Published in final edited form as:

Platelets. 2010 ; 21(4): 239–243. doi:10.3109/09537101003639931.

CalDAG-GEFI and platelet activation

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Abstract

Rapid activation of platelets at sites of vascular injury is a critical event in thrombosis and hemostasis. Here, we review recent findings, which a) identified CalDAG-GEFI (RasGRP2) at the nexus of the rapid, Ca^{2+} -dependent platelet activation, b) demonstrated a complex synergy between signaling provided by CalDAG-GEFI, protein kinase C, and the Gi-coupled receptor for ADP, P2Y₁₂, and c) suggested CalDAG-GEFI as a novel target for anti-platelet therapy.

Keywords

CalDAG-GEFI; RasGRP2; calcium signaling; P2Y₁₂; platelet activation; thrombosis

Introduction

The regulated activation of platelets is an essential component of thrombosis and hemostasis. Platelet stimulation through G protein-coupled and immunoglobulin-like receptors leads to the activation of phospholipase C (PLC) $\beta 2/3$ and PLC $\gamma 2$, respectively, which in turn mediate the generation of the second messengers calcium (Ca^{2+}) and diacylglycerol (DAG). DAG is critical for protein kinase C (PKC) activation, a key event in platelet granule release and integrin activation [1]. Ca^{2+} triggers many intracellular signaling processes important for various platelet responses such as integrin activation and the generation of autocrine agonists, such as ADP and TxA_2 . In our recent work, we identified the guanine nucleotide exchange factor, CalDAG-GEFI, as a critical molecule in Ca^{2+} signaling in platelets [2-5]. CalDAG-GEFI activates the small GTPase Rap1, a central molecular switch that drives platelet activation by directly regulating integrin-mediated aggregation [2,6,3,4] and the release of autocrine agonists [5]. Here, we review key findings that highlight how one molecule, CalDAG-GEFI, coordinates various Ca^{2+} -dependent platelet responses ensuring the rapid activation of platelets at the site of vascular injury.

Discovery

CalDAG-GEFI (RASGRP2, 11q13) was first cloned in 1998 in an effort to identify novel signaling molecules enriched in the striatum [7]. It is a member of the RasGRP family that includes four guanine nucleotide exchange factors, which differ for tissue expression and

target specificity (A, table). CalDAG-GEFI (609 amino acids) is predominantly expressed in the brain [7] and, within the hematopoietic lineage, in megakaryocytes and platelets as well as neutrophils [2]. A longer CalDAG-GEFI splice variant (62 additional N-terminal residues) with a different substrate specificity and a different intracellular localization has been proposed, [8] but the endogenous version has not been identified.

Regulation of CalDAG-GEFI

RasGRPs are second-messenger regulated activators of small-GTPases of the Ras family. GTPases cycle between an inactive GDP(guanosine diphosphate)-bound form, and an active GTP(guanosine triphosphate)-bound form. Guanine nucleotide exchange factors (GEFs) stimulate GTP loading and are antagonized by GTPase activating proteins (GAPs) that catalyze GTP hydrolysis. The GEF activity of RasGRPs is provided by two domains within the N-terminal catalytic region: a Ras exchange motif (REM) and a CDC25 domain, named for the prototypic Ras activator from *Saccharomyces cerevisiae*. The C-terminal regulatory region comprises two Ca^{2+} binding EF hand domains and a C1 domain [9] (A, upper panel). CalDAG-GEFI is the most divergent family member. Unlike RasGRP1 and -3, CalDAG-GEFI is found preferentially in the cytoplasm [8,10] and translocates toward juxtamembrane areas upon cellular stimulation [11]. It specifically activates Rap1 and Rap2 but lacks Ras exchange activity *in vivo* [7]. The C1 domain sequence is atypical and shows only weak affinity for DAG. Consequently, treatment of cells with DAG mimetics has only little effect on the subcellular localization and the activity of CalDAG-GEFI [12,13]. In contrast, it is well established that CalDAG-GEFI activates Rap1 in response to calcium ionophores [8] and it has been proposed that the direct association of its N-terminal domain with F-actin regulates the subcellular localization of CalDAG-GEFI [11].

Our studies confirm that Ca^{2+} , but not DAG, is the main second messenger regulating CalDAG-GEFI function in platelets. CalDAG-GEFI-deficient platelets show normal aggregation in response to the DAG mimetic PMA (phorbol 12-myristate 13-acetate), but fail to aggregate when stimulated with calcium ionophore [2] (B, upper panel). Moreover Rap1 activation in CalDAG-GEFI^{-/-} platelets is markedly delayed [4,5] suggesting that CalDAG-GEFI mediates the rapid but reversible activation of Rap1, which was previously identified as a Ca^{2+} -dependent mechanism [14] (B, middle panel).

CalDAG-GEFI and platelet signaling

Shattil and colleagues were the first to implicate CalDAG-GEFI in the activation of the platelet/megakaryocyte-specific integrin $\alpha\text{IIb}\beta 3$, when they demonstrated that the expression level of CalDAG-GEFI correlates with inside-out $\alpha\text{IIb}\beta 3$ activation in megakaryocytes [15]. Most of the later studies have been performed in a knockout mouse model, which has confirmed the importance of CalDAG-GEFI in Rap1 and integrin activation in both platelets [2] [16] and neutrophils [3] (B, lower panel). So far, CalDAG-GEFI is the only Rap1-GEF with documented activity in platelets. Other potential Rap regulators have been identified, such as CalDAG-GEFIII [17], PDZ-GEF1 [17] and Epac1 [18], but their relevance in platelet biology has not been investigated. Our studies demonstrate that platelets from CalDAG-GEFI-deficient mice have strongly impaired $\alpha\text{IIb}\beta 3$ -mediated aggregation in

response to all physiological agonists [2]. In the absence of CalDAG-GEFI, Rap1 and integrin activation require stimulation with PMA or high doses of strong agonists such as thrombin or collagen. Ca^{2+} /CalDAG-GEFI-independent Rap1 activation and integrin activation is mediated by PKC and co-signaling through the Gi-coupled receptor for ADP, P2Y12 [4] (B, middle panel). The two Rap1 activation pathways have complementary kinetics and fulfill different roles in thrombus growth. CalDAG-GEFI-dependent Rap1 activation is very rapid and ensures near-immediate integrin activation and platelet adhesion to a thrombogenic surface. In contrast, PKC/P2Y12 signaling leads to delayed but sustained Rap1 activation, a prerequisite for the formation of a stable platelet thrombus.

Independent of its role in integrin activation, the CalDAG-GEFI/Rap1 signaling module promotes the generation of thromboxane A_2 (TxA_2) through the MAPK/ERK-signaling cascade (C, upper panel). CalDAG-GEFI-dependent TxA_2 release provides crucial feedback especially in collagen-activated platelets. Compared to thrombin, collagen is not a potent activator of the PKC/P2Y12-dependent pathway, but co-signaling between collagen and the autocrine agonist TxA_2 supports PKC activation, granule release and P2Y12-mediated integrin activation [5] (C, lower panel).

The results from these studies are summarized schematically in section D of figure 1. The key elements of this model are: (1) the central role of one molecule, CalDAG-GEFI, in Ca^{2+} -dependent integrin activation, TxA_2 generation, and granule release, (2) the preferential activation of CalDAG-GEFI over PKC downstream of the collagen receptor GPVI, and (3) the kinetic differences between CalDAG-GEFI- and P2Y12-mediated Rap1 activation and the respective downstream signaling events.

The importance of CalDAG-GEFI signaling for platelet function was emphasized in experiments performed under physiological flow conditions both *in vitro* and *in vivo*. Flow chamber studies with anti-coagulated whole blood demonstrated that CalDAG-GEFI-/- platelets tether normally but fail to form thrombi on a collagen surface under flow [2] (E, upper panel). No thrombus formation was observed in CalDAG-GEFI-/- mice in a model of ferric chloride (FeCl_3)-induced arterial thrombosis [3] (E, lower left panel) and the mice were protected from collagen-induced systemic thrombosis [2]. CalDAG-GEFI-deficient mice also had problems to maintain hemostasis when challenged, with tail bleeding times being similar to those observed in wild-type mice treated with the P2Y12 inhibitor clopidogrel (E, lower right panel). It is currently not clear why signaling by PKC/P2Y12 was not sufficient to facilitate thrombus formation in CalDAG-GEFI-/- mice. Due to their defect in the rapid activation of Rap1, CalDAG-GEFI-deficient platelets may not be able to activate their integrins fast enough to allow adhesion under conditions of (high) shear stress. Supporting this idea, we found the most striking defects in the adhesion of CalDAG-GEFI-deficient neutrophils *in vivo* rather than under static conditions *in vitro* [3]. Alternatively, the levels of thrombin and/or collagen present at sites of vascular injury may not be high enough to trigger PKC activation.

CalDAG-GEFI as a target for anti-thrombotic therapy

Based on its central role in platelet activation, we suggest CalDAG-GEFI as a novel target for anti-platelet therapy. Inhibitors to CalDAG-GEFI are not expected to have many side-effects, as the expression of CalDAG-GEFI is restricted to few cell types (A, table). This limited expression pattern explains why CalDAG-GEFI^{-/-} mice appear completely normal when not challenged. Although CalDAG-GEFI is potentially important for motor control [19], no brain-related abnormalities have been reported for the knockout mice. Since the brain is also largely protected from drugs by the blood-brain-barrier, inhibition of neutrophil function will be the most likely side-effect of targeting CalDAG-GEFI in platelets. In our own studies, we have demonstrated that β 1 and β 2 integrin activation is impaired in CalDAG-GEFI^{-/-} neutrophils [3], resulting in protection of the mice in models of inflammatory disease. This combined defect in the inside-out activation of integrins in platelets and neutrophils led us to speculate that mice lacking CalDAG-GEFI may provide a mouse model for leukocyte adhesion deficiency type III (LAD-III), a clinical complication characterized by a similar phenotype. However, recent studies demonstrated that mutations in kindlin-3 (FERMT3), not CalDAG-GEFI, are the underlying cause for LAD-III [20]. Importantly, studies in dogs with naturally occurring mutations in CalDAG-GEFI confirmed its central role in platelet activation, while no defects in neutrophil function were reported [21]. Future studies will have to show how CalDAG-GEFI inhibition compares to current anti-platelet therapies such as treatment with aspirin and/or clopidogrel. It will also be interesting to see if targeting a regulatory domain within CalDAG-GEFI can facilitate partial inhibition of the protein, and if so, whether such an approach would improve the safety of anti-CalDAG-GEFI therapy.

Acknowledgments

This work was funded by the National Institute of Health (HL094594-01A1), the American Society of Hematology and the American Heart Association (SDG0630044N).

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Figure 1.

(A) **Upper panel:** Graphical summary of the structure of CalDAG-GEFI/RasGRP2 (*Homo sapiens*); **table:** CalDAG-GEFI/RasGRP family members differ for their response to second messengers, their target proteins and their expression in hematopoietic cells. (B) **Upper panel:** CalDAG-GEFI is regulated by calcium (Ca²⁺) but not by diacylglycerol (DAG) [2]; **middle panel:** Complementary kinetics of CalDAG-GEFI-dependent and P2Y₁₂-dependent Rap1 activation and α_{IIb}β₃-mediated aggregation [4]; **lower panel:** CalDAG-GEFI positively regulates activation of platelet integrins α_{IIb}β₃ [2] and α₆β₁ [3]. (C) **Upper panel:** CalDAG-GEFI promotes collagen-dependent Thromboxane A₂ (TxA₂) generation and ERK activation [5]; **lower panel:** Co-stimulation of the collagen receptor (GPVI) and the TxA₂ receptor (TP) restores aggregation and dense granule secretion in CalDAG-GEFI^{-/-} platelets [5]. (D) Schematic model of platelet activation over time upon stimulation with thrombin (**upper panel**) or collagen (**lower panel**). (E) Thrombosis and hemostasis in CalDAG-GEFI^{-/-} mice. **Upper panel:** flow chamber assay [2]; **lower left panel:** ferric chloride-induced arterial thrombosis model [3]; **lower right panel:** tail bleeding assay [2].