CalDAG-GEFI is at the Nexus of Calcium-dependent Platelet Activation

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Running title: CalDAG-GEFI in platelet calcium signaling
Abstract

The importance of the second messengers calcium (Ca\textsuperscript{2+}) and diacylglycerol (DAG) in platelet signal transduction was established more than 30 years ago. However, while protein kinase C (PKC) family members were discovered as the targets of DAG, little is known about the molecular identity of the main Ca\textsuperscript{2+} sensor(s). Here, we identify CalDAG-GEFI as a critical molecule in Ca\textsuperscript{2+}-dependent platelet activation. CalDAG-GEFI, through activation of the small-GTPase Rap1, directly triggers integrin activation and ERK-dependent thromboxane A\textsubscript{2} (TxA\textsubscript{2}) release. CalDAG-GEFI-dependent TxA\textsubscript{2} generation provides crucial feedback for the activation of PKC and granule release, particularly in cells activated with threshold agonist concentrations. PKC/P2Y12 signaling in turn mediates a second wave of Rap1 activation, which is necessary for sustained platelet activation and thrombus stabilization. Thus, our results lead to a revised model for platelet activation that establishes one molecule, CalDAG-GEFI, at the nexus of Ca\textsuperscript{2+}-induced integrin activation, TxA\textsubscript{2} 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 currently accepted view that DAG/PKC-dependent signaling events are crucial for the initiation of platelet adhesion.
Introduction

Platelet thrombus formation at sites of vascular injury is a complex and dynamic process that occurs in several phases. Platelet pre-activation and tethering (transient adhesion) is mediated by the interaction of GPIbα with matrix-bound von Willebrand factor (VWF), particularly in conditions of high shear. Platelet activation and firm adhesion is 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 co-stimulatory signaling provided by the second wave agonists, thromboxane A₂ (TxA₂) and ADP, which are released from activated platelets.

The central biochemical event in platelet aggregation is the agonist-induced inside-out activation of αIbb3 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. Early studies with calcium ionophores established a role of Ca²⁺ in integrin activation, TxA₂ generation and granule release. Initially, Ca²⁺-binding classical isoforms of protein kinase C (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 Ca\(^{2+}\)-sensitive signaling pathway that acts synergistically with PKC in the activation of integrin \(\alpha_{IIb}\beta_3\). Whereas the role of Ca\(^{2+}\) and DAG/PKC in various aspects of platelet activation has been well documented, the molecular nature of the Ca\(^{2+}\) sensor(s) and the interplay between Ca\(^{2+}\)- and DAG-driven signaling pathways have been less clearly defined.

In our recent work, we have identified Ca\(^{2+}\) and DAG regulated guanine nucleotide exchange factor I (CalDAG-GEFI, RasGRP2) as a key molecule regulating Ca\(^{2+}\)-dependent activation of integrins in platelets. CalDAG-GEFI contains binding sites for Ca\(^{2+}\) and DAG and a guanine nucleotide exchange factor (GEF) domain catalyzing the activation of small GTPases of the Ras family, in particular Rap1 and Rap2. In platelets, Rap1B accounts for 90% of the total Rap protein and its importance in \(\alpha_{IIb}\beta_3\) activation has recently been demonstrated in Rap1B-deficient mice. Importantly, studies in cell types other than platelets demonstrated only very weak affinity of the C1 domain of CalDAG-GEFI for DAG in platelets, suggesting that it is predominantly regulated by binding of Ca\(^{2+}\) to its EF hand domains. Several of our recent studies with platelets isolated from CalDAG-GEFI-deficient mice support this assumption. First, platelet aggregation induced by Ca\(^{2+}\) ionophores, but not by the phorbol ester PMA (DAG mimetic), was sensitive to CalDAG-GEFI expression. Second, CalDAG-GEFI was critical for the rapid but reversible activation of Rap1 in thrombin-activated platelets, a process that depends on increased [Ca\(^{2+}\)]\text{c}. And
third, CalDAG-GEFI-dependent integrin activation occurred independent of signaling by PKC/P2Y12, suggesting that CalDAG-GEFI is part of the Ca\(^{2+}\)-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 MAPK cascade\(^{24}\). Rap1 either interferes with Ras-mediated extracellular signal-regulated kinases (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-MAP kinases have been implicated in the phosphorylation of cytosolic phospholipase A\(_2\) (cPLA\(_2\))\(^{28}\), a key enzyme in the formation of arachidonic acid and thus TxA\(_2\)\(^{29-31}\). Interestingly, both Ca\(^{2+}\) 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 affect various aspects of platelet activation induced via stimulation of the collagen receptor, GPVI. Our studies demonstrate that CalDAG-GEFI is the main Ca\(^{2+}\) sensor that links increases in intracellular Ca\(^{2+}\) levels to the signaling pathways regulating integrin activation and ERK-MAPK signaling/TxA\(_2\) formation in stimulated platelets. CalDAG-GEFI mediates the first wave of platelet TxA\(_2\) 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, Bridgewater, NJ), heparin-coated capillaries (VWR, West Chester, PA), bovine serum albumin (BSA, fraction V), prostacyclin (PGI₂), human fibrinogen (type I) and U0126 (MEK inhibitor) (all from Sigma-Aldrich, St Louis, MO), 2-methylthio-AMP triethylammonium salt hydrate (2-MesAMP, P2Y12 inhibitor, BioLog, Bremen, Germany), complete protease inhibitor cocktail lacking ethylenediaminetetraacetic acid (Roche Applied Science, Indianapolis, IN), Ro31-8220 (protein kinase C inhibitor, EMD Chemicals, Gibbstown, NJ), fibrillar collagen type I (Chronlog, Havertown, PA), calcein AM (Invitrogen, Eugene, OR), RalGDS-RBD coupled to agarose beads and polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA), U46619 (Cayman Chemical, Ann Arbor, MI), acetylsalicylic acid (ASA, Bayer, Germany), and ³H-serotonin (Amersham Biosciences, Piscataway, NJ) were purchased. Convulxin was provided by K.J. Clemetson (Theodor Kocher Institute, University of Berne, Switzerland). Monoclonal blocking antibody to murine αIIbβ3, Leo.H4, and monoclonal antibody directed against the activated form of murine αIIbβ3, JON/A-PE, were purchased from emfret Analytics (Wuerzburg, Germany). Anti-phospho(Thr202/Tyr204)-ERK, anti-ERK and anti phospho-(Ser) PKC substrate antibody were purchased from Cell Signaling (Danvers, MA). Anti-Rap1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).
**Mice**

CalDAG-GEFI-/-\textsuperscript{16} and littermate control WT mice were obtained from the mouse facility at the 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 (PRP) was obtained by centrifugation at 100g for 5 minutes. PRP was centrifuged at 700g in the presence of PGI\textsubscript{2} (2μg/ml) for 5 minutes at room temperature. After two washing steps, pelleted platelets were resuspended at the concentration of 4-\texttimes10\textsuperscript{8} platelets/ml in modified Tyrode’s Buffer (137 mM NaCl, 0.3 mM Na\textsubscript{2}HPO\textsubscript{4}, 2mM KCl, 12 mM NaHCO\textsubscript{3}, 5 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 5 mM glucose, pH 7.3) containing 0.35% BSA and 1 mM CaCl\textsubscript{2}.

**Flow cytometry**

Washed platelets were diluted in Tyrodes buffer containing 1 mM CaCl\textsubscript{2}, activated with convulxin and/or U46619 in the presence of JON/A-PE\textsuperscript{33} for 10 minutes, and studied immediately by flow cytometry. MesAMP was added before platelet stimulation in order 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 over 10 minutes on a Chrono-log 4-channel optical aggregation system (Chrono-log, Havertown, PA).

**Thromboxane generation assay**

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

**Serotonin Release**

Washed platelets were incubated for 30 minutes at 37°C with ³H-serotonin (2 μCi [0.074 MBg]/mL). After one washing step, platelets were resuspended (4·10⁸ platelets/ml) in modified Tyrode’s Buffer containing 1 μM imipramine and 1 mM CaCl₂. Platelets were stimulated in standard aggregometry. At the indicated time points, 50 μl of sample were 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 $^3$H-serotonin. Total or 100% $^3$H-serotonin secretion was defined as the $^3$H-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 2x lysis buffer (100 mM Tris/HCl, pH 7.4, 400 mM NaCl, 5 mM MgCl$_2$, 2% Nonidet P-40, 20% glycerol and complete protease inhibitor cocktail lacking ethylenediaminetetraacetic acid). Cell lysis was completed on ice for 15 minutes. 200 μl of sample were 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. 200 μl of lysate were used to detect activated Rap1 according to the protocol previously described$^{16}$. Briefly, Rap1-GTP was precipitated from lysates using RalGDS-RBD beads (Millipore, Billerica, MA).

**Western Blotting**

Total protein lysates and precipitated proteins were separated on 4-12% or 4-20% SDS-PAGE gradient gels and transferred to PVDF membranes. Phosphorylated and non-phosphorylated 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, Burlingame, CA), immunoreactivity was detected by
Western Lightning enhanced chemiluminescence (G-Biosciences, Maryland Heights, MO).

**Flow chamber assay**

Whole blood anticoagulated with heparin (30 U/ml low molecular weight heparin, Lovenox) was perfused at a shear rate of 400 sec⁻¹ over bovine type I collagen-coated glass slides (PureCol, 300 μg/ml Inamed Biomaterials, Fremont, CA) in a parallel-plate flow chamber. Platelet adhesion was visualized with a Nikon Ti-U inverted microscope (Nikon Instruments Inc., Melville, NY) equipped with a Retiga EXL monochrome camera (QImaging, Surrey, Canada). Images were analyzed using Nikon NIS Elements software. After 5 minutes of perfusion, the number of firmly adherent platelets was determined in three separate areas (0.02 mm²). The experiment was repeated 3 times for each genotype.

**Statistics**

Results are reported as mean ± SEM. Statistical significance was assessed by unpaired 2-tailed Student t test. A P value less than 0.05 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 non-physiological agonist convulxin (Cvx). 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 ~3-fold lower concentrations in CalDAG-GEFI-/- platelets when compared to WT (Figure 1B).

Previous studies demonstrated that ADP/P2Y12 signaling is a major component of thrombin-induced TxA₂ generation. 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 TxA$_2$ generation showed a striking correlation with integrin activation as monitored by standard aggregometry (Figure 1C,D). Direct assessment of $\alpha$IIb$\beta$3 activation in platelets stimulated with low dose Cvx, using an antibody against the activated form of the receptor (JON/A-PE$^{33}$), demonstrated completely inhibited activation of $\alpha$IIb$\beta$3 in CalDAG-GEFI-/- platelets (Figure 1E). At high dose of Cvx, CalDAG-GEFI-/- platelets showed a small but significant increase in JON/A-PE binding, which was abolished in the presence of 2-MesAMP. To exclude the possibility that the defect in TxA$_2$ generation was secondary to the defect in integrin activation observed in knockout platelets, we determined TxA$_2$ release from platelets activated in the presence or absence of a blocking antibody to $\alpha$IIb$\beta$3. Integrin inhibition marginally reduced TxA$_2$ production in both WT and CalDAG-GEFI-/- platelets. However, there was a significant difference in TxA$_2$ release between CalDAG-GEFI-deficient and $\alpha$IIb$\beta$3-inhibited WT platelets ($P < 0.05$, Figure 1F), demonstrating that CalDAG-GEFI contributes to TxA$_2$ generation independent of its role in integrin inside-out signaling.

To further characterize the signaling pathways that lead to CalDAG-GEFI-dependent and -independent TxA$_2$ generation, we compared the kinetics of Rap1 activation to that of ERK-MAPK phosphorylation. Both ERK phosphorylation and Rap1 activation were completely abolished in CalDAG-GEFI-/- platelets activated with 100 ng/ml convulxin (Figure 2A). When activated with 500 ng/ml Cvx, activation of both Rap1 and ERK were 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 convulxin, we studied platelets stimulated with fibrillar type I collagen (Figure 3). Collagen activation led to ~40% more TxA2 generation in WT platelets when compared to convulxin. 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) to those obtained with WT platelets pretreated with the MEK inhibitor, U012634. Pretreatment with U0126 completely inhibited ERK phosphorylation (unpublished data) and TxA2 release in Cvx-activated WT platelets (Figure 3E). In collagen-activated platelets, U0126 abolished ERK phosphorylation (unpublished data) while it reduced TxA2 release by ~60%. Thus, the GPVI/Rap1/ERK-dependent signaling pathway accounts for ~60% of the TxA2 production in collagen-activated platelets.
Based on these results, we speculated that the defect in TxA\textsubscript{2} generation explains why CalDAG-GEFI/-/- platelets fail to aggregate in response to low concentrations of Cvx, and that addition of exogenous TxA\textsubscript{2} 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 co-stimulated 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, TxA\textsubscript{2} 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)\textsuperscript{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 convulxin 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 co-signaling by ADP, we next studied dense granule release in CalDAG-GEFI-deficient platelets. Release of $^3$H-labelled serotonin from platelets activated under stirring conditions was measured (Figure 4D). Low dose Cvx stimulation induced the rapid release of 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 TxA$_2$ 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 wild type platelets failed to restore aggregation of CalDAG-GEFI-/- platelets pretreated with a $\alpha$IIb$\beta$3-blocking antibody (5±1% of WT). Thus, TxA$_2$ released from the WT platelets provided a stimulatory signal required for $\alpha$IIb$\beta$3 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 αIIbβ3 blocking antibody, however, almost completely blocked the incorporation of knockout platelets.

In order to validate our findings under conditions of physiological flow, we tested the effect of exogenous thromboxane on the adhesion of CalDAG-GEFI-/- platelets to collagen in flow chamber studies (Figure 6A). While 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: (1) the central role of one molecule, CalDAG-GEFI, in Ca\textsuperscript{2+}-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 TxA\textsubscript{2} generation, (4) the critical role of CalDAG-GEFI in TxA\textsubscript{2} 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 Ca\textsuperscript{2+} 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 TxA\textsubscript{2} release. TxA\textsubscript{2} 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 TxA\textsubscript{2}. Higher concentrations of GPVI agonists lead to the concomitant activation of CalDAG-GEFI and PKC, facilitating integrin activation independent of feedback by endogenous TxA\textsubscript{2}. It is important to note that the G\textsubscript{\alpha}q-coupled receptor for ADP, P2Y1\textsubscript{1},\textsuperscript{36} also contributes to PLC.
signaling, thus supporting both CalDAG-GEFI- and PKC/P2Y12-dependent platelet activation (not shown).

Our studies identify CalDAG-GEFI as a long sought-after link between increased [Ca^{2+}]_{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 αIIbβ3-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 (unpublished data), demonstrating that CalDAG-GEFI-dependent TxA2 production is independent of CalDAG-GEFI-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 convulxin or low dose collagen, however, was restored by addition of exogenous TxA2 (Figure 4A, 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\textsuperscript{5,17,20,37} and
megakaryocytes. In other cell types, Rap1 also regulates cellular differentiation and proliferation via pathways that involve the regulation of the MAPK cascade. In platelets, ERK-MAPK play a critical role in the activation of cPLA₂, a key enzyme for the generation of arachidonic acid and thus TxA₂. 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 ~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, while 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.

Recent studies by Ginsberg and colleagues identified Rap1 interacting adapter molecule, RIAM, as the potential downstream effector of Rap1 that controls integrin activation in platelets. Their results further suggest that RIAM targets talin to the cytoplasmic domain of the integrin, a critical event in the activation of these adhesion receptors. 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\textsuperscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{46}. C-Raf expression in platelets has also been demonstrated\textsuperscript{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\textsuperscript{32}. This conclusion, however, was based on an \textit{in vitro} kinase activity assay but not on the functional 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 convulxin, we have also stimulated platelets with fibrillar type I collagen, a physiological agonist that binds to both GPVI and integrin $\alpha 2\beta 1$. In contrast to Cvx-activated platelets, collagen-dependent TxA\textsubscript{2}-release was only $\sim$60\% reduced in 2-MesAMP-treated CalDAG-GEFI\textsuperscript{-/-} platelets or MEK/ERK inhibitor-treated WT platelets. Correlating with
these results, we observed that WT platelets stimulated with fibrillar collagen produced ~40% more TxA₂ compared to cells activated with high dose convulxin. The most likely explanation for the Rap1 and ERK-independent formation of TxA₂ is outside-in signaling provided by α2β1. Several in vitro and in vivo studies suggested a supportive role of α2β1 in collagen-induced TxA₂ formation and platelet activation⁴⁸,⁴⁹. Notably, Heemskerk and colleagues demonstrated that platelets from α2 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 α2β1 plays a role in stabilizing the thrombi by enhancing GPVI-dependent TxA₂ release⁵⁰. In addition to ERK, the MAPK family members JNK1 and p38 are present in platelets⁵¹-⁵³. While p38 signaling has been linked to cPLA₂ phosphorylation in platelets stimulated by various agonists⁵⁴,⁵⁵, no such role has been described for JNK1. 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 cPLA₂α bound to the integrin⁵⁶. 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
TxA$_2^{57,58}$, we speculated that the aggregation/adhesion defects of CalDAG-GEFI/- platelets to collagen$^{16}$ could be overcome by adding exogenous TxA$_2$. Indeed, we were able to restore GPVI-dependent aggregation of CalDAG-GEFI/- platelets by adding U46619 (Figure 4A) or TxA$_2$ 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 physiological flow conditions (Figure 6). Exogenous TxA$_2$ 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 anti-platelet agents as well as for improving existing anti-platelets 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 anti-thrombotic effect, comparable to those observed with αIIbβ3 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 anti-platelet 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 TxA$_2$ 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 towards a better understanding of how P2Y12 signaling leads to Rap1 activation. In addition to CalDAG-GEFI, the Rap1-GEFs CalDAG-GEFIII,\textsuperscript{60} PDZ-GEF1,\textsuperscript{60} and Epac1 (cAMP-GEFI)\textsuperscript{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.\textsuperscript{60} In other cell types, G\textsubscript{a}i/o signaling was shown to promote ubiquitination and proteosomal degradation of Rap1GAP2, leading to enhanced Rap1 activity.\textsuperscript{62} However, the role of the above mentioned RapGEFs or Rap1GAP2 in P2Y12/G\textsubscript{a}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 anti-platelet therapy, as it would provide strategies for the development of an antidote to P2Y12 or CalDAG-GEFI inhibitors.
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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; W.B. designed the study and wrote the paper.

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Figure Legends

Figure 1

**CalDAG-GEFI is central to convulxin-dependent TxA2 generation.** Washed platelets, in the presence of 1mM Ca^{2+} and 50μg/ml fibrinogen, were stimulated under stirring conditions at 37°C (standard aggregometry) with low (LD, 100 ng/ml) (A, C) or high (HD, 500 ng/ml) (B, D) dose convulxin (Cvx). Samples were withdrawn at the indicated time points to measure the levels of TxB2, the stable product of the non-enzymatic hydration of TxA2. (A, B) TxB2 levels in the supernatant of WT (black line, circle) or CalDAG-GEFI-/-(KO, grey line, triangle) platelets activated in the presence (open symbol) or absence (filled symbol) of 75 μM 2-MesAMP (P2Y12 inhibitor). Data shown are mean±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 when compared to WT/MesAMP, KO, or KO/MesAMP platelets. (C, D) Aggregation traces representative of 3 independent experiments. (E) WT (black bars) or CalDAG-GEFI-deficient (grey bars) 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 αIIbβ3 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 (open bar) or absence (filled bar) of 40 μg/ml of the αIIbβ3 blocking antibody Leo.H4. Data shown are mean ± SEM (n=3), *P < 0.05, ** P < 0.01, *** P < 0.001.
Figure 2
CalDAG-GEFI and P2Y12 synergize in Cvx-dependent activation of Rap1 and ERK-MAPK. Washed WT or CalDAG-GEFI/- (KO) platelets were stimulated in standard aggregometry with 100 ng/ml (LD) or 500 ng/ml (HD) Cvx (B, C). Platelets were activated in the presence of 75 μM 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 (lower panel) were determined as a loading control. Results are representative of 3 individual experiments.

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/- (KO, grey line, triangle) platelets. Cells were stimulated with 25μg/ml fibrillar type I collagen under stirring conditions in the presence (open symbol) or absence (filled/full symbol) 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 when compared to KO or KO/MesAMP platelets. (B) Aggregation traces representative of 3 independent experiments. (C, D) Time course of ERK (upper 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 (HD Cvx), or
25μg/ml fibrillar collagen (HD collagen). Cells were activated in the presence (open bar) or absence (filled bar) 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 < 0.05, **P < 0.01, ***P < 0.001.

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-deficient platelets were stimulated with 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 αIIbβ3 activation by flow cytometry. n = 6. ** P < 0.001, * P < 0.05. (C) Detection of phosphorylated PKC substrates in platelet lysates (western blotting). WT (left lanes) and CalDAG-GEFI-/- (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 pleckstrin-1 (PLEK,
arrow), in WT platelets pre-treated with Ro31-8220 (10 μg/ml) or activated with PMA (100 nM) were determined as controls (right panel). Results are representative of 3 independent experiments. (D) ³H-serotonin release in WT (black line, circle) or CalDAG-GEF-/- (grey line, triangle) platelets activated with 100 ng/ml Cvx. After 3 minutes, 500 nM U46619 was added to CalDAG-GEF-/- platelets. Experiments were performed in the presence (open symbol) or absence (filled symbol) of the PKC inhibitor Ro31-8220. (n=3).

Figure 5

TxA₂ released from WT platelets restores aggregation of CalDAG-GEF-/- platelets. (A) Aggregation traces of platelets (10⁸ cells) activated with 10 μg/ml collagen. The aggregation response of WT (black line), CalDAG-GEF-/- (KO, light grey line), or CalDAG-GEF-/- platelets containing 10% WT platelets (dark grey line) was studied. WT platelets were pretreated with 1mM aspirin (ASA) before addition to CalDAG-GEF-/- platelets in order to inhibit TxA₂ release. CalDAG-GEF-/- platelets were pre-treated with 40 μg/ml of a blocking antibody to αIIbβ3 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-GEF-/-) 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 (400s⁻¹). (A) Representative images showing platelets firmly adherent to the collagen surface. U46619 (2 μM) was added to CalDAG-GEFI-/- (KO) whole blood immediately before the perfusion. 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<0.01.

Figure 7

**A revised model of platelet activation in response to low dose collagen.** (1) A rise in [Ca²⁺]i 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). Abbrs.: AA,
arachidonic acid; Gi, heterotrimeric G protein; P, residue phosphorylation; PL indicates phospholipids; TPα/β, thromboxane receptors.
Figure 1

A

LD CVX

- WT
- KO

**

![Graph showing TxB2 levels over time with LD CVX for WT and KO](image)

B

HD CVX

- WT
- KO
- WT/MesAMP
- KO/MesAMP

**

![Graph showing TxB2 levels over time with HD CVX for different genotypes](image)

C

Aggregation

WT

KO

D

Aggregation

WT

KO

KO/MesAMP

E

![Histogram showing mean fluorescence of activated αIibβ3](image)

F

![Bar chart showing TxB2 levels with HD CVx and αIibβ3 block + HD CVx for WT and KO](image)
Figure 3

A

HD Collagen

WT  KO  WT/MesAMP  KO/MesAMP

![Graph showing TxB2 production over time](image1)

B

Aggregation

![Graph showing aggregation over time](image2)

C

HD Collagen

WT  KO

0  40  180  600  0  40  180  600  sec

P-ERK  Rap1-GTP  ERK1/2

![Western blot images](image3)

D

2-MesAMP/HD Collagen

WT  KO

0  40  180  600  0  40  180  600  sec

P-ERK  Rap1-GTP  ERK1/2

![Western blot images](image4)

E

TxB2, % of maximum

HD Conv  HD Collagen

61  1  100  40

![Bar graph showing TxB2 levels](image5)
Figure 6

A

WT  KO  KO + U46619  KO/MesAMP + U46619

B

platelets/0.02 mm²

KO  KO  KO/MesAMP +
+ U46619  + U46619
Figure 7
CalDAG-GEFI is at the nexus of calcium-dependent platelet activation

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