. Our findings suggest that the major contribution of PLCγ2 following platelet interactions with VWF is to amplify GPIIb-IIIa-mediated outside-in signals.

2. Materials and methods

2.1. Platelet adhesion and spreading on VWF under static conditions

Adhesion and spreading of washed wild-type (WT) and PLCγ2-deficient [5] murine platelets on human VWF (huVWF) was performed essentially as described previously [18]. RGDW peptide, which effectively inhibits GPIIb-IIIa-mediated platelet spreading, was used to determine the surface area covered by attached, but not spread, platelets. Since rodent GPIIb-IIIa is relatively less sensitive to inhibition by RGD peptide than is human GPIIb-IIIa ($IC_{50} = 1.65$ versus 0.04 mM, respectively) [19,20], it was used at a concentration of 2 mM, which has previously been shown to inhibit VWF/botrocetin-induced aggregation and spreading of murine platelets [18].

2.2. Thrombus formation on VWF under conditions of arterial shear

Platelet thrombus formation on human VWF under flow was studied using a parallel-plate flow chamber as described previously [18]. Briefly, mepacrine-labeled, red blood cell-reconstituted platelets were perfused through the chamber using a constant rate syringe pump at a

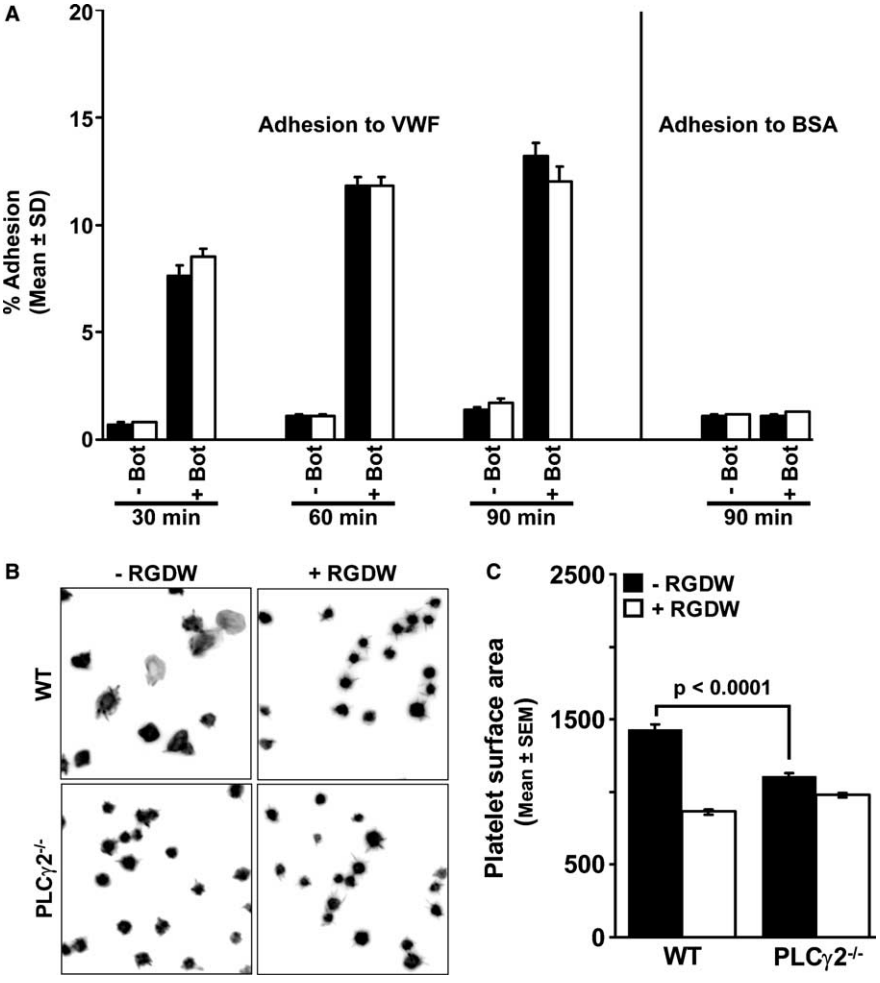

Fig. 1. PLCγ2-deficient murine platelets adhere to, but have impaired spreading on, immobilized VWF. (A) PLCγ2-deficient platelets exhibit normal adhesion to VWF. Calcein AM-labeled platelets were incubated under static conditions at 37 °C for indicated time points in microtiter wells that had been coated with 10 μg/mL human VWF or bovine serum albumin (BSA) in the presence or absence of 5 U/ml botrocetin (Bot). Percent adhesion was calculated by measuring the fluorescence of bound platelets ÷ total fluorescence × 100. Results are presented as percent adhesion ± S.D. of triplicate wells and are representative of two independent experiments. No appreciable difference in percent adhesion of WT versus $(PLC)\gamma2^{-/-}$ platelets to VWF was observed at any of the time points examined. (B) PLCγ2-deficient platelets exhibit impaired spreading on VWF. WT (upper panels) and $(PLC)\gamma2^{-/-}$ (lower panels) washed platelets were allowed to spread on glass slides coated with human VWF (10 μg/ml) in presence of 5 U/ml botrocetin under static conditions at 37 °C in the presence or absence of 2 mM RGDW. Images were taken 60 min following addition of platelets to VWF-coated slides. The panels shown are representative fields from two independent experiments. (C) Platelet surface area, defined as number of pixels covered by each platelet, was determined from digitized images using Metamorph software. At least 150–200 platelets per condition were examined. Results are presented as the mean surface area ± S.E.M. of two independent experiments. Note that the extent of spreading by $(PLC)\gamma2^{-/-}$ platelets was significantly ($P < 0.0001$) reduced compared to WT platelets.

calculated arterial shear rate of 1500 s^{-1} for five minutes. Thrombus formation was visualized at 40× magnification, recorded on videotape, digitized using Pinnacle Studio Software (Pinnacle Systems Inc., Mountain View, CA) and analyzed offline.

Thrombus size was analyzed using still images acquired from digitized recordings captured at 1, 2 and 3 min after initiation of flow. The number of pixels covered by the smallest platelet aggregate formed by PLC$\gamma2^{-/-}$ platelets after 1 min of flow was arbitrarily assigned as the cutoff value for definition of a thrombus. Surface areas of individual platelet aggregates and thrombi formed by WT and PLC$\gamma2^{-/-}$ platelets at 1, 2 and 3 min after initiation of flow were determined using Metamorph software. The mean size of individual WT and PLC$\gamma2^{-/-}$ platelet thrombi, as well as the combined surface areas of all thrombi in a given experiment, were normalized for each time point to that of the largest thrombus so that data from three separate experiments could be compared. Statistical analysis of means ± standard error was performed using an unpaired Student's *t* test.

3. Results and discussion

3.1. PLCγ2-deficient murine platelets adhere to, but spread poorly on, immobilized VWF

Adhesive interactions between human GPIb (huGPIb) and huVWF can be examined under static conditions by "activating" VWF immobilized on glass or plastic surfaces with conformational modifiers such as ristocetin or botrocetin. Previous studies [16,21] have shown that botrocetin is also able to support the binding of huVWF to murine platelets, resulting in shape change and aggregation. As shown in Fig. 1A, wild-type and PLC$\gamma2^{-/-}$ murine platelets bound to a similar extent to immobilized huVWF/botrocetin, confirming a recent report [16] that initial adhesive interactions between GPIb and VWF do not require PLCγ2. This group also found that PLCγ2-deficient platelets are slower to extend filopodia following platelet binding to immobilized VWF than are their wild-type counterparts. To examine the longer-term effects of PLCγ2 deficiency on GPIb-mediated platelet activation, we evaluated both filopodia and lamellipodia formation 60 min after VWF binding. Though the number of filopodia was not statistically different at this later time point (data not shown), lamellipodia formation was significantly inhibited in PLC$\gamma2^{-/-}$ platelets, with spreading reduced to the degree achieved by wild-type platelets in the presence of RGDW peptide (Fig. 1B and C), which inhibits GPIIb-IIIa-mediated adhesive interactions. Since lamellipodia formation and platelet spreading on VWF require interaction of activated GPIIb-IIIa with the VWF C1

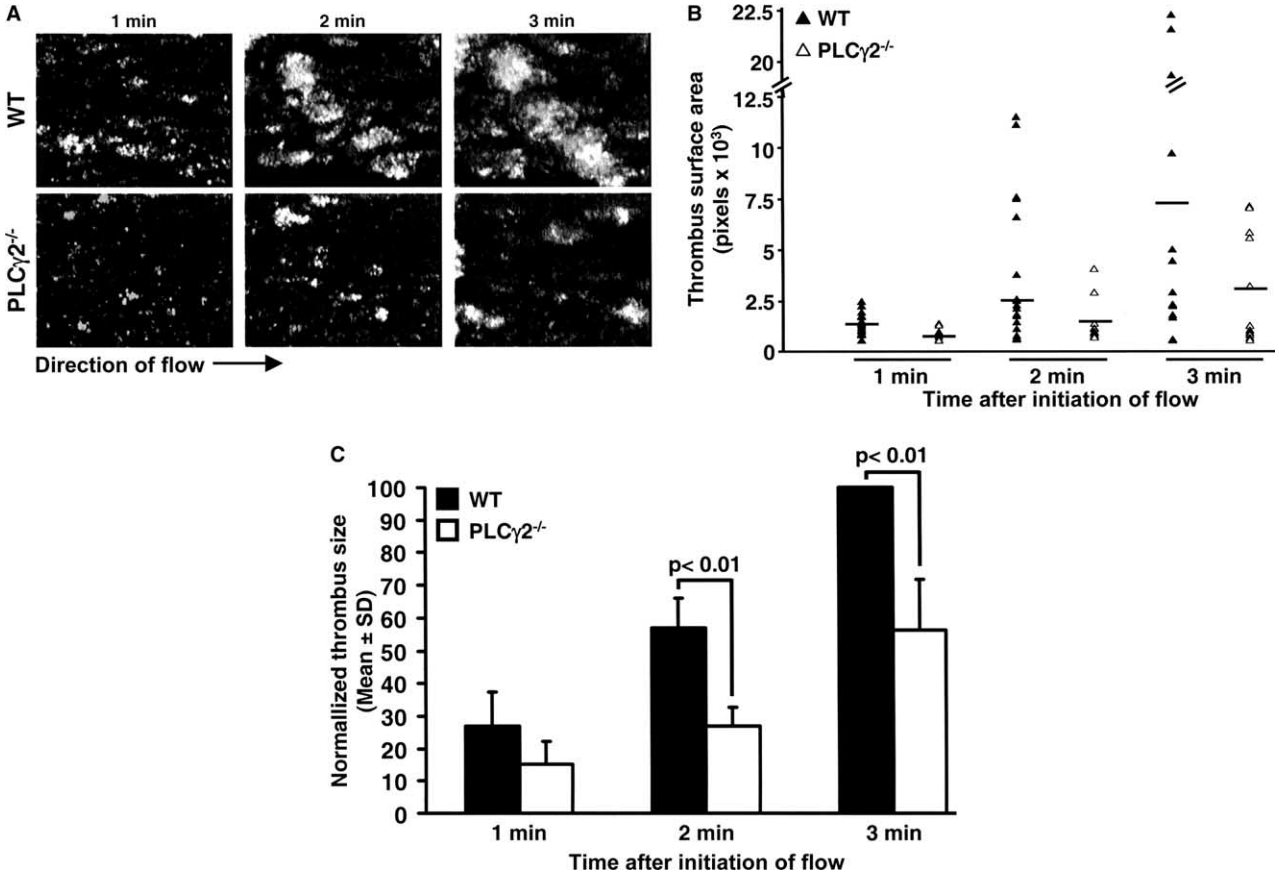

Fig. 2. PLCγ2-deficiency affects the rate and extent of murine platelet thrombus formation on VWF under conditions of arterial shear. (A) Thrombus formation by WT (upper panels) and (PLC)$\gamma2^{-/-}$ (lower panels) platelets. Washed and RBC-reconstituted platelets were perfused over glass slides pre-coated with 40 μg/ml human VWF at arterial wall shear rates of 1500 s^{-1} in a parallel-plate flow chamber. The images shown are frames frozen at 1, 2 and 3 min after initiation of flow and are representative of three independent experiments. Note that thrombi formed more rapidly and grew larger in wild-type versus (PLC)$\gamma2^{-/-}$ platelets. (B) Photographic images were digitized and the surface area covered by each thrombus was determined as described in Section 2. Results are presented as a scatter plot of the sizes of individual thrombi formed by WT (filled triangles) and (PLC)$\gamma2^{-/-}$ (open triangles) platelets at 1, 2 and 3 min following initiation of flow and are representative of three independent experiments. Horizontal lines represent mean thrombus size. (C) Thrombus sizes in each of three different experiments were normalized and subjected to statistical analysis as described in Section 2. Results are presented as mean thrombus size ± S.E.M. of three independent experiments. Note that (PLC)$\gamma2^{-/-}$ platelets formed significantly smaller thrombi ($P < 0.01$) after 2 and 3 min of flow, relative to wild-type platelets.

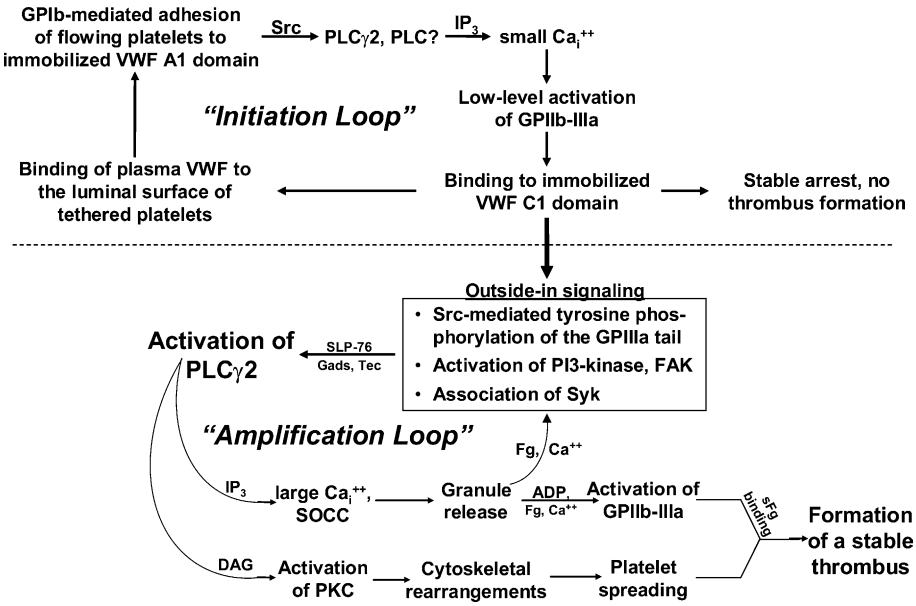

Fig. 3. A model showing events in GPIb-mediated thrombus formation to which PLCγ2 contributes.

domain [14,15] our data support the notion that PLCγ2, while not important for initial GPIb-mediated platelet adhesion to immobilized VWF, contributes importantly in amplifying GPIIb-IIIa mediated platelet spreading and lamellipodia formation on VWF.

3.2. PLCγ2 deficiency affects the rate and extent of murine platelet thrombus formation on VWF under conditions of arterial shear

We have recently shown that washed, red blood cell-reconstituted murine platelets can tether to, become activated by, and form thrombi on huVWF under conditions of arterial shear [18], thereby obviating the need for added botrocetin as a conformational modifier. These conditions are similar to those previously employed to examine huVWF/huGPIb interactions [22,23], and likely involve GPIb-mediated activation events that lead to secretion of (1) soluble agonists such as ADP and (2) adhesive proteins such as VWF and fibrinogen that together support thrombus formation in the absence of exogenously added plasma proteins. To examine the effects of PLCγ2 deficiency on thrombus formation under these conditions, we perfused wild-type and PLCγ2$^{-/-}$ murine platelets over immobilized huVWF at arterial shear rates. As shown in Fig. 2A, wild-type platelets began to form aggregates within one minute following the initiation of flow, and these aggregates rapidly coalesced to form large, stable thrombi. In contrast, PLCγ2$^{-/-}$ platelets were observed to form platelet-platelet contacts only transiently, limiting both the rate and extent of thrombus growth in a statistically significant fashion (Fig. 2B and C). These data demonstrate that PLCγ2 contributes importantly to the growth of platelet thrombi on VWF under conditions of arterial shear.

The observation that PLCγ2-deficient platelets can still form thrombi following exposure to immobilized VWF, albeit smaller and more transiently (see supplemental online video to Fig. 2A) than those formed by wild-type platelets, suggests that other PLC isoforms may be involved in this process. Consistent with this notion, we found that pharmacological inhibition of total PLC activity by U73122, a PLC antagonist [24] while not affecting stable adhesion, completely abolished thrombus formation by human platelets on VWF (data not shown). Since PLCβ isoforms do not appear to be involved in GPIb-mediated platelet activation [16], it is likely that PLCγ1, though expressed at much lower levels than is PLCγ2 [12], may compensate for the absence of PLCγ2 in supporting thrombus formation on VWF. In fact, PLCγ1 has recently been shown to function downstream of GPVI following platelet exposure to collagen [12]. The contribution of PLCγ1 to GPIb- and GPIIb-IIIa-mediated signal transduction using genetic models of PLCγ1 deficiency is currently under investigation.

In conclusion, our data extend the recent findings of Mangin et al. [16] and strongly support the concept that PLCγ2, while playing only a minor role in initial platelet adhesion to immobilized VWF, contributes importantly to platelet spreading and platelet thrombus formation on VWF under conditions of arterial shear. Since efficient thrombus formation involves secretion of secondary mediators such as ADP and binding of soluble fibrinogen and VWF to activated GPIIb-IIIa complexes, it would appear that the major contribution of PLCγ2 is as an amplifier of outside-in signals emanating from GPIIb-IIIa (see schematic in Fig. 3). Further studies will be required to delineate the potential for other PLC isoforms to contribute to platelet activation, adhesion, and thrombus formation in vivo.

Acknowledgements: The authors are grateful to Bob Montgomery for supplying human VWF, to Tim Nichols for botrocetin, and to Cheryl Hillery for help with flow chamber studies. This work was supported by Grant HL-44612 from the Heart, Lung and Blood Institute of the National Institutes of Health.

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