A PLCγ2-independent platelet collagen aggregation requiring functional association of GPVI and integrin $\alpha_2\beta_1$

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Abstract The role of the phospholipase C (PLC)γ2 isotype in platelet activation was evaluated by studying PLCγ2 −/− mice. These mice have a prolonged bleeding time but their platelets respond normally to non-collagenous agonists. PLCγ2-null platelets show residual aggregation response to collagen fibres (6% versus 74% for wild-type) with minimal granule secretion and no shape change. A delayed shape change is observed at later aggregation times. Specific activation by glycoprotein (GP)VI agonists (convulxin, collagen-related peptide and GPVI crosslinking) is, however, abolished. Antibodies against integrin $\alpha_2\beta_1$ and GPVI each inhibit the residual collagen response, implying a role of $\alpha_2\beta_1$ in platelet activation and a functional association with GPVI. These responses are also prevented by blocking integrin $\alpha_{IIb}\beta_3$ and phosphoinositide 3-kinase, whereas aspirin treatment and ADP receptor blockade only inhibit shape change. These results provide evidence for a PLCγ2-independent collagen activation pathway requiring cooperation between GPVI and $\alpha_2\beta_1$ leading to $\alpha_{IIb}\beta_3$-dependent aggregation and shape change by released ADP and thromboxane A_2.

Key words: Collagen; Integrin $\alpha_2\beta_1$; Phospholipase C-γ2; Knockout mouse; Platelet; Signal transduction

1. Introduction

Collagen fibres exposed at sites of vascular injury play a crucial role in haemostasis and thrombosis by initiating platelet adhesion and activation. Several collagen receptors have been identified on platelets, the two most important being the integrin $\alpha_2\beta_1$ (glycoprotein (GP)Ia–IIa, VLA2) and the immunoglobulin (Ig) superfamily protein GPVI [1,2]. Although the different contributions of these two receptors are still subject to debate, the currently favoured mechanism ascribes roles to integrin $\alpha_2\beta_1$ in platelet adhesion and to GPVI in platelet activation [1,3]. GPVI-dependent signalling, which has been studied in more detail, follows a pathway requiring the associated FcRγ chain and culminates after a series of phosphorylation steps in activation of phospholipase C (PLC)γ2 [4]. Studies with FcRγ-chain-deficient platelets, which lack GPVI, or with platelets in which GPVI was depleted in vivo by antibody treatment strongly suggest an essential role of this receptor for collagen-induced activation [5,6]. The existence of an $\alpha_2\beta_1$ signalling pathway remains controversial with reports of weak $\alpha_{IIb}\beta_3$ activation, or the recent proposal of a minor role of $\alpha_2\beta_1$ in collagen responses based on studies in knockout mice lacking α_2 or β_1 [7–9]. Analysis of collagen-induced activation is further complicated by amplification loops involving agonists like ADP and thromboxane A_2 (TXA_2) which are released from platelets and act on G protein-coupled receptors linked to PLCβ [10].

Seven PLC isozymes belonging to the PLCβ, γ and δ families have been identified in platelets, the major isoforms being PLCβ2, PLCβ3 and PLCγ2 [11]. A large number of platelet agonists including ADP, thrombin and TXA_2 activate PLCβ isozymes through $G\alpha_q$ protein-coupled receptors [12]. Collagen and immune complexes acting on GPVI and FcRγIIA respectively appear to activate PLCγ2 on the basis of observations of PLCγ2 phosphorylation or the presence of PLCγ2 in signalling complexes following platelet activation [13,14]. On the other hand, the role of PLCγ1 has been questioned, at least with regard to collagen-induced activation, owing to its lack of phosphorylation [13].

A straightforward analysis of the role of PLCγ2 in collagen-triggered responses and more generally in agonist-induced platelet activation has become feasible with the recent development of PLCγ2 knockout mice [15,16]. These animals are viable, despite defects in B cell maturation and signalling and an intraperitoneal and gastrointestinal bleeding tendency [15]. Platelet aggregation and secretion were found to be normal in PLCγ2-deficient mice in response to ADP or thrombin, but abolished in response to collagen [15].

The aim of the present study was to examine in more detail the contribution of PLCγ2 to platelet responses to collagen and other physiological agonists. Collagen-induced platelet activation and aggregation were severely decreased but not fully abolished in PLCγ2-deficient mice, which responded normally to agonists acting through G protein-coupled receptors but had a prolonged bleeding time. The residual activation was found to be dependent on both GPVI and integrin $\alpha_2\beta_1$

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Abbreviations: CRP, collagen-related peptide; GP, glycoprotein; Ig, immunoglobulin; mAb, monoclonal antibody; PI3K, phosphoinositide 3-kinase; PLC, phospholipase C; vWF, von Willebrand factor

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and, through activation of phosphoinositide 3-kinase (PI3K) and integrin $\alpha_{IIb}\beta_3$, to lead to an ADP/TXA_2-dependent response.

2. Materials and methods

2.1. Materials

ADP was purchased from Roche Molecular Biochemicals (Meylan, France). U46619, serotonin (5-HT), adrenaline, thrombin, arachidonic acid, MRS 2179 and insoluble fibrillar type I bovine collagen were from Sigma (Saint-Quentin Fallavier, France). Horm fibrillar type I collagen from equine tendons was from Nycomed (Munchen, Germany). Human fibrinogen was from Kabi (Stockholm, Sweden) and [^{3}H]serotonin from Dupont NEN (Boston, MA, USA). Potato apyrase [17] and human von Willebrand factor (HvWF) [18] were purified according to published procedures. The specific ADP/ATPase ratio of the apyrase is 1/8 with a 60 μmol min^{-1} mg^{-1} specific ADPase activity. AR-C69931MX was provided by Astra Charwood (Loughborough Leics, UK) while botrocetin was a gift from S. Jackson (Australian Centre for Blood Diseases, Box Hill, Australia). Aspirin was purchased from Synthelabo (Fallavier, France), LY294002 and Wortmannin were from Calbiochem (La Jolla, CA, USA) and the integrin $\alpha_{IIb}\beta_3$ antagonist Ro 43-5054 was provided by B. Steiner (Hoffmann LaRoche, Basel, Switzerland). HMα2, a monoclonal antibody (mAb) against mouse integrin $\alpha_2\beta_1$, was from PharMingen (La Jolla, CA, USA) while rat anti-mouse integrin $\alpha_{IIb}\beta_3$ (RAM.2), GPIbβ (RAM.1), GPIbα (RAM.6), CD9 (RAM.4), and GPVI (JAQ1) were produced in our laboratories as described previously [6,19,20]. Hamster anti-mouse GPV was kindly provided by Dr J. Fujimoto (National Children's Medical Research Center, Tokyo, Japan). Rabbit anti-rat IgG was from Jackson Laboratories (West Grove, PA, USA), collagen-related peptide (CRP) was provided by Dr R. Farndale (University of Cambridge, UK) [21] and convulxin was a gift from Dr M. Jandrot-Perrus (Faculté Xavier Bichat, Paris, France) [22].

2.2. Mouse strains

A PLCγ2-deficient mouse colony was established in the animal facilities of the Etablissement Français du Sang-Alsace by breeding heterozygotes provided by Pr. J. Ihle (St. Jude Children's Research Hospital, Memphis, TN, USA). FcRγ-chain-deficient mice were obtained from Taconic (Germantown, NY, USA).

2.3. Bleeding time

The bleeding time was measured by severing a 3 mm segment from the distal tail of 8–10 week old mice. The amputated tail was immediately immersed in isotonic saline at 37°C and the time required for the blood flow to stop was recorded. If bleeding did not cease after 30 min, the tail was cauterised and ≥1800 s was noted as the bleeding time.

2.4. Platelet aggregation and secretion

Blood drawn into ACD-anticoagulant from the abdominal aorta of four to six mice was pooled and platelets were washed and adjusted to 2.10^5/μl in Tyrode's buffer (12 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, pH 7.3, 137 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose) containing 5 mM HEPES and 0.35% albumin [19]. Platelet aggregation was measured turbidimetrically (Payton, Scarborough, ON, Canada) [17]. A 450 μl aliquot of platelet suspension was stirred at 1100 rpm and activated by addition of different agonists (ADP, arachidonic acid, collagen or U46619), in the presence of human fibrinogen (55 μg ml^{-1}) and in a final volume of 500 μl. Secretion of the dense granule contents during aggregation was determined as previously described using platelets loaded with [^{3}H]serotonin in the washing procedure [10].

2.5. Scanning electron microscopy

Platelets were fixed in the aggregation cuvette by addition of 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3, 305 mOsm kg^{-1}) containing 2% sucrose and incubation for 45 min at room temperature. The fixed cells were allowed to adhere to poly-L-lysine-coated slides. After three washes in 0.9% saline and dehydration in graded ethanol solutions, the samples were air-dried with hexadimethyldisilazane, sputtered with gold and examined under a Hitachi S-800 scanning electron microscope (Hitachi, Tokyo, Japan) (5 kV) [10].

2.6. Statistical analysis

The statistical significance of differences between means was evaluated using Student's *t*-test for paired samples and *P* values of less than 0.05 were considered to be significant.

3. Results

3.1. PLCγ2-deficient mice have a prolonged bleeding time

In an initial study PLCγ2-deficient mice were reported to have a tendency to spontaneous bleeding [15]. To evaluate more precisely a possible defect of primary haemostasis, standardised tail bleeding times were determined. The bleeding time was significantly prolonged in PLCγ2 −/− mice as compared to heterozygotes or wild-type mice with a mixed genetic background (median: 536 s versus 70 and 75 s respectively, $P < 0.0001$) (Fig. 1).

3.2. Platelet aggregation responses of PLCγ2-deficient mice

The requirement for PLCγ2 in platelet responses to agonists acting through different signalling pathways was assessed in aggregation experiments. High concentrations of bovine or equine fibrillar type I collagen induced severely decreased aggregation in PLCγ2 −/− platelets (Fig. 2A and data not shown). In contrast to the total lack of collagen-induced aggregation reported by Wang et al., we nevertheless observed a weak but highly reproducible aggregation response (6.1 ± 0.7% amplitude at 3 min for bovine collagen) in the absence of a turbidimetrically measurable shape change. This was followed after 4–5 min by a decrease in light transmission and loss of scattering power, indicating a delayed platelet shape change.

Normal aggregation was observed in PLCγ2 −/− platelets activated with low or high concentrations of ADP (0.25 and 1 μM) or thrombin (0.05 and 0.5 U ml^{-1}), arachidonic acid (100 μM) or the TXA_2 analogue U46619 (2 μM) (Fig. 2A and data not shown). The shape change induced by serotonin and

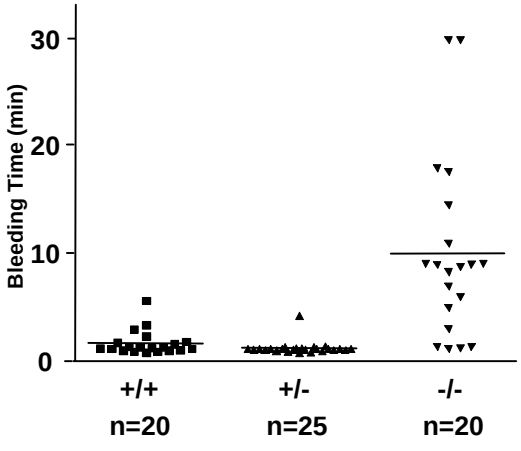

Fig. 1. PLCγ2-deficient mice have a prolonged bleeding time. Tail bleeding times were measured in PLCγ2 +/+, +/− and −/− mice as described in Section 2. Symbols represent individual mice and the median value for each population is indicated with a horizontal bar. The bleeding time was significantly prolonged in PLCγ2 −/− as compared to PLCγ2 +/− or PLCγ2 +/+ mice (median: 536 s ($n = 20$) versus 70 s ($n = 25$) and 75 s ($n = 20$) respectively, ***$P < 0.0001$).

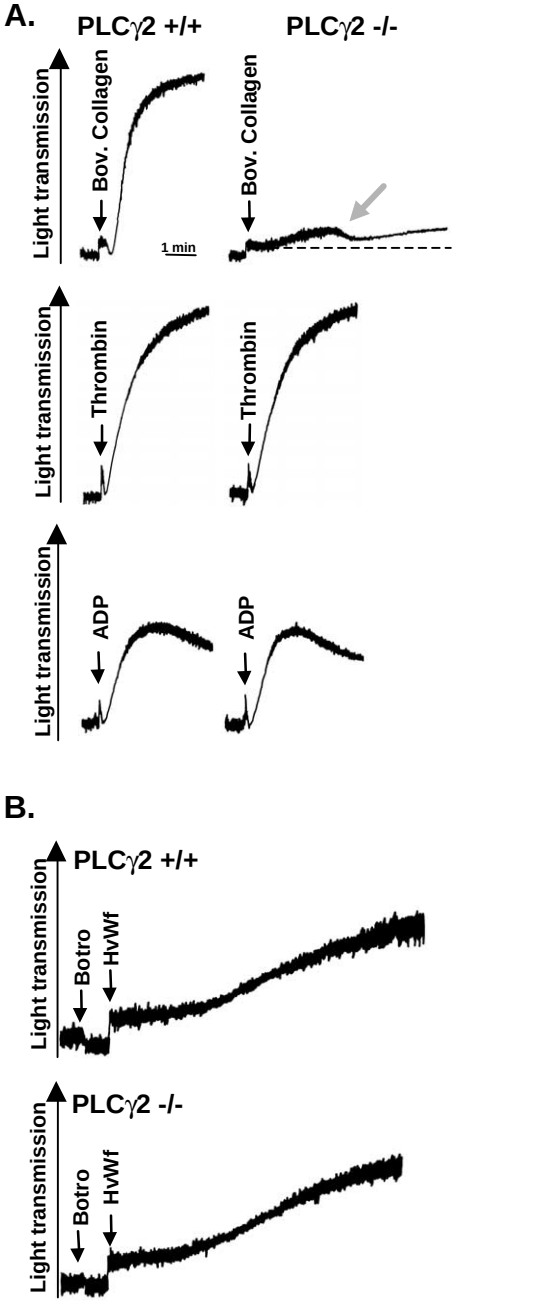

Fig. 2. Platelet aggregation responses of PLCγ2-deficient mice. Aggregation and agglutination responses of washed platelets from PLCγ2 −/− and +/+ mice. A: Aggregation induced by 50 μg ml^{-1} bovine collagen was strongly decreased in PLCγ2 deficiency and a delayed shape change (grey arrow) was observed, while responses to 1 μM ADP and 0.5 U ml^{-1} thrombin were not affected. The baseline is represented by a brocken line. B: Platelet agglutination induced by 5 μg ml^{-1} vWF and 2 μg ml^{-1} botrocetin in the presence of an $\alpha_{IIb}\beta_3$ integrin blocker (10 μM Ro 43-5054) was normal in PLCγ2 −/− mice. The aggregation profiles are representative of three separate experiments.

the potentiating effect of adrenaline on ADP-induced aggregation were also comparable in PLCγ2 +/+ and PLCγ2 −/− cells (data not shown). Finally, PLCγ2-deficient platelets agglutinated normally in response to vWF and botrocetin (Fig. 2B).

3.3. Morphological changes of PLCγ2-deficient platelets during collagen-induced activation

PLCγ2 −/− platelets challenged with collagen were analysed by scanning electron microscopy to better characterise their morphological changes during low amplitude aggregation. Resting PLCγ2 −/− platelets displayed a normal size and disc-shaped structure as compared to PLCγ2 +/+ cells (Fig. 3A,D). PLCγ2 +/+ platelets activated with 50 μg ml^{-1} collagen underwent a rapid (30 s) disc to sphere transformation, extended filopodia upon adhesion to the collagen fibres (Fig. 3B) and after 3 min formed large aggregates containing tightly packed cells (Fig. 3C). Small aggregates of mostly discoid platelets were observed in suspensions from PLCγ2 −/−

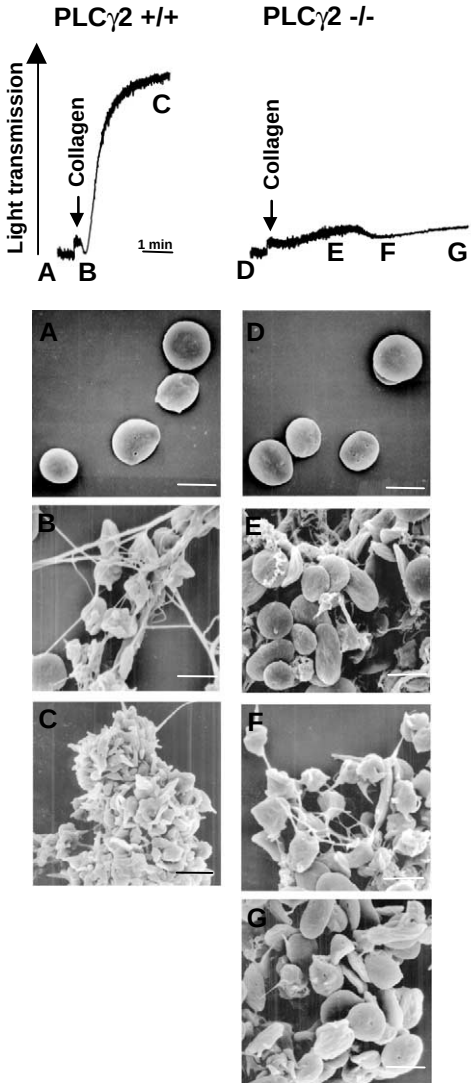

Fig. 3. Morphological changes during collagen-induced activation of PLCγ2-deficient platelets. Platelets from PLCγ2 +/+ or −/− mice were activated with 50 μg ml^{-1} collagen in the aggregometer cuvette (upper panel) and fixed at the times indicated by the letters A–G for scanning electron microscopy. At time 0, PLCγ2 +/+ (A) and PLCγ2 −/− (D) cells displayed a similar discoid shape typical of resting platelets. Collagen induced a rapid shape change (B) and formation of tightly packed aggregates (C) in wild-type platelets. PLCγ2-deficient cells did not change shape during the first 3 min following collagen activation and formed comparatively small aggregates (E). A late and reversible shape change was observed in these platelets after 4–5 min (F,G). The scale bar corresponds to 2 μm.

mice for 3–4 min following collagen addition (Fig. 3E). After 4–5 min, concomitant with the drop in light transmission, the platelets became more spherical and extended filopodia (Fig. 3F). These morphological changes were nevertheless transient, since at 5–6 min the cells recovered their discoid shape (Fig. 3G).

3.4. *Collagen-induced granule secretion is strongly decreased in PLCγ2-deficient platelets*

The role of PLCγ2 in platelet secretion was evaluated using [^{3}H]serotonin-loaded platelets. Maximal [^{3}H]serotonin release was obtained in both PLCγ2 −/− (83 ± 11%) and PLCγ2 +/+ (84 ± 2%) cells upon stimulation with 0.5 U ml^{-1} thrombin (Fig. 4). However, PLCγ2-deficient platelets displayed little secretion (1.25 ± 0.75%) in response to bovine fibrillar type I collagen, whereas wild-type platelets released 49 ± 0,65% of their dense granule contents.

3.5. *Platelet aggregation induced by selective GPVI ligands is abolished in PLCγ2 −/− mice*

Direct stimulation of GPVI with CRP (50 μg ml^{-1}), convulxin (4.4 nM) or JAQ1 (5 μg ml^{-1}) crosslinked by an anti-rat second antibody (20 μg ml^{-1}) failed to induce platelet aggregation in PLCγ2-deficient mice (Fig. 5).

3.6. *Analysis of the residual collagen-induced aggregation of PLCγ2-deficient platelets*

The failure of PLCγ2 −/− platelets to aggregate in response to GPVI ligands suggested the involvement of another collagen receptor, possibly integrin $\alpha_2\beta_1$. Incubation with a mAb (Hmα2) against mouse $\alpha_2\beta_1$ completely blocked aggregation and shape change in PLCγ2 −/− platelets (Fig. 6A and Table 2). To evaluate the possible activation by a pathway involving GPVI/FcRγ and independent of PLCγ2, aggregation studies were performed in PLCγ2 −/− platelets in the presence of blocking concentrations of JAQ1 (Fig. 6A) and in FcRγ chain −/− platelets, which also lack GPVI [23] (Fig. 6B). Aggregation in response to collagen was completely abolished under both conditions (Fig. 6 and Table 2). Treatment with a mAb against integrin $\alpha_{IIb}\beta_3$ or with two unrelated PI3K inhibitors (LY294002 (25 μM) or Wortmannin (100 nM)), also strongly and significantly inhibited aggregation of PLCγ2 −/− platelets (∼1% as compared to ∼6% at 5 min, Fig. 6A and Table 2). Finally, the involvement of released ADP and TXA_2 in the PLCγ2-independent platelet response was evaluated by blocking the $P2Y_1$ and $P2Y_{12}$ receptors simultaneously with the selective ADP antagonists MRS-2179 (100 μM) and AR-C69931MX (5 μM) [24,25] or the TXA_2 pathway by treating the cells with 10 mM aspirin. In both cases, although the shape change typically observed at 4–5 min was blunted, the early aggregation triggered by collagen was not significantly affected (Fig. 6A and Table 2).

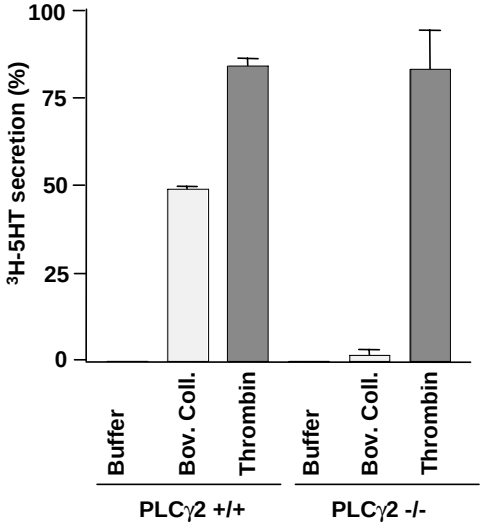

Fig. 4. Collagen-induced granule secretion is strongly decreased in PLCγ2-deficient platelets. PLCγ2 +/+ and −/− platelets, washed and loaded with [^{3}H]serotonin, were stimulated or not with 50 μg ml^{-1} type I collagen or 0.5 U ml^{-1} thrombin in the aggregometer cuvette. [^{3}H]serotonin secretion was measured in the supernatant after 3 min and results are the mean ± S.E.M. values from two separate experiments. Comparable levels of secretion were observed following thrombin activation whereas collagen-induced secretion was severely decreased in PLCγ2-deficient platelets but not abolished.

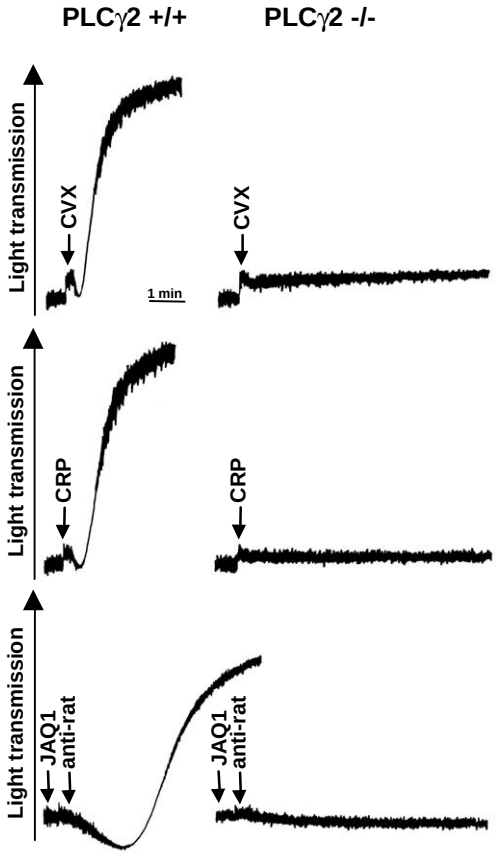

Fig. 5. Platelet aggregation in response to GPVI-selective agonists is abolished in PLCγ2-deficient mice. Washed platelets from PLCγ2 +/+ or −/− mice were stimulated with 4.4 nM convulxin, 50 μg ml^{-1} CRP or 5 μg ml^{-1} crosslinked JAQ1. These GPVI-selective agonists induced a full aggregation response in PLCγ2 +/+ platelets but did not activate PLCγ2 −/− cells. These results are from one experiment, representative of three.

4. Discussion

The main finding of this study is the existence of a PLCγ2-independent response to collagen in platelets. Although collagen-induced aggregation was severely impaired in PLCγ2-deficient platelets, some residual activation still occurred. On the other hand, selective stimulation of GPVI with convulxin,

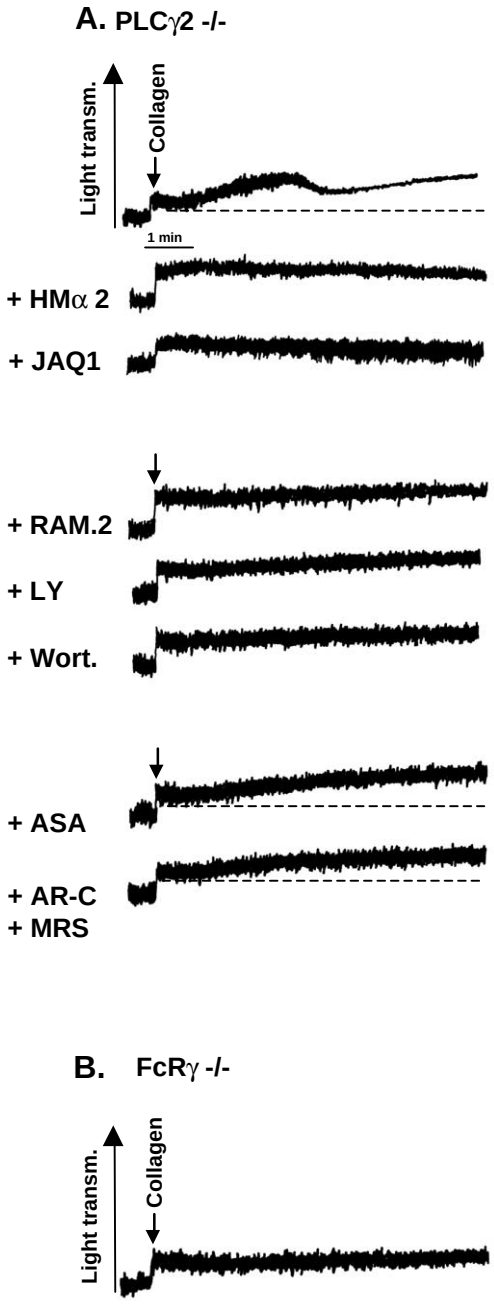

Fig. 6. Study of the receptors and signalling partners involved in collagen-induced aggregation of PLCγ2-deficient platelets. A: Washed PLCγ2 −/− platelets were stimulated with 50 μg ml^{-1} collagen in the aggregometer cuvette after pretreatment with inhibitors or antibodies blocking specific receptors or signalling partners: 10 μg ml^{-1} Hmα2 (mAb blocking $\alpha_2\beta_1$), 10 μg ml^{-1} JAQ1 (mAb blocking GPVI), 10 μg ml^{-1} RAM.2 (mAb blocking $\alpha_{IIb}\beta_3$), 25 μM LY294002 or 100 nM Wortmannin (PI3K inhibitors), 10 mM aspirin (ASA) or 100 μM MRS-2179 and 5 μM AR-C69931MX ($P2Y_1$ and $P2Y_{12}$ antagonists respectively). Typical aggregation curves are shown. The baseline is represented by a brocken line. B: Washed FcRγ-deficient platelets were stimulated with 50 μg ml^{-1} collagen.

CRP or crosslinked JAQ1 mAb completely failed to induce platelet activation in PLCγ2 −/− mice. The low level aggregation in response to collagen required mobilisation of both GPVI and integrin $\alpha_2\beta_1$ and led to integrin $\alpha_{IIb}\beta_3$ activation and to a late shape change triggered by TXA_2 production and ADP secretion. Agonists acting through G protein-coupled receptors were also found to activate platelets independently of the PLCγ2 pathway.

Table 1
Surface expression of different glycoproteins on wild-type and PLCγ2 −/− platelets

	PLCγ2 +/+	PLCγ2 −/−
$\alpha_2\beta_1$	5.4 ± 0.2	6.3 ± 1
GPVI	6 ± 0.3	5.1 ± 0.5
GPV	12.4 ± 1.5	15.5 ± 2.1
GPIbα	15 ± 6	21 ± 7
GPIbβ	36 ± 5.6	44 ± 5.2
$\alpha_{IIb}\beta_3$	53 ± 1.3	53 ± 4.7
CD9	92 ± 32	94 ± 15

Surface expression of the glycoproteins was measured by flow cytometry on PLCγ2 +/+ and PLCγ2 −/− platelets. Results are expressed as mean fluorescence intensity values ± S.D. from three platelet preparations for each group of mice.

We confirmed the previous study of Wang et al. [15], and observed 33% perinatal mortality in PLCγ2 −/− mice often accompanied by spontaneous gastrointestinal and intraperitoneal bleeding. Our work further revealed the existence of a prolonged bleeding time, pointing to a defect of primary haemostasis. This defect could not be attributed to a decreased platelet count or abnormal platelet structure (data not shown). Moreover, PLCγ2 deficiency did not affect the cell surface expression of various adhesive receptors including GPVI and integrin $\alpha_2\beta_1$ (Table 1), indicating that the haemostatic abnormality more likely resided at the signalling level. Bleeding times in PLCγ2 −/− mice were moderately increased compared to those reported in integrin β_3 −/− [26] and GPIbα

Table 2
Aggregation responses to 50 μg ml^{-1} bovine collagen of PLCγ2 +/+ and PLCγ2 −/− platelets and effects of specific inhibitors on PLCγ2 −/− residual aggregation

PLCγ2 +/+	73.8±2.7%	n=5	p<0.001
PLCγ2 -/-	6.1±0.7%	n=7	
+ HMα 2	0.5±0.5%	n=3	p<0.0003
+ JAQ1	1.0±1.0%	n=4	p<0.0002
+ RAM.2	1.2±0.5%	n=4	p<0.0003
+ LY	1.0±0.3%	n=3	p<0.0006
+ Wort.	1.3±0.3%	n=3	p<0.001
+ ASA	6.0±1.9%	n=3	p=0.776
+ AR-C + MRS	4.3±0.3%	n=3	p=0.102

The amplitude of aggregation was expressed as the mean percentage in light transmission ± S.E.M. at 3 min. The statistical significance of differences between PLCγ2 +/+ and PLCγ2 −/− platelets (upper bracket) and between untreated and treated PLCγ2 −/− samples was determined by a Student's *t*-test. *n* indicates the number of separate experiments.

−/− mice [27] but were comparable with those reported in mice immunologically depleted of GPVI [6]. These results illustrate the importance of the GPVI/PLCγ2 activation pathway for normal haemostasis and as a potential pharmacological target. Integrin $\alpha_2\beta_1$-deficient mice had normal bleeding time [7,9] in agreement with a secondary role of this receptor for full platelet activation.

The major defect of PLCγ2 −/− platelets was a profound decrease in collagen-induced aggregation, which was observed even for high concentrations of collagen. This confirmed the importance of this pathway in collagen-triggered platelet activation [4]. However, contrary to an initial report [15], a residual collagen response was observed with slight progressive aggregation but no initial shape change. This was not merely due to trapping of platelets by the collagen fibres as platelet to platelet contacts were observed by electron microscopy. Further indication of a true aggregation response was its blockade by two integrin $\alpha_{IIb}\beta_3$ antagonists, the mAb RAM.2 and the Ro 43-5054 compound (data not shown), and in the absence of added fibrinogen. One remarkable feature of this aggregation was that it occurred without obvious platelet shape change during the initiation phase.

It was reported very recently that PLCγ2-deficient platelets aggregated fully in response to high concentrations of collagen [28]. No information was available on the nature of the platelet aggregates and their structure. Although our study agrees with the existence of a PLCγ2-independent response to collagen, a much lower level of aggregation was observed in PLCγ2 deficiency even after strong collagen stimulation. This discrepancy is not easily explained as both studies were performed with the same mouse strain. One possible reason for the different aggregation response could reside in the platelet preparation, since we used washed platelets and Cho et al. [28] citrated platelet-rich plasma. Enhanced aggregation to weak agonists is known to occur when $[Ca^{2+}]_i$ is decreased to micromolar [29]. The difference was not explained by the source of collagen as we also observed a weak aggregation with the same commercial source of equine collagen. The aggregation response to equine collagen at 10 μg ml^{-1} was comparable to that obtained with 50 μg ml^{-1} bovine collagen (9% versus 7% amplitude at 4 min) and showed similar sensitivities to antagonists such as the PI3K inhibitor LY294002. However this collagen was not used in further studies as it gave in our hands less consistent responses between experiments. In addition, with concentrations above 10 μg ml^{-1} this reagent significantly decreased light transmission baseline values preventing correct calibration of the aggregometer and amplitude measurements and precluding meaningful dose–response studies. Aggregation experiments with bovine collagen responses have therefore been presented throughout this study. Cho et al. [28] interpreted their results as evidence for separate responses to low and high collagen concentrations with the latter being independent of PLCγ2. From the lack of sensitivity of the response to TXA_2 and ADP receptor blockade, they suggested the existence of a secretion independent signalling pathway specific for high collagen concentrations. The present results of a low level of aggregation in PLCγ2-deficient platelets and the use of GPVI-specific ligands and antagonists of GPVI and integrin $\alpha_2\beta_1$ are better explained by signalling mechanism triggered by the functional association of the two main collagen receptors.

However, this pathway only plays a synergistic role compared to the strong activation mediated by the GPVI/PLCγ2-dependent pathway. The failure of PLCγ2 −/− platelets to respond to GPVI agonists such as CRP, convulxin or antibody-crosslinked JAQ1 reveals, unlike in collagen stimulation, an absolute need for PLCγ2 in a pathway immediately downstream of GPVI, in agreement with a model proposed by several authors [1,4,30]. Since no response was observed even at concentrations of convulxin (a stronger agonist than collagen) inducing maximal aggregation and secretion in wild-type platelets, its absence could not be attributed to insufficient stimulation. This also agrees with the conventional role of GPVI in a full collagen response, known to involve signalling through PLCγ2, but does not provide an explanation for the collagen-induced aggregation and shape change observed in PLCγ2 −/− platelets.

Collagen differs from GPVI-specific ligands in that it is multivalent, which allows it to bind to other receptors. Earlier studies have implicated integrin $\alpha_2\beta_1$ in platelet activation [31,32] and patients lacking this integrin present defects of collagen-induced platelet adhesion and aggregation [33]. The recent inactivation of $\alpha_2\beta_1$ in mice played down the importance of this receptor for triggering of a full response, but did not rule out a role of $\alpha_2\beta_1$, particularly in view of the observed defective aggregation and adhesion of $\alpha_2\beta_1$ −/− platelets on soluble collagen [7]. The blockade of the collagen response in PLCγ2 −/− platelets by a mAb against integrin $\alpha_2\beta_1$ strongly supports an additional contribution of this receptor to collagen-dependent signalling.

Despite the lack of response to selective GPVI ligands, the fact that JAQ1 blocked collagen activation in PLCγ2 deficiency nevertheless points to the involvement of GPVI. This result, together with the absence of a collagen response in platelets depleted of GPVI by chronic JAQ1 treatment and in FcRγ −/− platelets [5,6,30,34], suggests that $\alpha_2\beta_1$ needs to be activated by GPVI for inducing a collagen signalling leading to integrin $\alpha_{IIb}\beta_3$ mobilisation and aggregation. The requirement for GPVI/FcRγ in a response distinct from the classical GPVI//FcRγ/PLCγ2 signalling pathway was unexpected. It raises the question of a possible separate functional association involving GPVI, FcRγ and $\alpha_2\beta_1$, as for example in raft microdomains requiring crosslinking by collagen fibres. GPVI/FcRγ association with raft formation has been demonstrated following stimulation with convulxin, which was apparently insufficient to trigger activation in PLCγ2 −/− platelets, but not following treatment with collagen [35,36]. Use of LY294002 and Wortmannin indicated a key role of PI3K activities in $\alpha_{IIb}\beta_3$-dependent collagen-triggered activation in the absence of PLCγ2. PI3K could also be implicated in GPVI-dependent activation of $\alpha_2\beta_1$, as suggested by Jung and Moroi from studies of the binding of soluble collagen to this integrin [37].

ADP and TXA_2 were not involved in the pathway leading to $\alpha_{IIb}\beta_3$ activation but were required for delayed shape change. Levels of TXA_2 and ADP production were not measured but were probably very low in view of the limited aggregation and minimal serotonin secretion in response to collagen. This level of secretion was calculated to produce ~35 nM ADP in the aggregation cuvette, a concentration sufficient to induce shape change of resting washed platelet. Secretion and shape change were abolished when the platelets were pretreated with RAM.2, suggesting that these responses resulted at least in part from $\alpha_{IIb}\beta_3$ outside-in signalling.

Many important physiological agonists such as ADP, thrombin, TXA_2 or serotonin signal through seven transmembrane domain receptors coupled to Gαq proteins which subsequently activate PLCβ [12]. Our results agree with this model in that they showed normal responses to all these agonists in PLCγ2-deficient mice. There have been reports that thrombin could also act through PLCγ2, notably on the basis of its phosphorylation after stimulation of platelets with thrombin [38]. Our findings do not support this hypothesis. Although some studies have implicated PLCγ2 in GPIb/vWF signalling [39], we observed no abnormalities of platelet agglutination in response to vWF, which suggests that PLCγ2 is not involved in regulating GPIb binding. Its role in GPIb-mediated intracellular signalling nevertheless remains to be determined.

In conclusion, this study has demonstrated an absolute requirement for PLCγ2 in GPVI signalling and its crucial but incomplete requirement for collagen-triggered responses. The PLCγ2-independent collagen signalling pathway requires GPVI in association with integrin $\alpha_2\beta_1$ and leads to activation of integrin $\alpha_{IIb}\beta_3$, but the nature of the link between GPVI and $\alpha_2\beta_1$ and details of the pathway leading to $\alpha_{IIb}\beta_3$ remain to be explored. The existence of multiple receptors and signalling pathways for collagen-induced platelet activation could be a way of avoiding severe bleeding in phenotypes bearing a genetic defect in one of these membrane proteins or intracellular enzymes.

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