

CalDAG-GEFI is at the nexus of calcium-dependent platelet activation

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The importance of the second messengers calcium (Ca²⁺) and diacylglycerol (DAG) in platelet signal transduction was established more than 30 years ago. Whereas protein kinase C (PKC) family members were discovered as the targets of DAG, little is known about the molecular identity of the main Ca²⁺ sensor(s). We here identify Ca²⁺ and DAG-regulated guanine nucleotide exchange factor I (CalDAG-GEFI) as a critical molecule in Ca²⁺-dependent platelet activation. CalDAG-GEFI, through activation of the small GTPase Rap1, directly triggers inte-

grin activation and extracellular signal-regulated kinase-dependent thromboxane A₂ (TxA₂) release. CalDAG-GEFI-dependent TxA₂ generation provides crucial feedback for PKC activation and granule release, particularly at threshold agonist concentrations. PKC/P2Y₁₂ signaling in turn mediates a second wave of Rap1 activation, necessary for sustained platelet activation and thrombus stabilization. Our results lead to a revised model for platelet activation that establishes one molecule, CalDAG-GEFI, at the nexus of Ca²⁺-induced integrin activation, TxA₂

generation, and granule release. The preferential activation of CalDAG-GEFI over PKC downstream of phospholipase C activation, and the different kinetics of CalDAG-GEFI- and PKC/P2Y₁₂-mediated Rap1 activation demonstrate an unexpected complexity to the platelet activation process, and they challenge the current model that DAG/PKC-dependent signaling events are crucial for the initiation of platelet adhesion. (Blood. 2009; 114:2506-2514)

Introduction

Platelet thrombus formation at sites of vascular injury is a complex and dynamic process that occurs in several phases.^{1,2} Platelet preactivation and tethering (transient adhesion) are mediated by the interaction of glycoprotein (GP) Iba with matrix-bound von Willebrand factor, particularly in conditions of high shear. Platelet activation and firm adhesion are then triggered by collagen exposed in the vessel wall and/or locally generated thrombin. Sustained integrin activation and the formation of stable thrombi further depend on persistent costimulatory signaling provided by the second wave agonists, thromboxane A₂ (TxA₂) and adenosine 5'-diphosphate (ADP), which are released from activated platelets.³⁻⁵

The central biochemical event in platelet aggregation is the agonist-induced inside-out activation of $\alpha_{IIb}\beta_3$ integrin. Most platelet agonists initiate intracellular signaling through the activation of phospholipase C (PLC), followed by the generation of the second messengers calcium (Ca²⁺) and diacylglycerol (DAG). DAG is critical for protein kinase C (PKC) activation, a key event in platelet granule release and integrin activation. Platelets express at least 6 PKC isoforms, which have activating as well as inhibitory roles in platelet activation.⁶⁻¹⁰ Early studies with calcium ionophores established a role of Ca²⁺ in integrin activation, TxA₂ generation, and granule release.¹¹⁻¹³ Initially, Ca²⁺-binding classical isoforms of PKC were suggested as the cell's main signal integrators for elevated intracellular Ca²⁺ concentrations ([Ca²⁺]_i). However, more recent studies using calcium chelators in combination with broad range PKC inhibitors identified an independent Ca²⁺-sensitive signaling pathway that acts synergistically with PKC in the activation of integrin $\alpha_{IIb}\beta_3$.^{14,15} Whereas the role of Ca²⁺ and DAG/PKC in various aspects of platelet activation has been well documented, the molecular nature of the Ca²⁺ sensor(s)

and the interplay between Ca²⁺- and DAG-driven signaling pathways have been less clearly defined.

In our recent work, we have identified Ca²⁺- and DAG-regulated guanine nucleotide exchange factor I (CalDAG-GEFI, RasGRP2) as a key molecule regulating Ca²⁺-dependent activation of integrins in platelets.^{16,17} CalDAG-GEFI contains binding sites for Ca²⁺ and DAG and a GEF domain catalyzing the activation of small GTPases of the Ras family, in particular Rap1 and Rap2.¹⁸ In platelets, Rap1B accounts for 90% of the total Rap protein,¹⁹ and its importance in $\alpha_{IIb}\beta_3$ activation has recently been demonstrated in Rap1B-deficient mice.²⁰ Importantly, studies in cell types other than platelets demonstrated only very weak affinity of the C1 domain of CalDAG-GEFI for DAG in platelets,^{21,22} suggesting that it is predominantly regulated by binding of Ca²⁺ to its hand domains. Several of our recent studies with platelets isolated from CalDAG-GEFI-deficient mice support this assumption. First, platelet aggregation induced by Ca²⁺ ionophores, but not by the phorbol ester phorbol myristate acetate (DAG mimetic), was sensitive to CalDAG-GEFI expression.¹⁶ Second, CalDAG-GEFI was critical for the rapid, but reversible activation of Rap1 in thrombin-activated platelets, a process that depends on increased [Ca²⁺]_i.¹⁷ And third, CalDAG-GEFI-dependent integrin activation occurred independent of signaling by PKC/P2Y₁₂, suggesting that CalDAG-GEFI is part of the Ca²⁺-dependent pathway of platelet activation, which synergizes with signaling by PKC in integrin activation.^{14,15}

Rap1 has been extensively studied for its role in the regulation of cell adhesion.²³ In cells other than platelets, Rap1 also regulates cellular differentiation and proliferation via pathways that involve the regulation of the mitogen-activated protein kinase (MAPK) cascade.²⁴ Rap1 either interferes with Ras-mediated extracellular

Submitted April 27, 2009; accepted July 2, 2009. Prepublished online as *Blood* First Edition paper, July 23, 2009; DOI 10.1182/blood-2009-04-218768.

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signal-regulated kinase (ERK) activation or it activates ERK independently of Ras in a cell context-dependent manner.²⁵ In certain neurons, regulation of Rap1-dependent MAPK activation has been found to be directly downstream of CalDAG-GEFI.^{26,27} In platelets, ERK-MAPKs have been implicated in the phosphorylation of cytosolic phospholipase A₂ (cPLA₂),²⁸ a key enzyme in the formation of arachidonic acid and thus TxA₂.²⁹⁻³¹ Interestingly, both Ca²⁺ and PKC signaling contribute to ERK1/2 activation.³²

In the present study, we investigated the mechanisms by which signaling by CalDAG-GEFI and PKC affects various aspects of platelet activation induced via stimulation of the collagen receptor, GPVI. Our studies demonstrate that CalDAG-GEFI is the main Ca²⁺ sensor that links increases in intracellular Ca²⁺ levels to the signaling pathways regulating integrin activation and ERK-MAPK signaling/TxA₂ formation in stimulated platelets. CalDAG-GEFI mediates the first wave of platelet TxA₂ formation, which provides critical feedback for the activation of PKC and granule release. P2Y₁₂/Gi-dependent signaling induces a second wave of platelet activation, which is required for the formation of stable platelet aggregates.

Methods

Reagents and antibodies

Lovenox (enoxaparin sodium; Sanofi-Aventis), heparin-coated capillaries (VWR), bovine serum albumin (fraction V), prostacyclin, human fibrinogen (type I), and U0126 (mitogen-activated protein kinase kinase [MEK] inhibitor; all from Sigma-Aldrich), 2-methylthio-adenosine 5'-monophosphate triethylammonium salt hydrate (2-MesAMP, P2Y₁₂ inhibitor; BioLog), complete protease inhibitor mixture lacking ethylenediaminetetraacetic acid (Roche Applied Science), Ro31-8220 (PKC inhibitor; EMD Chemicals), fibrillar collagen type I (Chronlog), calcein AM (Invitrogen), RalGDS-RBD coupled to agarose beads and polyvinylidene fluoride membranes (Millipore), U46619 (Cayman Chemical), acetylsalicylic acid (Bayer), and ³H-serotonin (Amersham Biosciences) were purchased. Convulxin (Cvx) was provided by K. Clemetson (Theodor Kocher Institute, University of Berne). Monoclonal blocking antibody to murine α_{IIb}β₃, Leo.H4, and monoclonal antibody directed against the activated form of murine α_{IIb}β₃, JON/A-PE, were purchased from Emfret Analytics. Anti-phospho(Thr²⁰²/Tyr²⁰⁴)-ERK, anti-ERK, and anti-phospho-(Ser) PKC substrate antibody were purchased from Cell Signaling Technology. Anti-Rap1 was purchased from Santa Cruz Biotechnology.

Mice

CalDAG-GEFI^{-/-} mice¹⁶ on a C57B1/6 background were obtained from the lab of Ann Graybiel (Massachusetts Institute of Technology) and were bred in the mouse facility of Thomas Jefferson University. Experimental procedures were approved by the Animal Care and Use Committee of Thomas Jefferson University.

Platelet preparation

Blood was drawn from the retroorbital plexus into heparinized tubes. Platelet-rich plasma was obtained by centrifugation at 100g for 5 minutes. Platelet-rich plasma was centrifuged at 700g in the presence of prostacyclin (2 μg/mL) for 5 minutes at room temperature. After 2 washing steps, pelleted platelets were resuspended at the concentration of 4 × 10⁸ platelets/mL in modified Tyrode buffer (137 mM NaCl, 0.3 mM Na₂HPO₄, 2 mM KCl, 12 mM NaHCO₃, 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 5 mM glucose, pH 7.3) containing 0.35% bovine serum albumin and 1 mM CaCl₂.

Flow cytometry

Washed platelets were diluted in Tyrode buffer containing 1 mM CaCl₂, activated with Cvx and/or U46619 in the presence of JON/A-PE³³ for

10 minutes, and studied immediately by flow cytometry. MesAMP was added before platelet stimulation to block P2Y₁₂ receptor signaling.

Aggregometry

Light transmission was measured in washed platelets activated in the presence of 50 μg/mL fibrinogen under stirring conditions at 37°C. Inhibitors and agonists were added at the indicated concentrations, and light transmission was recorded more than 10 minutes on a Chrono-log 4-channel optical aggregation system (Chrono-log).

Thromboxane generation assay

Platelets were stimulated in standard aggregometry. At different time points, 50 μL of sample was withdrawn and 5 mM EDTA (ethylenediaminetetraacetic acid)/1 mM aspirin was added. The cells were removed by centrifugation, and thromboxane B₂ (TxB₂) levels were determined in the supernatant by enzyme immunoassay (Assay Designs). TxB₂ is the stable product of the nonenzymatic hydration of TxA₂, which itself has a half-life of only 37 seconds under physiologic conditions.

Serotonin release

Washed platelets were incubated for 30 minutes at 37°C with ³H-serotonin (2 μCi [0.074 MBq]/mL). After one washing step, platelets were resuspended (4 × 10⁸ platelets/mL) in modified Tyrode buffer containing 1 μM imipramine and 1 mM CaCl₂. Platelets were stimulated in standard aggregometry. At the indicated time points, 50 μL of sample was withdrawn, and the reaction was stopped with an equal volume of 0.1 M EDTA/2% formaldehyde. The samples were then centrifuged for 5 minutes at 10 000g, and the supernatants were used for scintillation counting of ³H-serotonin. Total or 100% ³H-serotonin secretion was defined as the ³H-serotonin in samples lysed with 0.5% Triton X-100.

Rap1 and ERK activation

Platelets were stimulated in standard aggregometry for various times. Reactions were stopped with ice-cold 2× lysis buffer (100 mM Tris/HCl, pH 7.4, 400 mM NaCl, 5 mM MgCl₂, 2% Nonidet P-40, 20% glycerol, and complete protease inhibitor mixture lacking ethylenediaminetetraacetic acid). Cell lysis was completed on ice for 15 minutes. A total of 200 μL of sample was immediately solubilized in sample buffer (75 mM Tris/HCl, pH 6.8, 10% sodium dodecyl sulfate, 5% 2-mercaptoethanol, 0.004% bromophenol blue) for the detection of phosphorylated ERK2 and total ERK1/2 levels. A total of 200 μL of lysate was used to detect activated Rap1 according to the protocol previously described.¹⁶ Briefly, Rap1-adenosine triphosphate (GTP) was precipitated from lysates using RalGDS-RBD beads (Millipore).

Western blotting

Total protein lysates and precipitated proteins were separated on 4% to 12% or 4% to 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gradient gels and transferred to polyvinylidene fluoride membranes. Phosphorylated and nonphosphorylated ERK1/2 and Rap1 were detected with rabbit polyclonal antibodies. PKC-dependent protein phosphorylation was evaluated using a rabbit anti-phospho-(Ser) PKC substrate antibody. After incubation with anti-rabbit antibodies, conjugated to horseradish peroxidase (Vector Laboratories), immunoreactivity was detected by Western Lightning enhanced chemiluminescence (G-Biosciences).

Flow chamber assay

Whole blood anticoagulated with heparin (30 U/mL low molecular weight heparin; Lovenox) was perfused at a shear rate of 400 seconds⁻¹ over

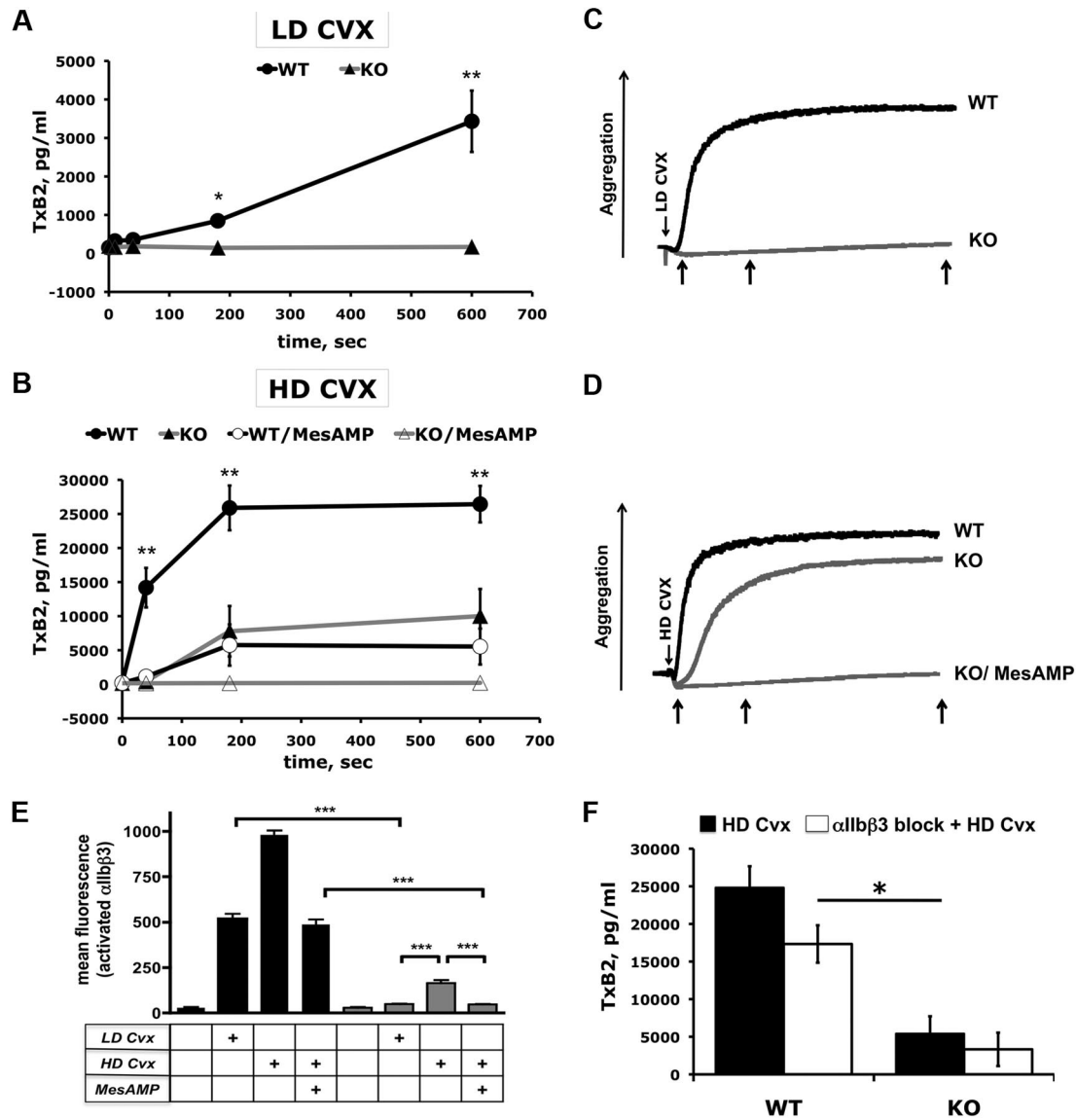


Figure 1. CalDAG-GEFI is central to Cvx-dependent TxA₂ generation. Washed platelets in the presence of 1 mM Ca²⁺ and 50 $\mu\text{g}/\text{mL}$ fibrinogen were stimulated under stirring conditions at 37°C (standard aggregometry) with (A,C) low (LD, 100 ng/mL) or (B,D) high (HD, 500 ng/mL) dose Cvx. Samples were withdrawn at the indicated time points to measure the levels of TxB₂, the stable product of the nonenzymatic hydration of TxA₂. (A-B) TxB₂ levels in the supernatant of WT (black line, ●) or CalDAG-GEFI^{-/-} (knockout [KO], gray line, ▲) platelets activated in the presence (open symbols) or absence (filled symbols) of 75 μM 2-MesAMP (P2Y12 inhibitor). Data shown are mean \pm SEM (n = 4). At a high dose of Cvx (B), significantly higher levels of TxB₂ were detected at all time points in the supernatant of activated WT platelets compared with WT/MesAMP, KO, or KO/MesAMP platelets. (C-D) Aggregation traces representative of 3 independent experiments. (E) WT (■) or CalDAG-GEFI^{-/-} (□) platelets were stimulated with LD (100 ng/mL) or HD (750 ng/mL) Cvx in the presence or absence of 2-MesAMP. Binding of JON/A-PE was measured to determine the level of $\alpha\text{IIb}\beta_3$ activation by flow cytometry. n = 6. (F) TxB₂ release from WT and CalDAG-GEFI^{-/-} (KO) platelets stimulated for 10 minutes with HD Cvx in the presence (□) or absence (■) of 40 $\mu\text{g}/\text{mL}$ $\alpha\text{IIb}\beta_3$ blocking antibody Leo.H4. Data shown are mean \pm SEM (n = 3), **P* < .05, ***P* < .01, ****P* < .001.

bovine type I collagen-coated glass slides (PureCol, 300 $\mu\text{g}/\text{mL}$; Inamed Biomaterials) in a parallel-plate flow chamber. Platelet adhesion was visualized with a 20 \times /0.45 PlanFluor objective on a Nikon Ti-U inverted microscope (Nikon Instruments Inc) equipped with a Retiga EXL monochrome camera (QImaging). Images were analyzed using Nikon NIS Elements AR 3.0 software and processed with Photoshop CS3. After 5 minutes of perfusion, the number of firmly adherent platelets was determined in 3 separate areas (0.02 mm²). The experiment was repeated 3 times for each genotype.

Statistics

Results are reported as mean plus or minus SEM. Statistical significance was assessed by unpaired 2-tailed Student *t* test. A *P* value less than .05 was considered significant.

Results

The generation of TxA₂ and ADP is crucial for platelet aggregation in response to stimulation of the main collagen receptor, GPVI. To test whether CalDAG-GEFI is critical for GPVI-induced formation of second wave mediators, we first studied TxA₂ generation in CalDAG-GEFI^{-/-} platelets activated with the nonphysiologic agonist. As shown in Figure 1A, TxA₂ generation, as measured by the formation of its stable analog TxB₂, was completely abolished in knockout platelets stimulated with low dose Cvx (100 ng/mL). In response to high dose (500 ng/mL) of the agonist, TxA₂ production was delayed and reached approximately 3-fold lower

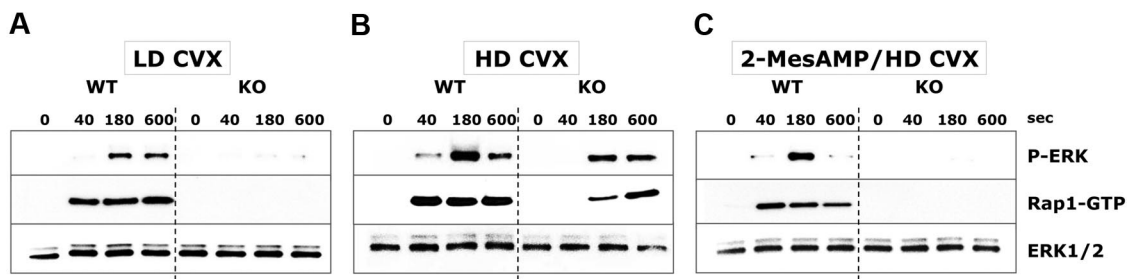


Figure 2. CalDAG-GEFI and P2Y12 synergize in Cvx-dependent activation of Rap1 and ERK-MAPK. Washed WT or CalDAG-GEFI^{-/-} (knockout [KO]) platelets were stimulated in standard aggregometry with (A) 100 ng/mL (low dose [LD]) or (B-C) 500 ng/mL (high dose [HD]) Cvx. Platelets were activated in the presence (C) of 75 μ M 2-MesAMP to inhibit signaling by P2Y12. Rap1-GTP and phospho-ERK were determined in samples withdrawn at the indicated time points. Total ERK1/2 levels (bottom panel) were determined as a loading control. Results are representative of 3 individual experiments.

concentrations in CalDAG-GEFI^{-/-} platelets compared with WT (Figure 1B).

Previous studies demonstrated that ADP/P2Y12 signaling is a major component of thrombin-induced TxA₂ generation.³¹ Thus, we investigated the role of the ADP/P2Y12 signaling pathway downstream of the collagen receptor GPVI. The P2Y12 inhibitor, 2-MesAMP, impaired TxA₂ release from Cvx-activated WT platelets to a similar extent as the absence of CalDAG-GEFI. Moreover, 2-MesAMP completely abolished the residual TxA₂ release observed in Cvx-stimulated CalDAG-GEFI^{-/-} platelets (Figure 1B), demonstrating a synergistic role of P2Y12-dependent and CalDAG-GEFI-dependent signaling in GPVI-dependent TxA₂ generation.

The time course of TxA₂ generation showed a striking correlation with integrin activation as monitored by standard aggregometry (Figure 1C-D). Direct assessment of $\alpha_{IIb}\beta_3$ activation in platelets stimulated with low dose Cvx, using an antibody against the activated form of the receptor (JON/A-PE³³), demonstrated completely inhibited activation of $\alpha_{IIb}\beta_3$ in CalDAG-GEFI^{-/-} platelets (Figure 1E). At high dose of Cvx, CalDAG-GEFI^{-/-} platelets showed a small, but significant increase in JON/A-PE binding, which was abolished in the presence of 2-MesAMP. To exclude the possibility that the defect in TxA₂ generation was secondary to the defect in integrin activation observed in knockout platelets, we determined TxA₂ release from platelets activated in the presence or absence of a blocking antibody to $\alpha_{IIb}\beta_3$. Integrin inhibition marginally reduced TxA₂ production in both WT and CalDAG-GEFI^{-/-} platelets. However, there was a significant difference in TxA₂ release between CalDAG-GEFI-deficient and $\alpha_{IIb}\beta_3$ -inhibited WT platelets ($P < .05$; Figure 1F), demonstrating that CalDAG-GEFI contributes to TxA₂ generation independent of its role in integrin inside-out signaling.

To further characterize the signaling pathways that lead to CalDAG-GEFI-dependent and CalDAG-GEFI-independent TxA₂ generation, we compared the kinetics of Rap1 activation with that of ERK-MAPK phosphorylation. Both ERK phosphorylation and Rap1 activation were completely abolished in CalDAG-GEFI^{-/-} platelets activated with 100 ng/mL Cvx (Figure 2A). When activated with 500 ng/mL Cvx, activation of both Rap1 and ERK was delayed (Figure 2B), and the residual activation observed in knockout platelets was abolished upon pretreatment of the cells with 2-MesAMP (Figure 2C).

In addition to Cvx, we studied platelets stimulated with fibrillar type I collagen (Figure 3). Collagen activation led to approximately 40% more TxA₂ generation in WT platelets compared with Cvx. TxA₂ release in collagen-activated CalDAG-GEFI-deficient platelets was markedly impaired and delayed (Figure 3A). The kinetics of TxA₂ release correlated well with those of Rap1 and ERK activation (Figure 3C), suggesting that CalDAG-GEFI is crucial for

the first wave of TxA₂ release. Pretreatment of knockout platelets with 2-MesAMP markedly reduced, but did not abolish collagen-induced TxA₂ release (Figure 3A), although activation of both ERK and Rap1 was completely inhibited under these experimental conditions (Figure 3D).

To confirm the role of ERK signaling in Rap1-dependent TxA₂ generation, we compared our results with 2-MesAMP-pretreated CalDAG-GEFI^{-/-} platelets (Rap1 activation completely blocked) with those obtained with WT platelets pretreated with the MEK inhibitor, U0126.³⁴ Pretreatment with U0126 completely inhibited ERK phosphorylation (not shown) and TxA₂ release in Cvx-activated WT platelets (Figure 3E). In collagen-activated platelets, U0126 abolished ERK phosphorylation (not shown), whereas it reduced TxA₂ release by approximately 60%. Thus, the GPVI/Rap1/ERK-dependent signaling pathway accounts for approximately 60% of the TxA₂ production in collagen-activated platelets.

Based on these results, we speculated that the defect in TxA₂ generation explains why CalDAG-GEFI^{-/-} platelets fail to aggregate in response to low concentrations of Cvx, and that addition of exogenous TxA₂ could restore aggregation of Cvx-stimulated knockout platelets. As expected, aggregation was observed in CalDAG-GEFI^{-/-} platelets stimulated with threshold concentrations of the thromboxane mimetic, U46619, and low dose Cvx, but not with either agonist alone (Figure 4A). Aggregation was reversed by pretreatment with a broad range PKC inhibitor, Ro31-8220, or the P2Y12 inhibitor 2-MesAMP. Consequently, binding of JON/A-PE to mutant platelets costimulated with Cvx and U46619 was significantly higher than in mutant platelets activated with either agonist alone, or platelets activated with Cvx and U46619 in the presence of 2-MesAMP (Figure 4B). Thus, in CalDAG-GEFI^{-/-} platelets activated with a low dose of a GPVI agonist, TxA₂ is required to trigger integrin activation through the CalDAG-GEFI-independent, but PKC/P2Y12-dependent pathway.

To confirm this hypothesis, we evaluated the phosphorylation state of downstream targets of PKC, such as pleckstrin-1 (PLEK),³⁵ in stimulated platelets. In WT platelets, PLEK phosphorylation was observed in response to threshold doses of Cvx or U46619. In contrast, PLEK phosphorylation was markedly reduced in CalDAG-GEFI-deficient platelets activated with low dose Cvx or low dose U46619. However, PLEK phosphorylation was restored in CalDAG-GEFI^{-/-} platelets activated with both Cvx and U46619 (Figure 4C). To test whether impaired integrin activation at low Cvx concentrations could be the result of defective release and cosignaling by ADP, we next studied dense granule release in CalDAG-GEFI-deficient platelets. Release of ³H-labeled serotonin from platelets activated under stirring conditions was measured (Figure 4D). Low dose Cvx stimulation induced the rapid release of

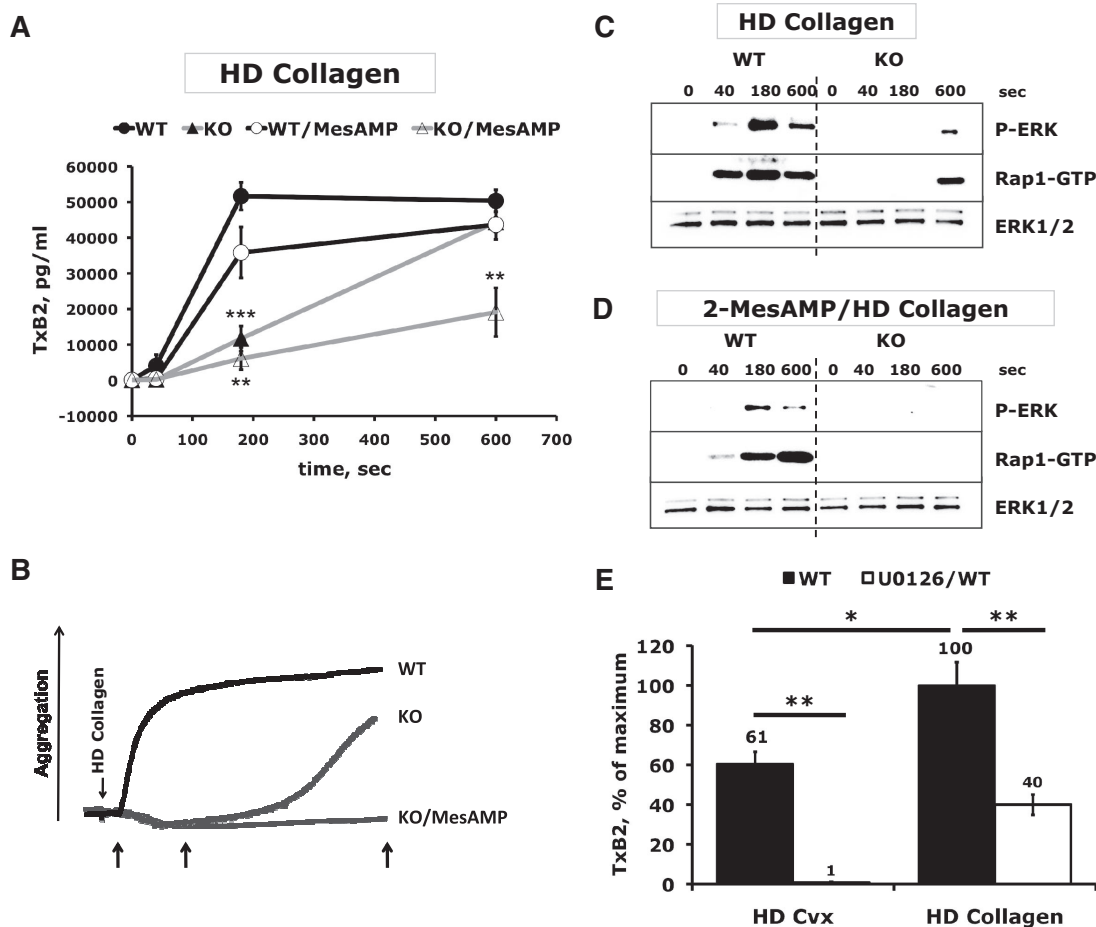


Figure 3. CalDAG-GEFI is critical for collagen-dependent TxA₂ generation. (A) Time course of TxB₂ release from WT (black line, circle) or CalDAG-GEFI^{-/-} (knockout [KO], gray line, triangle) platelets. Cells were stimulated with 25 μg/mL fibrillar type I collagen under stirring conditions in the presence (open symbols) or absence (filled symbols) of 75 μM 2-MesAMP. Data shown are mean ± SEM (n = 4). Significantly higher levels of TxB₂ were detected at indicated time points in the supernatant of activated WT platelets compared with KO or KO/MesAMP platelets. (B) Aggregation traces representative of 3 independent experiments. (C-D) Time course of ERK (top panel) and Rap1 (middle panel) activation in platelets activated with collagen in the presence (D) or absence (C) of 75 μM 2-MesAMP. Representative of 3 independent experiments. (E) TxB₂ levels in the supernatant of WT platelets stimulated for 10 minutes with 500 ng/mL Cvx (high dose [HD] Cvx), or 25 μg/mL fibrillar collagen (HD collagen). Cells were activated in the presence (□) or absence (■) of 50 μM U0126 (MEK inhibitor). U0126 totally abolished ERK phosphorylation in response to Cvx or collagen (data not shown). Data are shown as mean ± SEM. The amount of TxB₂ released from collagen-activated platelets was defined as 100%. n = 3. *P < .05, **P < .01, ***P < .001.

serotonin from WT, but not CalDAG-GEFI^{-/-}, platelets. However, a threshold dose of U46619 could partially restore Cvx-induced secretion. Secretion under these conditions was dependent on PKC activation, as it was blocked by the broad range PKC inhibitor, Ro31-8220.

Based on these findings, we speculated that TxA₂ released from WT platelets might be able to induce the aggregation of CalDAG-GEFI^{-/-} platelets activated with low dose collagen. As shown in Figure 5A, addition of 10% WT platelets partially restored aggregation of CalDAG-GEFI^{-/-} platelets (62% ± 23% of WT). In contrast, WT platelets pretreated with aspirin were unable to restore aggregation of knockout platelets (0% of WT). Furthermore, 10% of WT platelets failed to restore aggregation of CalDAG-GEFI^{-/-} platelets pretreated with a α_{IIb}β₃ blocking antibody (5% ± 1% of WT). Thus, TxA₂ released from the WT platelets provided a stimulatory signal required for α_{IIb}β₃ activation in CalDAG-GEFI^{-/-} platelets activated with low dose collagen. To confirm the generation of aggregates consisting of both WT and CalDAG-GEFI^{-/-} platelets, we repeated these studies with calcein green-labeled WT and calcein red-labeled CalDAG-GEFI^{-/-} platelets (Figure 5B). Fixed aggregates were visualized by fluorescence microscopy. As expected, green-labeled WT platelets recruited a

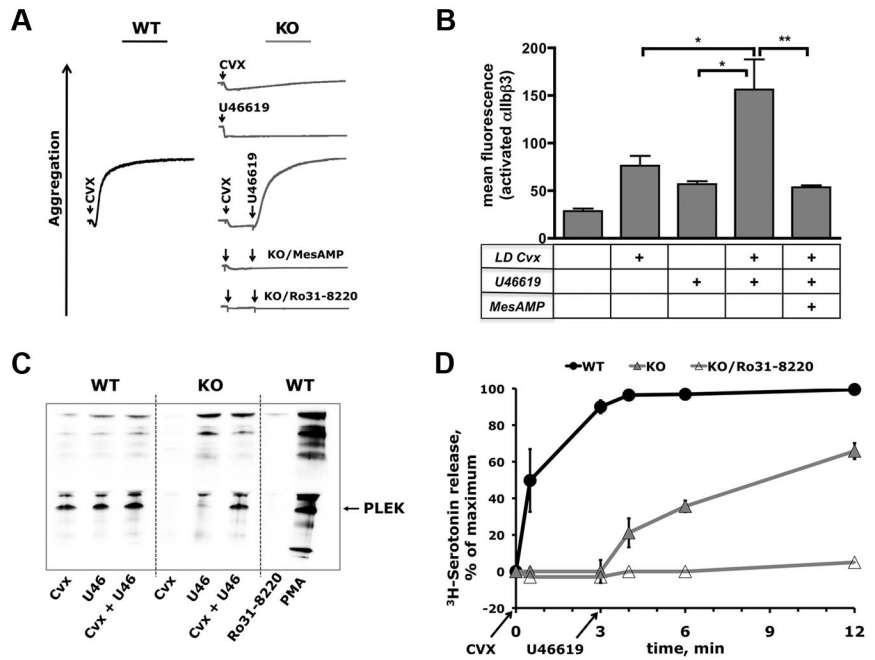
large number of red-labeled CalDAG-GEFI^{-/-} platelets into the aggregates. Pretreatment with an α_{IIb}β₃ blocking antibody, however, almost completely blocked the incorporation of knockout platelets.

To validate our findings under conditions of physiologic flow, we tested the effect of exogenous thromboxane on the adhesion of CalDAG-GEFI^{-/-} platelets to collagen in flow chamber studies (Figure 6A). Whereas WT platelets formed thrombi on the collagen surface, only few CalDAG-GEFI^{-/-} platelets were able to firmly adhere to collagen under these conditions. Addition of U46619 led to significantly more firm adhesion of CalDAG-GEFI^{-/-} platelets (Figure 6B). The effect of U46619 was reversed by pretreating CalDAG-GEFI^{-/-} platelets with 2-MesAMP, suggesting that exogenous thromboxane promotes adhesion of knockout platelets via an ADP/P2Y₁₂-dependent mechanism.

Discussion

Our studies establish a revised model for platelet activation by collagen (Figure 7). The key elements of this new model are as follows: (1) the central role of one molecule, CalDAG-GEFI, in

Figure 4. Exogenous TxA₂ restores aggregation, PKC activation, and dense granule secretion in Cvx-stimulated CalDAG-GEFI^{-/-} platelets. (A) Left: Aggregation of WT platelets stimulated with low dose Cvx (100 ng/mL). Right: Aggregation traces for CalDAG-GEFI^{-/-} platelets activated with 100 ng/mL Cvx, 500 nM U46619, or the combination of both agonists. Platelet aggregation was studied in the presence of 75 μM 2-MesAMP or 5 μg/mL Ro31-8220 to block signaling by P2Y12 and PKC, respectively. Results are representative of 5 individual experiments. (B) CalDAG-GEFI^{-/-} platelets were stimulated with low dose (LD) Cvx (100 ng/mL) and/or U46619 (3 μM) in the presence or absence of 2-MesAMP. Binding of JON/A-PE was measured to determine the level of α_{IIb}β₃ activation by flow cytometry. n = 6. **P < .001, *P < .05. (C) Detection of phosphorylated PKC substrates in platelet lysates (Western blotting). WT (left lanes) and CalDAG-GEFI^{-/-} (knockout [KO], central lanes) platelets were stimulated for 10 minutes with 100 ng/mL Cvx, 500 nM U46619, or the combination of both agonists. Phosphorylation of PKC substrates, including PLEK (←), in WT platelets pretreated with Ro31-8220 (10 μg/mL) or activated with phorbol myristate acetate (100 nM) were determined as controls (right panel). Results are representative of 3 independent experiments. (D) ³H-serotonin release in WT (black line, ●) or CalDAG-GEFI^{-/-} (gray line, ▲) platelets activated with 100 ng/mL Cvx. After 3 minutes, 500 nM U46619 was added to CalDAG-GEFI^{-/-} platelets. Experiments were performed in the presence (open symbols) or absence (filled symbols) of the PKC inhibitor Ro31-8220 (n = 3).



Ca²⁺-dependent platelet activation; (2) the preferential activation of CalDAG-GEFI over PKC downstream of PLC activation; (3) the importance of Rap1, activated by CalDAG-GEFI and P2Y12 signaling, in integrin activation and TxA₂ generation; (4) the critical role of CalDAG-GEFI in TxA₂ release and subsequent PKC activation, degranulation, and activation of P2Y12/Gi signaling; and (5) the kinetic differences between CalDAG-GEFI- and P2Y12-mediated Rap1 activation and the respective downstream signaling events.

In platelets activated with threshold concentrations of GPVI agonists, CalDAG-GEFI serves as a highly sensitive response element to Ca²⁺ that allows for the rapid activation of Rap1. CalDAG-GEFI-mediated Rap1 activation triggers a first wave of integrin activation and ERK (MAPK) signaling, followed by TxA₂ release. TxA₂ provides crucial feedback for the activation of PKC and granule/ADP release. ADP in turn triggers the second, P2Y12-dependent wave of Rap1-mediated signaling

events, leading to the sustained activation of integrins and further release of TxA₂. Higher concentrations of GPVI agonists lead to the concomitant activation of CalDAG-GEFI and PKC, facilitating integrin activation independent of feedback by endogenous TxA₂. It is important to note that the Gαq-coupled receptor for ADP, P2Y1,³⁶ also contributes to PLC signaling, thus supporting both CalDAG-GEFI- and PKC/P2Y12-dependent platelet activation (data not shown).

Our studies identify CalDAG-GEFI as a long sought-after link between increased [Ca²⁺]_i and the signaling mechanisms regulating integrin activation and TxA₂ release. To ensure an independent role of CalDAG-GEFI in both processes, we blocked integrin outside-in signaling or TxA₂ generation in platelets. The α_{IIb}β₃ blocking antibody, Leo.H4, only marginally reduced TxA₂ production in WT or knockout platelets (Figure 1F) and did not have any effect on ERK phosphorylation, demonstrating that CalDAG-GEFI-dependent TxA₂ production is independent of CalDAG-GEFI-

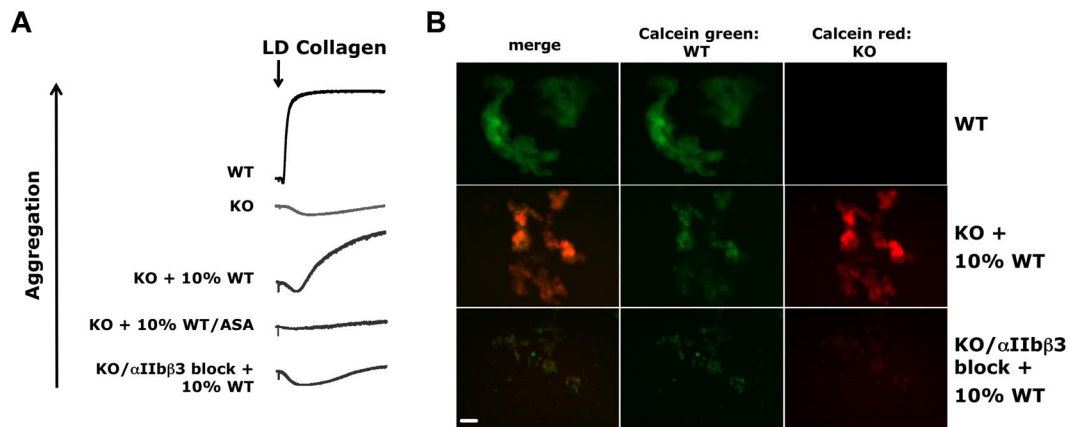


Figure 5. TxA₂ released from WT platelets restores aggregation of CalDAG-GEFI^{-/-} platelets. (A) Aggregation traces of platelets (10⁸ cells) activated with 10 μg/mL collagen. The aggregation response of WT (black line), CalDAG-GEFI^{-/-} (knockout [KO], light gray line), or CalDAG-GEFI^{-/-} platelets containing 10% WT platelets (dark gray line) was studied. WT platelets were pretreated with 1 mM aspirin (acetylsalicylic acid) before addition to CalDAG-GEFI^{-/-} platelets to inhibit TxA₂ release. CalDAG-GEFI^{-/-} platelets were pretreated with 40 μg/mL blocking antibody to α_{IIb}β₃ to demonstrate that the aggregation was integrin-dependent. Traces are representative of 3 individual experiments. (B) Representative images showing aggregates of calcein green-labeled WT and/or calcein red-labeled KO (CalDAG-GEFI^{-/-}) platelets fixed with 3.7% formaldehyde 10 minutes after stimulation and visualized with fluorescence microscopy. Scale bar = 25 μm.

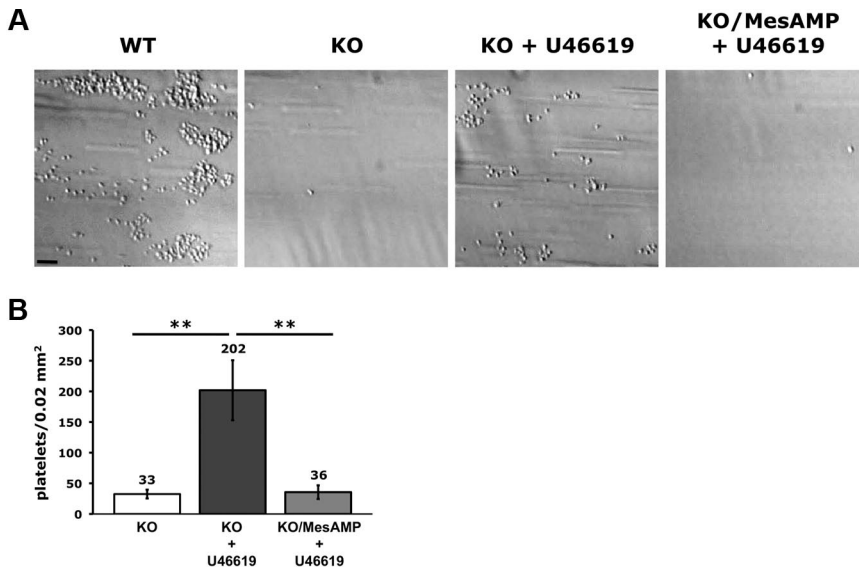


Figure 6. Exogenous TxA₂ induces firm adhesion of CalDAG-GEFI^{-/-} platelets to collagen. Whole blood from WT or CalDAG-GEFI^{-/-} mice was perfused over a collagen surface at venous shear rates (400 seconds⁻¹). (A) Representative images showing platelets firmly adherent to the collagen surface. U46619 (2 μM) was added to CalDAG-GEFI^{-/-} (knockout [KO]) whole blood immediately before the perfusion. A total of 100 μM 2-MesAMP was added 10 minutes before the perfusion. Scale bar = 10 μm. (B) Quantification of adherent platelets. The data are presented as the mean ± SEM of the absolute number of platelets per 0.02 mm² surface area. n = 9; 3 different blood preparations. **P < .01.

mediated integrin activation. In contrast, the defect in integrin activation in CalDAG-GEFI^{-/-} platelets was in part secondary to impaired TxA₂ generation observed in these cells. At high agonist concentrations, aggregation was mediated by CalDAG-GEFI and P2Y12 signaling in a TxA₂-independent manner. Aggregation of knockout platelets activated with low dose Cvx or low dose collagen, however, was restored by addition of exogenous TxA₂ (Figures 4A and 5). TxA₂ added important feedback for the activation of PKC and thus granule/ADP release (Figure 4C-D).

The small GTPase Rap1 is the major substrate of CalDAG-GEFI in platelets. We and others have demonstrated that CalDAG-GEFI, P2Y12, and Rap1 are critical to integrin activation in platelets^{5,17,20,37} and megakaryocytes.^{38,39} In other cell types, Rap1 also regulates cellular differentiation and proliferation via path-

ways that involve the regulation of the MAPK cascade.^{24,25} In platelets, ERK-MAPK play a critical role in the activation of cPLA₂,^{30,31} a key enzyme for the generation of arachidonic acid and thus TxA₂.²⁹ Our results provide the first evidence of a Rap1/ERK signaling pathway in platelets and of its involvement in TxA₂ generation. We found that GPVI-dependent TxA₂ generation relies entirely on the Rap1/ERK pathway, as inhibition of Rap1 activation (CalDAG-GEFI^{-/-} platelets + 2-MesAMP) or ERK signaling (WT platelets + U0126) abolished Cvx-dependent TxA₂ generation (Figure 3E). CalDAG-GEFI deficiency or inhibition of P2Y12 alone reduced TxA₂ release by approximately 70%. However, a marked difference in the kinetics of CalDAG-GEFI- and P2Y12-mediated ERK activation was observed. CalDAG-GEFI facilitated the rapid, but reversible activation of ERK, whereas P2Y12 signaling led to a delayed, but sustained generation of phospho-ERK. The latter findings confirm recent studies that identified a critical role of P2Y12 signaling for ERK activation and TxA₂ generation in thrombin-activated platelets.³¹

Recent studies by Ginsberg and colleagues identified Rap1-interacting adapter molecule as the potential downstream effector of Rap1 that controls integrin activation in platelets. Their results further suggest that Rap1-interacting adapter molecule targets talin to the cytoplasmic domain of the integrin, a critical event in the activation of these adhesion receptors.^{40,41} Little is known about the downstream target of Rap1 that mediates the activation of ERK. In fibroblasts, Rap1 was shown to antagonize MAPK signaling by sequestering the serine/threonin kinase, C-Raf.^{42,43} In contrast, studies in neurons demonstrated that recruitment of B-Raf to Rap1-GTP results in the sequential activation of MEK and ERK.⁴⁴ Thus, the activating or inhibitory effect of Rap1 on ERK signaling depends on the relative abundance of B-Raf and C-Raf found in a particular cell type.^{25,26,45} B-Raf is expressed in megakaryocytes and platelets, and the B-Raf/Rap1 pathway was shown to stimulate the ERK cascade in a model of megakaryocyte differentiation.⁴⁶ C-Raf expression in platelets has also been demonstrated.⁴⁷ Our studies suggest that Rap1/B-Raf/ERK signaling may be the predominant pathway in platelets. To our knowledge, there has been only one study on the role of Raf kinases in platelet activation, which suggested that ERK activation in response to thrombin relies on PKC, but not on Raf kinases.³² This conclusion, however, was based on an in vitro kinase activity assay, but not on the functional

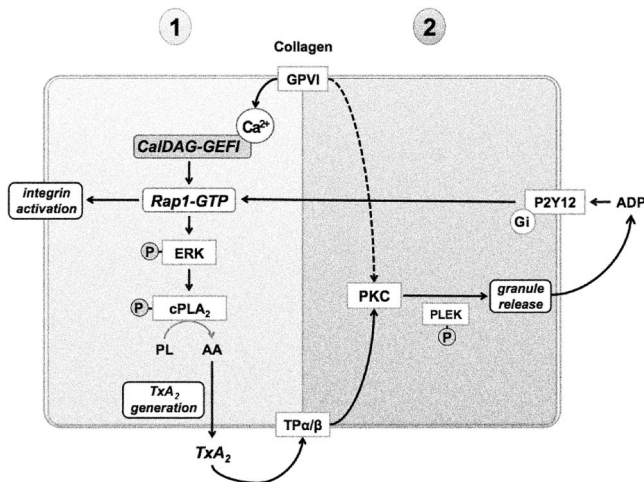


Figure 7. A revised model of platelet activation in response to low dose collagen. (1) A rise in [Ca²⁺], stimulates the CalDAG-GEFI-dependent first wave of Rap1 and integrin activation. Activated Rap1 also triggers ERK-dependent generation of TxA₂, which in turn provides critical feedback for the activation of PKC. (2) In a next step, PKC triggers the release of granule contents, including ADP. Released ADP signals through P2Y12/Gi to stimulate the second wave of Rap1/integrin activation and TxA₂ generation, which is required for the formation of stable platelet-platelet contacts. Higher concentrations of GPVI agonists lead to the concomitant activation of CalDAG-GEFI and PKC, facilitating integrin activation independent of feedback by endogenous TxA₂ (dashed line). AA indicates arachidonic acid; Gi, heterotrimeric G protein; P, residue phosphorylation; PL, phospholipids; and TPα/β, thromboxane receptors.

blockade of the kinases. Furthermore, these studies do not exclude the possibility of a B-Raf–dependent activation in platelets stimulated by agonists other than thrombin. Further studies are necessary to understand the role of Raf kinases in Rap1-dependent MAPK activation in platelets.

In addition to the GPVI-specific agonist Cvx, we have also stimulated platelets with fibrillar type I collagen, a physiologic agonist that binds to both GPVI and integrin $\alpha_2\beta_1$. In contrast to Cvx-activated platelets, collagen-dependent TxA₂ release was only approximately 60% reduced in 2-MesAMP–treated CalDAG-GEFI^{-/-} platelets or MEK/ERK inhibitor-treated WT platelets. Correlating with these results, we observed that WT platelets stimulated with fibrillar collagen produced approximately 40% more TxA₂ compared with cells activated with high dose Cvx. The most likely explanation for the Rap1- and ERK-independent formation of TxA₂ is outside-in signaling provided by $\alpha_2\beta_1$. Several in vitro and in vivo studies suggested a supportive role of $\alpha_2\beta_1$ in collagen-induced TxA₂ formation and platelet activation.^{48,49} Notably, Heemskerk and colleagues⁵⁰ demonstrated that platelets from α_2 integrin-deficient mice and aspirin-treated WT platelets have similar defects in their adhesion to collagen under flow. Based on their results, they concluded that integrin $\alpha_2\beta_1$ plays a role in stabilizing the thrombi by enhancing GPVI-dependent TxA₂ release.⁵⁰ In addition to ERK, the MAPK family members c-Jun N-terminal kinase 1 and p38 are present in platelets.^{51–53} Whereas p38 signaling has been linked to cPLA₂ phosphorylation in platelets stimulated by various agonists,^{54,55} no such role has been described for c-Jun N-terminal kinase 1. In addition to MAPK signaling, Shattil and colleagues recently demonstrated that integrin outside-in signaling could directly affect the enzymatic activity of a pool of cPLA₂ bound to the integrin.⁵⁶ Independent of the mechanisms regulating this second pathway for collagen-induced TxA₂ formation, our studies demonstrate that GPVI-dependent TxA₂ release requires signaling by CalDAG-GEFI and P2Y₁₂, which synergize at the level of Rap1 and ERK activation.

A key finding of this study is the critical role of CalDAG-GEFI signaling in GPVI-dependent TxA₂ release (Figure 1). Based on previous reports showing that collagen-induced aggregation strongly depends on feedback by endogenous TxA₂,^{57,58} we speculated that the aggregation/adhesion defects of CalDAG-GEFI^{-/-} platelets to collagen¹⁶ could be overcome by adding exogenous TxA₂. Indeed, we were able to restore GPVI-dependent aggregation of CalDAG-GEFI^{-/-} platelets by adding U46619 (Figure 4A) or TxA₂ released from WT platelets (Figure 5). Furthermore, addition of U46619 significantly increased the ability of CalDAG-GEFI^{-/-} platelets to firmly adhere to a collagen surface under physiologic flow conditions (Figure 6). Exogenous TxA₂ supported CalDAG-GEFI-independent, but P2Y₁₂-dependent platelet adhesion by providing critical feedback for GPVI-induced activation of PKC and thus granule/ADP release (Figure 4C–D). These findings may have important implications for the development of novel antiplatelet agents as well as for improving existing antiplatelet strategies. Our data identify CalDAG-GEFI as a powerful new target. Due to its

central role in the early phase of platelet activation, inhibitors of CalDAG-GEFI will have a strong antithrombotic effect, comparable with those observed with $\alpha_{IIb}\beta_3$ inhibitors. On strong thrombogenic surfaces, however, inhibition of CalDAG-GEFI can be partially overcome by PKC/P2Y₁₂ signaling, resulting in the firm adhesion of platelets, and thus reduced bleeding. Our studies also provide new insights on the mechanisms by which P2Y₁₂ inhibitors like clopidogrel, currently one of the most successful antiplatelet strategies in the clinic,⁵⁹ prevent thrombosis. Platelets treated with P2Y₁₂ inhibitors rely on signaling by CalDAG-GEFI for Rap1 activation. CalDAG-GEFI-mediated Rap1 activation occurs rapidly, but it is reversible. Consequently, P2Y₁₂ inhibitors markedly reduce GPVI-induced TxA₂ release, and integrin activation is transient. Under flow conditions, transient Rap1 activation does not allow stable platelet aggregates to form.

Future studies should be directed toward a better understanding of how P2Y₁₂ signaling leads to Rap1 activation. In addition to CalDAG-GEFI, the Rap1-GEFs CalDAG-GEFIII,⁶⁰ PDZ-GEF1,⁶⁰ and Epac1 (cyclic adenosine 5'-monophosphate-GEFI)⁶¹ have been identified in platelets and may thus be involved. Rap1 activation is also regulated by GTPase-activating proteins (GAPs). In platelets, Rap1GAP2 was recently identified.⁶⁰ In other cell types, G α i/o signaling was shown to promote ubiquitination and proteosomal degradation of Rap1GAP2, leading to enhanced Rap1 activity.⁶² However, the role of the above-mentioned RapGEFs or Rap1GAP2 in P2Y₁₂/G α i-dependent Rap1 activation in platelets has not been addressed. Another area of interest will be studies aimed at a better understanding of how Rap1 activation can be sustained in the absence of P2Y₁₂ signaling, or how to speed up Rap1 activation in the absence of CalDAG-GEFI signaling. Success in these studies could lead to a marked improvement of antiplatelet therapy, as it would provide strategies for the development of an antidote to P2Y₁₂ or CalDAG-GEFI inhibitors.

Acknowledgments

We thank Michael Holinstat for helpful discussions.

This work was supported by Scientist Development Grant 0630044N from the American Heart Association (W.B.).

Authorship

Contribution: L.S. designed the study, performed many of the experiments, and wrote the paper; R.C.R. maintained the mouse colony and helped with the experiments; and W.B. designed the study and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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