

REVIEW ARTICLE

Cyclic nucleotide-dependent inhibitory signaling interweaves with activating pathways to determine platelet responses

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Abstract

Platelets are regulated by extracellular cues that impact on intracellular signaling. The endothelium releases prostacyclin and nitric oxide which stimulate the synthesis of cyclic nucleotides cAMP and cGMP leading to platelet inhibition. Other inhibitory mechanisms involve immunoreceptor tyrosine-based inhibition motif-containing receptors, intracellular receptors and receptor desensitization. Inhibitory cyclic nucleotide pathways are traditionally thought to represent a passive background system keeping platelets in a quiescent state. In contrast, cyclic nucleotides are increasingly seen to be dynamically involved in most aspects of platelet regulation. This review focuses on crosstalk between activating and cyclic nucleotide-mediated inhibitory pathways highlighting emerging new hub structures and signaling mechanisms. In particular, interactions of plasma membrane receptors like P2Y₁₂ and GPIb/IX/V with the cyclic nucleotide system are described. Furthermore, differential regulation of the RGS18 complex, second messengers, protein kinases, and phosphatases are presented, and control over small G-proteins by guanine-nucleotide exchange factors and GTPase-activating proteins are outlined. Possible clinical implications of signaling crosstalk are discussed.

KEYWORDS

14-3-3 protein, cyclic AMP, G-protein, kinase, protein kinase A, RGS18

Essentials

- Cyclic nucleotide-dependent inhibition and platelet activation are depicted as separate pathways.
- Emerging evidence suggests that these pathways are intertwined at multiple levels.
- Platelet inhibition and activation often regulate the same signaling nodes in opposite directions.
- Better understanding of cross-inhibition between these pathways will advance antiplatelet therapy.

1 | INTRODUCTION

Platelets play a key role in vascular homeostasis. Platelet functions are tightly controlled by signaling pathways that respond to diverse extracellular cues. Healthy endothelium releases short-lived mediators prostacyclin (PGI₂) and nitric oxide (NO). PGI₂ binds to a G-protein

coupled receptor (GPCR) which is linked to the G_s heterotrimeric G-protein that stimulates cAMP synthesis by adenylate cyclase (AC). cAMP binds to the regulatory domains of cAMP-dependent protein kinase (isoforms PKA I and II) leading to activation of the PKA catalytic subunits and to phosphorylation of a multitude of substrate proteins (Figure 1).¹ NO diffuses through the plasma membrane and

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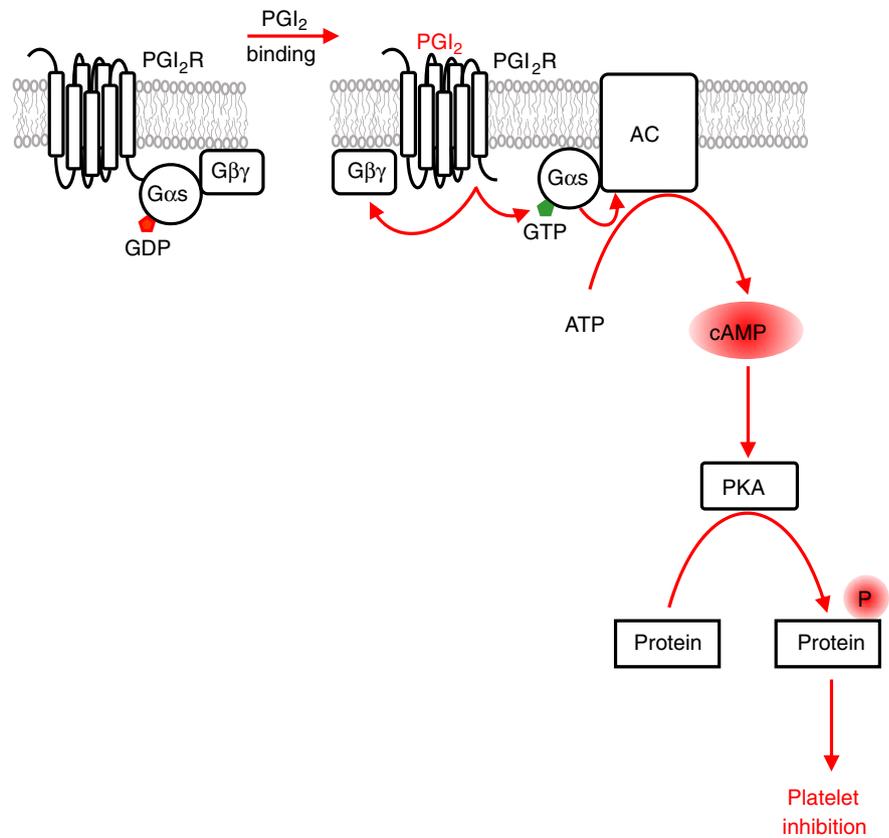


FIGURE 1 Prostacyclin signaling. Prostacyclin (PGI₂) is released by healthy endothelial cells. The prostacyclin receptor on platelets couples to the stimulatory heterotrimeric Gs protein complex. Prostacyclin binding leads to a conformational change in the receptor activating its guanine-nucleotide exchange factor (GEF) activity towards Gαs resulting in the exchange of GDP by GTP. Gαs-GTP binds to and activates the transmembrane protein adenylate cyclase (AC) to synthesize cAMP from ATP. The second messenger cAMP has only one major target in platelets which is the cAMP-dependent protein kinase (PKA) family. cAMP binding to the regulatory subunits of PKA leads to activation of the catalytic subunits and to the phosphorylation of numerous substrate proteins resulting in a profound inhibition of most platelet functions

activates guanylate cyclase (sGC, NO-GC) directly to produce cGMP. cGMP activates cGMP-dependent protein kinase (isoform PKG Iβ) which phosphorylates substrate proteins that are often identical to the ones phosphorylated by PKA (Figure 2).^{1,2} Cytosolic levels of the cyclic nucleotides are further controlled by phosphodiesterases (PDE) that degrade cAMP and cGMP. The signaling pathways induced by PGI₂ and NO are thought to keep platelets in a quiescent state in the absence of damage.

In contrast, exposure of the extracellular matrix protein collagen and binding of von Willebrand factor (VWF) after vascular injury initiates platelet activation. Collagen stimulates the immunoreceptor tyrosine-based activation motif (ITAM)-containing glycoprotein VI (GPVI)-Fc receptor γ chain complex which signals via Src family and Syk tyrosine kinases, whereas VWF binds to the GPIb/IX/V complex. In parallel, vascular damage also triggers the clotting cascade leading to thrombin generation which directly activates platelets via thrombin receptors and converts soluble fibrinogen into a fibrin mesh. Activated platelets release ADP and thromboxane A₂ (TxA₂) which bind to specific GPCRs. The signaling events downstream of these receptors include the activation of diverse kinases and GPCR-linked heterotrimeric G-proteins as well as small G-proteins of the Ras and Rho families. Furthermore, calcium ions (Ca²⁺) are released from intracellular stores leading to further signal amplification ultimately inducing membrane and cytoskeletal reorganization, granule release and integrin activation.³⁻⁵ In addition to endothelium-dependent PGI₂ and NO pathways, platelet activation is limited by negative feedback through immunoreceptor

tyrosine-based inhibition motif (ITIM)-containing receptors G6b-B and PECAM-1,⁶ serine/threonine phosphatases, intracellular receptors and mechanisms of receptor desensitization and cleavage.⁵ The ectonucleotidase CD39 expressed by endothelial cells hydrolyses ATP and ADP, thus limiting the availability of platelet agonists. The inhibitory functions of these molecules are often deduced from the phenotypes of the corresponding knockout mouse models (ie, hyper-reactive platelets).

Cyclic nucleotide-mediated inhibition and platelet activation are commonly described as separate systems. However, increasing evidence suggests an alternative model whereby simultaneous regulation of both systems determines platelet function. For example, to prevent platelet activation, inhibitory signaling targets key nodes in activating pathways.⁷ On the other hand, platelet activation requires active blockage of endothelium-dependent inhibitory pathways,⁸⁻¹¹ including ADP-mediated inhibition of cAMP production via P2Y₁₂ and thrombin-mediated cAMP degradation via PDE type 3A. Interestingly, both P2Y₁₂ and PDE3A are targets of multiple clinically used drugs which reduce thrombosis.¹² Once initiated, cyclic nucleotides are also able to reverse platelet aggregation leading to complete disaggregation of already formed platelet aggregates.¹³⁻¹⁶ Given the powerful activity of endothelium-derived inhibitors combined with the relative abundance of endothelial cells compared to platelets,¹⁷ cyclic nucleotide pathways are likely to contribute more significantly to the control of platelet activation than is usually appreciated. Recent comprehensive phosphoproteome studies indicate an increasing complexity in signaling events following binding

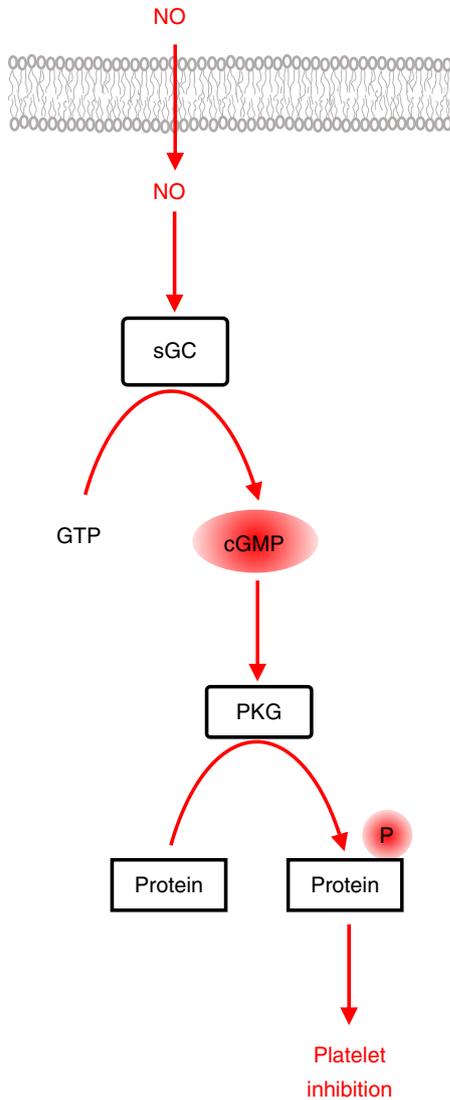


FIGURE 2 Nitric oxide signaling. Nitric oxide (NO) is released by healthy endothelial cells. NO is a small gaseous molecule that diffuses through the plasma membrane of platelets. The main target of NO in platelets is the soluble guanylate cyclase (sGC) which is stimulated by NO binding to synthesize the second messenger cGMP from GTP. The sole target of cGMP in platelets is the I β isoform of cGMP-dependent protein kinase (PKG). cGMP binding to PKG I β activates the kinase domain to phosphorylate many substrate proteins resulting in a profound inhibition of most platelet functions

of single agonists or combinations of agonists to platelet receptors activating each of these pathways.^{18,19}

In this paper, we focus on the interdependence of cyclic nucleotide-dependent inhibitory and platelet activation pathways. To modulate each other's actions, these pathways constantly interact at multiple levels. We will describe these interactions highlighting recently identified protein-protein assemblies involved in platelet control like the RGS18 complex. The potential clinical relevance of crosstalk between endothelium-dependent inhibition and platelet activation will also be considered.

2 | INTERACTIONS AT RECEPTOR LEVEL

2.1 | Gi signaling

The plasma membrane contains multiple receptor and regulatory proteins required for the initiation of signaling events leading to platelet inhibition and/or activation. Major receptors for platelet activators like ADP, epinephrine, and prostaglandin E2 (PGE2) work, at least in part, by inhibiting cAMP production, and thus by interfering with inhibitory pathways. Binding of ADP to the P2Y12 receptor causes a conformational change in the receptor allowing it to act as a guanine-nucleotide exchange factor (GEF) and activate the membrane-associated heterotrimeric G-protein of the Gi-family. The active GTP-bound G α i-subunit dissociates from the β and γ subunits and binds to AC leading to diminished cAMP synthesis (Figure 3 and Table 1). Simultaneously Gi triggers the phosphatidylinositol 3-kinase (PI3K) pathway, which converts phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate at the plasma membrane leading to the activation of the kinase Akt and inhibition of the Rap1-GAP RASA3, key activating pathways in platelets.²⁰⁻²² The synergism between P2Y12 function and blockage of cyclic nucleotide pathways has been shown for both, cAMP and cGMP components, although cGMP regulation by P2Y12 is not well understood.^{9,23} These studies indicate that P2Y12-mediated inhibition of cyclic nucleotide signaling is mandatory to achieve full platelet activation. Accordingly, knockout of the P2Y12 receptor in mice, congenital deficiency of the receptor in humans, or expression of constitutively active G α i2 in a mouse model confirm the importance of P2Y12 signaling for platelet aggregation and thrombus formation.^{10,24,25} In thrombosis studies, the establishment of a tight vascular seal has been suggested to depend on P2Y12 function,²⁶ and thus on cross-inhibition of cAMP/cGMP pathways. P2Y12 and Gi signaling also contribute to the formation of the outer shell structure of hemostatic thrombi.²⁷

This type of crosstalk is not restricted to the P2Y12 receptor alone. The α 2A-adrenergic and EP3 receptors for epinephrine and PGE2, respectively, are also coupled to Gi-family proteins. PGE2 can be released from activated platelets providing positive feedback on platelet activation at least in part through inhibition of cAMP synthesis.²⁸ Mathematical modelling suggests that the EP3 receptor might be even more potent than the P2Y12 receptor in inhibiting cAMP production.²⁹ Another example of a Gi-coupled receptor in platelets is the chemokine receptor CXCR4.^{30,31} The importance of costimulatory Gi signaling was recently highlighted in a study of matrix metalloproteinase 2 which stimulates protease-activated receptor PAR1 without being able to induce platelet aggregation in itself because of a lack of concomitant Gi activation.³²

2.2 | RGS18 complex

GPCR signaling is terminated by hydrolysis of GTP bound to G α -subunits of heterotrimeric G-proteins. GTP hydrolysis is enabled by a specific family of GTPase-activating proteins (GAPs) called

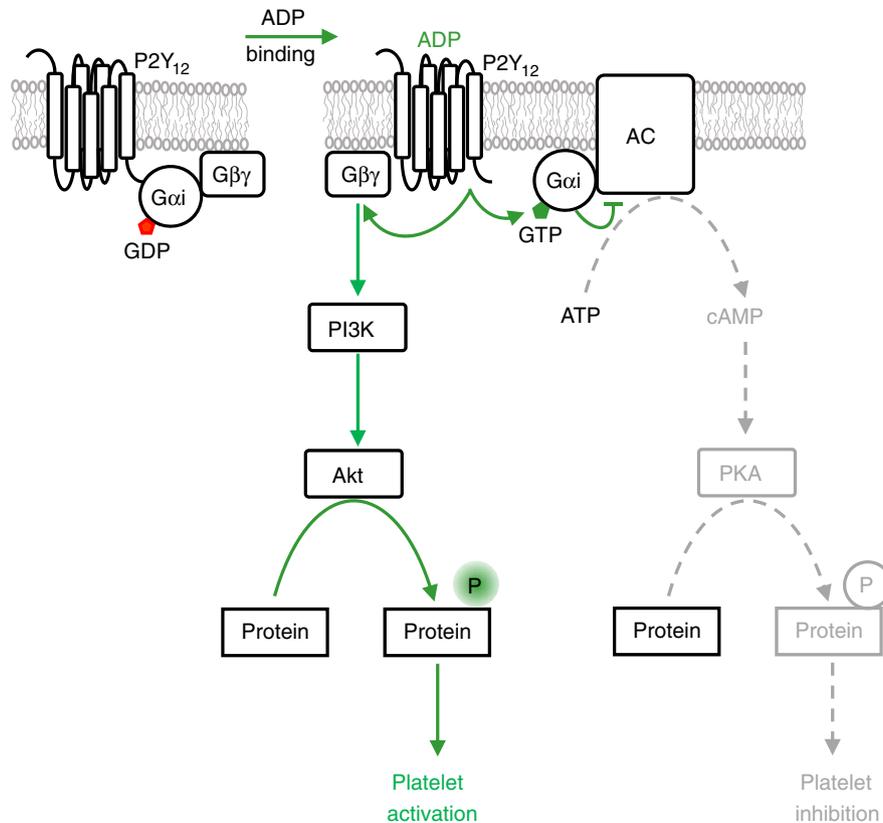


FIGURE 3 Gi signaling. The P2Y₁₂ receptor for ADP is coupled to an inhibitory heterotrimeric Gi protein complex composed of Gα_i and Gβγ subunits. ADP binding leads to exchange of GDP by GTP on the Gα_i subunit and to the dissociation of the Gβγ subunit. The Gα_i-GTP subunit binds to and inhibits the function of adenylate cyclase (AC) resulting in a reduction of prostacyclin and cAMP/PKA signaling and diminished platelet inhibition. The Gβγ subunit binds to and activates phosphatidylinositol 3-kinase β and γ (PI3K) to synthesize the membrane lipid phosphatidylinositol 3,4,5-trisphosphate (PIP₃) from phosphatidylinositol 4,5-bisphosphate. PIP₃ serves as docking site to recruit and activate the kinase Akt which phosphorylates substrate proteins contributing to platelet activation

regulators of G-protein signaling (RGS) which accelerate the intrinsic GTPase activity of Gα subunits. RGS18 is a highly expressed GAP of Gα_i and Gα_q in platelets (Figure 4). Deletion of RGS18 in mice results in platelet activation marked by enhanced thrombus formation, platelet granule release and aggregation particularly in response to thrombin and TxA₂.^{33,34} RGS18 and its associated proteins have emerged as a highly regulated hub structure that coordinates cyclic nucleotide-mediated inhibitory signaling and platelet activation. In resting platelets, RGS18 interacts with the scaffold protein spinophilin (neurabin-2, PPP1R9B), the tyrosine phosphatase SHP-1, and the phospho-serine/threonine binding adapter protein 14-3-3 (Figure 5, RGS18 complex in transition i). Platelet activation by thrombin and TxA₂ induces a dissociation of RGS18 and SHP-1 from spinophilin³⁵ and increases binding of 14-3-3γ to RGS18.⁷ 14-3-3 bound RGS18 is less active leading to enhanced Gq signaling and Ca²⁺-release (Figure 5, RGS18 complex inactive). Loss of SHP-1 during platelet activation leads to binding of the serine/threonine phosphatase PP1 to spinophilin which is associated with reduced PP1 activity towards myosin light chain (MLC) potentially contributing to enhanced MLC phosphorylation and platelet activation.³⁶ Cyclic nucleotide inhibitory pathways also disrupt the RGS18/spinophilin/SHP-1 complex,³⁷ however, they inhibit binding of 14-3-3γ to RGS18 (Figure 5,

RGS18 complex active).⁷ This free RGS18 binds more effectively to Gα_{i2} and attenuates Gq signaling leading to reduced Ca²⁺-release and thus contributing to platelet inhibition.^{7,37} The reorganization of RGS18 complexes is mediated by multiple de-/phosphorylation events. Thrombin and TxA₂ induce SHP-1 Y536 phosphorylation leading to phosphatase activation and de-phosphorylation of tyrosines on spinophilin thus contributing to dissolution of the spinophilin/SHP-1/RGS18 complex.³⁷ The role of SHP-1 phosphatase activity in inducing complex dissociation is supported by pharmacological studies using a nonselective SHP-1/SHP-2 inhibitor (NSC-87877).³⁵ Thrombin, TxA₂, and ADP induce phosphorylation of RGS18 on S49 resulting in enhanced 14-3-3 binding to RGS18.³⁸ In contrast, PKA phosphorylates S216 of RGS18 leading to de-phosphorylation of S49 and S218, presumably by PP1 (Figure 5, RGS18 complex in transition a), and to detachment of 14-3-3 from RGS18.³⁸ The PKA-induced release of RGS18 from spinophilin is supported by phosphorylation of spinophilin on S94.³⁷

Interestingly, spinophilin knockout platelets exhibit reduced Ca²⁺-release and aggregation which could, at least in part, be due to dysregulated RGS18 function.³⁵ Absence of spinophilin also results in reduced cAMP synthesis in response to PGI₂, highlighting multiple functions of this scaffold protein in platelets. Studies on

Protein	Effect of platelet activators	Effect of cyclic nucleotide-dependent inhibitors
Adenylate cyclase	Gi mediated inhibition (induced by P2Y ₁₂ , ²³ α 2A-adrenergic, and EP3 receptors ²⁸)	Gs mediated activation (prostacyclin receptor)
Soluble guanylate cyclase	Thrombospondin-induced inhibition, ⁶³ VWF-induced partial activation ⁵¹	NO-induced maximal activation ⁵⁰
RGS18	Activation and reduction of Gq signaling, ³⁵ inhibition leading to increased Ca ²⁺ levels ⁷	Activation leading to reduced Ca ²⁺ levels ^{7,37}
GPIb α receptor	Receptor shedding	Inhibition of shedding ⁴⁶
GPVI receptor	Dimerization	Inhibition of dimerization ⁴⁸
IP ₃ -receptor	Opening and Ca ²⁺ -release	Inhibition ¹¹³
PDE3A	Activation leading to reduced cAMP levels ¹¹	Inhibition by cGMP leading to elevated cAMP levels, ⁷³ activation by cAMP ⁷¹
ERK	Activation	Inhibition ⁷⁸
p38 MAPK	Activation	Inhibition ⁷⁸
Rap1B	Activation	Inhibition ¹¹⁴
Rap1GAP2	Inhibition leading to enhanced cell adhesion ⁹²	Activation leading to reduced adhesion ⁹²
Rac1	Activation ⁹⁵	Inhibition ^{94,95}
RhoA	Activation ⁹⁵	Inhibition ⁹⁵
Arf6	Inhibition ⁹⁸	Activation ⁹⁸
Myosin light chain	Increased phosphorylation	Decreased phosphorylation ⁷⁶

TABLE 1 Examples of proteins differentially regulated by platelet activators and cyclic nucleotide-dependent inhibitors

SHP-1-deficient mice have identified a main role of SHP-1 in regulating GPVI and integrin α IIb β 3 signaling but did not reveal any defect in GPCR signaling.³⁹ Because SHP-1 is ablated during megakaryocyte differentiation in these mice, it is possible that the role of SHP-1 in regulating GPCR signaling is not apparent because of compensatory mechanisms and/or redundancy with the closely related SHP-2 phosphatase. Indeed, platelets deficient in both SHP-1 and SHP-2, display marked defects in PAR4 receptor signaling, suggesting a level of redundancy.³⁹ Whether a spinophilin/RGS18 complex exists in SHP-1 deficient platelets remains to be determined.

2.3 | GPIb/IX/V complex

The GPIb/IX/V complex is a highly and specifically expressed VWF receptor on the platelet membrane with a crucial role in platelet adhesion at high shear rates. The cytosolic region of the GPIb α subunit interacts with the actin-binding protein filamin A and with 14-3-3.⁴⁰ Filamin A has emerged as a critical link between GPIb α and the actin cytoskeleton playing an important role in platelet stability and adhesion under high shear conditions.⁴¹ Filamin A also interacts with integrin α IIb β 3⁴² and has been involved in tyrosine kinase signaling during platelet activation possibly through

anchoring of the tyrosine kinase Syk near the plasma membrane. PKA might impact indirectly on the GPIb α /filamin interaction by phosphorylating filamin A leading to its protection against proteolysis.¹ A key site in GPIb α that is phosphorylated in resting platelets and dephosphorylated during activation by an unknown kinase and phosphatase, respectively, is S609.⁴³ GPIb α S609 phosphorylation is required for 14-3-3 binding and receptor signaling.⁴⁴ The GPIb β subunit can be phosphorylated by PKA on S166 possibly contributing to binding of 14-3-3 to the GPIb/IX/V complex.¹ S166 phosphorylation has been suggested to depend on localization of PKA to lipid rafts possibly via the A-kinase anchoring protein moesin.⁴⁵ The functional role of this phosphorylation is uncertain, as mutations of S166 have resulted in enhanced, as well as reduced receptor function,¹ and the role of phosphorylated S166 for 14-3-3 binding has been questioned.⁴⁰ PKA has been described to inhibit shedding of extracellular regions of GPIb α .⁴⁶ Similarly, PKA inhibits shedding of the semaphorin family member Sema4D, a receptor that contributes to thrombus growth and stability.⁴⁷ These shedding processes are mediated by the metalloproteinase ADAM17, however, the mechanisms involved in PKA-induced inhibition of ADAM17 are not known. These data indicate a possible new role of PKA in the long-term stabilization of certain membrane receptors which could contribute to thrombus growth.

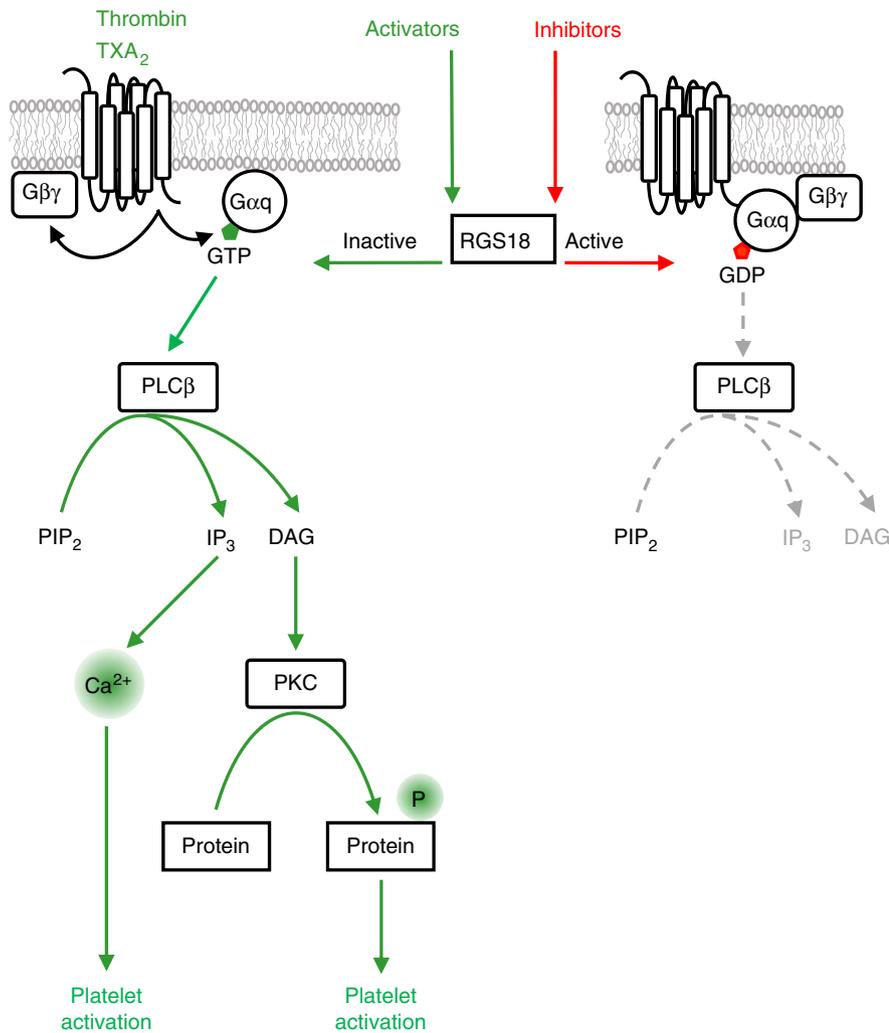


FIGURE 4 Regulation of Gq signaling by RGS18. Binding of the platelet activators thrombin and thromboxane A₂ (TXA₂) to their respective receptors induces the formation of active Gαq-GTP which binds to and activates phospholipase Cβ (PLCβ) leading to the generation of inositol-3-phosphate (IP₃) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP₂). IP₃ triggers the release of calcium ions (Ca²⁺) from intracellular stores which are essential for platelet activation. DAG activates protein kinase C (PKC) isoforms which phosphorylate many proteins supporting platelet activation. Regulator of G-protein signaling 18 (RGS18) is a GTPase-activating protein (GAP) which accelerates the intrinsic GTPase activity of Gαq. RGS18 enables the hydrolysis of GTP bound to Gαq resulting in the formation of inactive Gαq-GDP and in the termination of Gq signaling. Platelet activators like thrombin and TXA₂ inhibit RGS18 thus support Gq signaling, whereas platelet inhibitors stimulate RGS18 function to stop Gq signaling

2.4 | GPVI

The GPVI-Fc receptor γ chain complex plays a key role in platelet activation by collagen. Dimerization of the GPVI receptor is essential for binding of collagen to GPVI. GPVI activity depends on Gi signaling, indicating an inhibitory role of PKA. Indeed, cAMP and cGMP pathways inhibit GPVI dimerization.⁴⁸ This suggests that inhibitory pathways might need to be down-regulated in order to enable collagen binding to GPVI. Mathematical models of GPVI signaling have so far not taken this negative regulation by cyclic nucleotides into account.⁴⁹

2.5 | TxA₂ receptor

The TxA₂ receptor is a GPCR coupling to Gq and G13 and mediates platelet activation by TxA₂. TxA₂ is synthesized by cyclooxygenase-1, the key target of the antiplatelet drug aspirin. The receptor can be phosphorylated by PKA and PKG leading to inhibited receptor signaling, although only limited evidence for the presence of this phosphorylation in platelets is available.¹ An alternative mechanism of regulating TxA₂-induced Gq signaling involves the RGS18 complex described above. PKA-induced phosphorylation of Gα13

linked to the TxA₂ receptor might contribute to RhoA inhibition by cAMP.¹

2.6 | NO and sGC

The classical pathway for the activation of cGMP production involves endothelium-derived NO stimulating platelet sGC. sGC represents the main receptor for endothelial NO in platelets and NO-binding stimulates cGMP production up to 100-fold.⁵⁰ Interestingly, VWF can induce a 3-fold increase in platelet cGMP levels,⁵¹ which has been attributed to an activation of sGC by Src kinase mediated Y192 phosphorylation⁵¹ and which might involve Akt kinase. Akt has also been suggested to play a role in lipopolysaccharide-induced cGMP elevation.⁵² Thrombin and collagen might increase cGMP levels slightly involving Lyn kinase, although this has not been confirmed by other studies.^{1,53} Platelet-derived NO might contribute to sGC activation and platelet NO synthesis has recently been observed by microscopy.⁵⁴ Stimulation of NO production by L-nebivolol results in reduced thrombosis in mice,⁵⁵ however, the presence of any NO-synthase in platelets has been disputed,^{51,56} indicating that NO might be released from other currently unknown sources. Cross-activation of cGMP production by platelet activators like VWF has

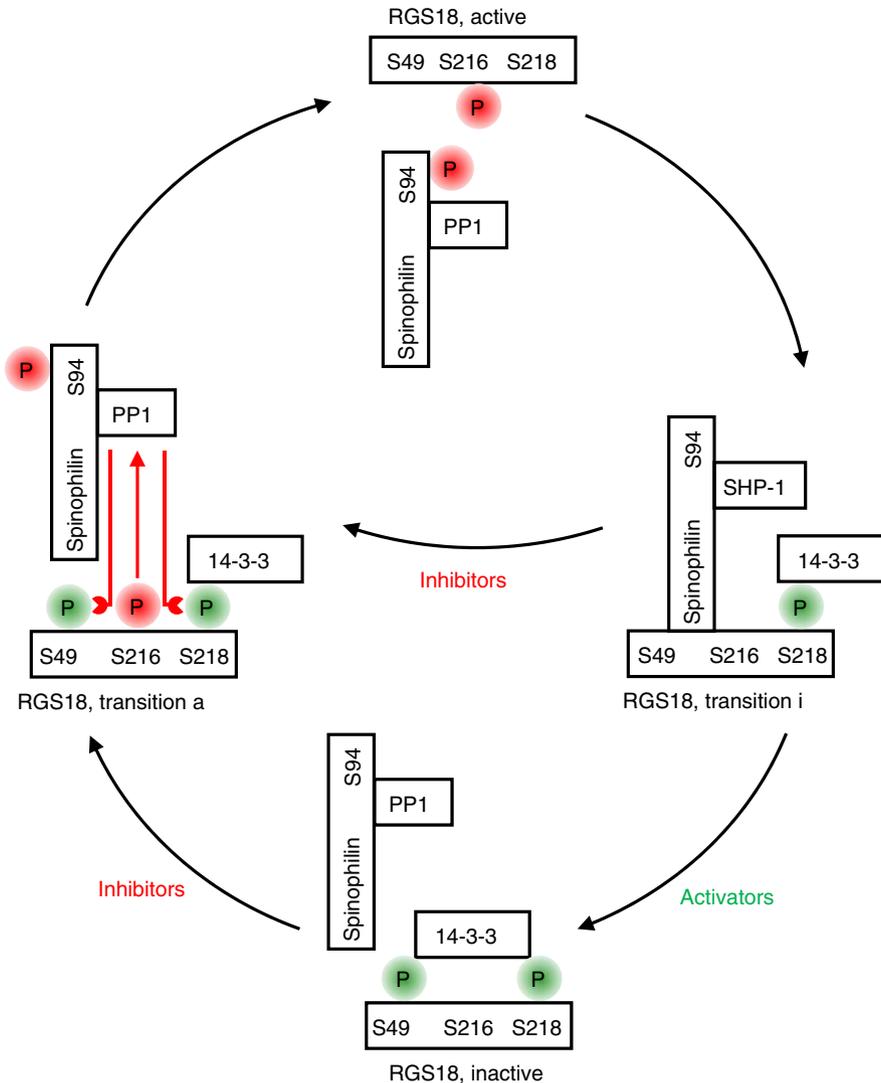


FIGURE 5 Activation/inactivation cycle of RGS18. Regulator of G-protein signaling 18 (RGS18) terminates Gq signaling and is regulated by both platelet activating and inhibitory pathways. In freshly isolated resting platelets RGS18 is found in a complex with the adapter protein spinophilin, the tyrosine phosphatase SHP-1 and the phospho-serine/threonine binding protein 14-3-3 γ attached to phosphorylated serine 218 of RGS18 (transition state on the way towards inactivation, transition i). Platelet activators like thrombin and TXA₂ induce the phosphorylation of serine 49 of RGS18 generating a second 14-3-3 binding site leading to enhanced 14-3-3 binding. Simultaneously, SHP-1 is activated and detaches from spinophilin (involving de-phosphorylation of tyrosine residues on spinophilin), the serine/threonine phosphatase PP1 binds to spinophilin instead, and spinophilin dissociates from RGS18. In this state the RGS18 complex is inactive. Platelet inhibitors like prostacyclin and nitric oxide induce the phosphorylation of serine 216 on RGS18 and serine 94 on spinophilin which lead to the activation of PP1 and detachment of spinophilin from RGS18 (transition state on the way towards activation, transition a). Active PP1 removes phosphate groups from serines 49 and 218 of RGS18 leading to the loss of 14-3-3 binding. This state of the RGS18 complex is characterized by free catalytically active RGS18 which can hydrolyse G α q-GTP to form inactive G α q-GDP (Figure 4). Phosphorylation sites linked to platelet inhibition are highlighted in red, whereas sites linked to platelet activation are marked in green

been interpreted in different ways, either as evidence of negative feedback or as an indication of a role for cGMP in platelet activation. A possible functional role of sGC during platelet activation is supported by studies of platelet specific sGC knockout mice and of patients lacking sGC expression. In both cases NO-induced platelet inhibition is abolished, as expected.⁵⁷⁻⁵⁹ However, sGC knockout platelets aggregate less well in response to low agonist concentrations, they show reduced Akt and ERK activation, and sGC knockout mice exhibit prolonged bleeding and impaired thrombus formation.⁵⁷ Similarly, platelets from patients lacking sGC expression do

not aggregate well in response to low ADP concentrations, and platelet adhesion to collagen and VWF is attenuated.⁵⁹ However, reduced sGC expression has also been linked to an increased risk of myocardial infarction possibly due to enhanced platelet activation.⁶⁰ Interestingly, low NO concentrations have been seen to slightly potentiate thrombin-induced Ca²⁺-elevations thus potentially contributing to platelet activation.^{61,62} Platelet activators like thrombospondin-1 (TSP1) have been shown to cross-inhibit sGC through some unknown mechanism.⁶³ Further studies are required to clarify interactions between different receptors, NO, sGC, cGMP, and Ca²⁺

keeping in mind the methodological issues that might affect experiments on NO and cGMP pathways.^{64,65}

3 | INTERACTIONS AT THE LEVEL OF SECOND MESSENGERS

3.1 | Calcium

Gq-coupled receptor and GPVI signaling trigger phospholipase C activation, leading to an increase in cytosolic inositol-3-phosphate (IP₃) levels. IP₃ binds to Ca²⁺ channels on the dense tubular system, called IP₃-receptors, resulting in Ca²⁺-release into the cytosol followed by store-operated calcium entry through the plasma membrane ultimately contributing to platelet activation.⁶⁶ Steady state, ADP- and thrombin-induced elevation of cytosolic Ca²⁺ levels have been modelled resulting in the validation of many static and kinetic parameters of the enzymes and ion channels involved.^{67,68} The well established antagonism between activators and endothelial inhibitors in the regulation of Ca²⁺ levels has recently been confirmed in a systematic study comparing effects of ADP, thrombin, convulxin and U46619 in combination with activators of cAMP and cGMP pathways.⁶¹ The Gq GAP RGS18 contributes to differential control of Ca²⁺ levels by platelet activators and inhibitors.^{7,35} PKA also inhibits IP₃-induced Ca²⁺-release through phosphorylation of the IP₃-receptor and the IP₃-receptor-associated G-kinase substrate (IRAG, MRV1).¹ In the absence of IRAG cGMP is less potent in inhibiting platelet aggregation and dense granule release.⁶⁹ The IP₃-receptor/PKG complex includes PDE type 5 which might support spatial control of cGMP signaling.¹

3.2 | Cyclic nucleotides

In addition to the cyclic nucleotide-synthesizing enzymes AC and sGC, PDEs control the spatial and temporal distribution of cAMP and cGMP. Platelets contain three PDEs, two of which degrade cAMP (PDE2A and PDE3A) and one is cGMP-specific (PDE5A).¹ PDE3A inhibition by cilostazol or JF959602 leads to increased cAMP levels and inhibits the initial accumulation of platelets at sites of injury as well as attachment and detachment of platelets from growing thrombi in vivo.⁷⁰ Thrombin stimulates the enzymatic function of PDE3A resulting in reduced cAMP levels.¹¹ Thus, costimulation of Gq and Gi during platelet activation leads to PDE3A activation and AC inhibition resulting in a pronounced decrease in intracellular cAMP levels. Stimulation of PDE3A by thrombin is mediated by protein kinase C (PKC) which phosphorylates serines 312, 428, 438, 465, and 492 of PDE3A.⁷¹ A similar phosphorylation pattern is induced by treatment of platelets with collagen-related peptide or by U46619. Phosphorylated S428 has been suggested to play a role as gate-keeper for binding of 14-3-3 to PDE3A (Figure 6). In contrast, the cAMP/PKA pathway triggers the phosphorylation of S312 alone which is not linked to 14-3-3 binding but which also stimulates the enzymatic function of PDE3A. These data indicate that activating pathways rapidly phosphorylate and stimulate PDE3A to reduce cAMP levels below an inhibitory threshold whereas inhibitory PKA pathways may use PDE3A in a negative

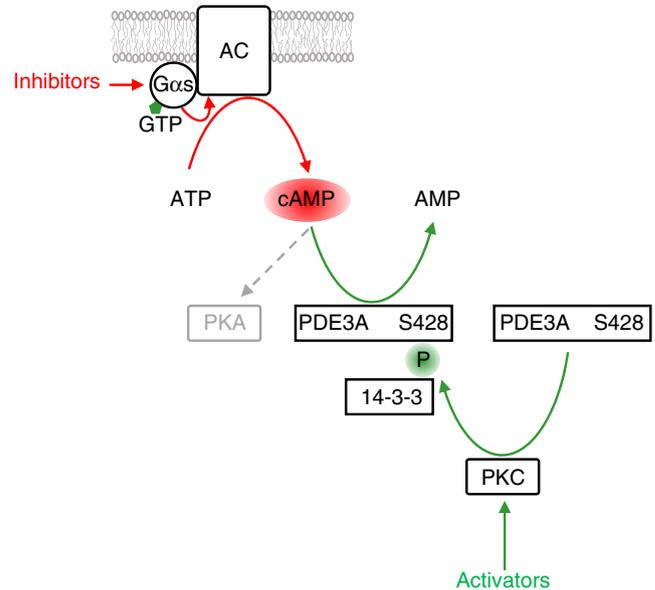


FIGURE 6 PDE3A signaling. Upon binding of active G α s-GTP adenylate cyclase (AC) is stimulated to synthesize cAMP from ATP. cAMP is required for the activation of cAMP-dependent protein kinase (PKA). cAMP levels can be reduced by phosphodiesterase PDE3A which specifically degrades cAMP to AMP. PDE3A is regulated by multiple phosphorylation events. For example, platelet activation leads to activation of protein kinase C isoforms (PKC, Figure 4) which phosphorylate PDE3A on serine 428 leading to attachment of the phospho-serine/threonine binding protein 14-3-3 and to stimulation of PDE3A function. In this way platelet activation interferes with inhibitory cAMP pathways

feedback loop. 14-3-3 binding to PDE3A might help to discriminate between activating and inhibitory pathways.⁷¹ Modelling of cyclic nucleotide levels confirms that PDEs are highly regulated exhibiting very low basal catalytic activity.⁷² These studies indicate that cAMP does not influence total cGMP levels. In contrast, cGMP might stimulate PKA function through an inhibition of PDE3A.⁷³ In fact, a synergistic inhibitory role for NO/cGMP in the cAMP/PKA pathway has been proposed.^{74,75} Subcellular compartmentalization of cyclic nucleotide signaling by scaffolding proteins like kinase anchoring proteins is likely to have an important role in determining crosstalk between NO/cGMP and cAMP pathways.¹

4 | INTERACTIONS INVOLVING KINASES AND PHOSPHATASES

4.1 | Kinases

Opposite regulation of MLC kinase and MLC phosphorylation levels by activating and cyclic nucleotide-mediated inhibitory pathways has been known for a long time,⁷⁶ and this regulation is likely to contribute to the control of actin dynamics, platelet adhesion, and aggregation.⁷⁷ Extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) activation induced by VWF, thrombin, collagen and TxA₂ are generally thought to be

blocked by cyclic nucleotide pathways,⁷⁸ although PKG-mediated activation of p38 MAPK has also been described.¹ Thrombin and collagen are able to activate PKA by releasing the catalytic PKA subunit from an NFκB complex possibly involving PI3K resulting in negative feedback and dampened platelet activation.⁷⁹ Recently, PKA activation was detected during thrombus formation. Using biosensors based on the principle of Förster resonance energy transfer expressed in mouse platelets, Hiratsuka et al⁸⁰ observed ERK activation throughout the developing thrombus after laser injury of subcutaneous arterioles, as expected. Surprisingly, PKA activation was detectable inside the thrombus as well, both in the vasculature *in vivo* and in a flow chamber in the absence of endothelium. PKA appeared to be more active on the downstream side of the aggregate, possibly matching the outer shell structure where P2Y12 signaling has been suggested to be involved in regulating thrombus size.²⁷ Thus, PKA might be activated during thrombus formation by an endothelium-independent mechanism to limit thrombus growth.

Platelet activation by oxidized low-density lipoproteins has been suggested to involve Src kinases, PKC, p47^{phox} phosphorylation, NADPH oxidase 2, and the production of reactive oxygen species ultimately leading to an inhibition of PKG.⁸¹ This mechanism might be similar to TSP1-induced inhibition of PKA which has also been suggested to involve Src family kinases.⁸² Src family kinases are crucial for initiating and propagating signals from major platelet receptors,⁸³ supporting the concept that platelet activation requires blockage of cyclic nucleotide signaling. Recent mass spectrometry based proteome studies indicate that many kinases are phosphorylated upon PGI₂ and/or ADP treatment of platelets probably leading to complex inhibition/activation patterns.^{18,19,84}

4.2 | Phosphatases

Proteome studies document de-phosphorylation of many sites confirming an important role for phosphatases in platelet control.^{18,85} Certain kinases are able to activate phosphatases, as shown for thrombin-induced phosphorylation and activation of SHP-1 leading to de-phosphorylation of spinophilin,³⁵ and for PKA-mediated activation of PP1 leading to detachment of 14-3-3 from RGS18.³⁸ Although tyrosine phosphatases are important during platelet activation,⁸⁶ their role in cyclic nucleotide-mediated platelet inhibition remains unclear. Interestingly, of the many phosphorylation sites induced by ADP treatment only half are reversed upon activation of cAMP pathways while the remaining sites affected by ADP are not altered. However, these cAMP-induced changes are sufficient to reverse ADP-induced platelet aggregation.¹⁸

5 | INTERACTIONS INVOLVING SMALL G-PROTEINS

5.1 | Rap1

Small G-proteins of the Ras and Rho families like Rap1, Rac1, and RhoA are critical regulators of platelet function.⁸⁷ Similarly to

heterotrimeric G-proteins, small G-proteins are dependent on specific GEFs and GAPs for activation and inhibition, respectively.^{88,89} These GEFs and GAPs have been shown to be controlled by activation pathways as well as by cyclic nucleotide signaling leading to differential changes in small G-protein activity. Rap1 is the most highly expressed small G protein in platelets and is the main activator of the fibrinogen receptor integrin αIIbβ3. CalDAG-GEFI (CD-GEFI, RasGRP2), an important GEF of Rap1, is activated by Gq-coupled receptor signaling leading to increased Rap1-GTP levels and integrin αIIbβ3 activation (Figure 7). PKA inhibits CalDAG-GEFI by phosphorylation of S116, S117, and S587 resulting in lowered Rap1-GTP.^{90,91} ADP treatment is also able to reduce S587 phosphorylation thus reversing PKA effects.¹⁸ A corresponding regulation of Rap1GAP2, a GAP of Rap1, contributes to the control of Rap1 activity. Rap1GAP2 function is blocked by platelet activators and activated by cyclic nucleotide pathways which involves 14-3-3 binding. During platelet activation Rap1GAP2 S9 phosphorylation enhances 14-3-3 binding, whereas PKA/PKG-mediated S7 phosphorylation does the opposite (Figure 7).⁹² Platelet activation has also been shown to inhibit the function of RASA3, a highly expressed GAP of Rap1 in platelets, in a PI3K-dependent manner.^{21,22} Vasodilator-stimulated phosphoprotein (VASP) is an actin filament and focal adhesion binding protein and was one of the first substrates of PKA/PKG to be identified in platelets. PKA and PKG phosphorylate VASP preferentially on serines 157 and 239, respectively.¹ Recent data suggest that VASP plays a positive role in the activation of the small G-protein Rap1. Treatment of VASP-deficient mouse platelets with thrombin, ADP or U46619 results in reduced Rap1-GTP levels compared to normal platelets.⁹³ VASP-dependent Rap1 activation might be mediated through an interaction of VASP with Crk-like, an activator of C3G (RapGEF1), which is a GEF of Rap1. PKA-dependent VASP S157 phosphorylation inhibits the VASP/Crk interaction thus potentially contributing to platelet inhibition.⁹³

5.2 | Rac1

Rac1 integrates signals from several platelet activation pathways and is involved in platelet lamellipodia formation and thrombus stability. Similar to Rap1, parallel regulation of the Rac1 regulating proteins ArhGEF6 and ArhGAP17 has been described whereby PKA and PKG simultaneously inhibit ArhGEF6 and stimulate ArhGAP17 leading to attenuated Rac1 function.⁹⁴ These effects are mediated by ArhGEF6 S684 phosphorylation and 14-3-3 binding, and by ArhGAP17 S702 phosphorylation resulting in detachment of the actin regulator Cdc42-interacting protein 4, CIP4.

5.3 | RhoA

RhoA is activated downstream of G13 and is an essential regulator of platelet shape change. RhoA is also known to be differentially regulated by cyclic nucleotides and platelet activators.⁹⁵ RhoA inhibition by PKA/PKG involves RhoA S188 phosphorylation and reduced

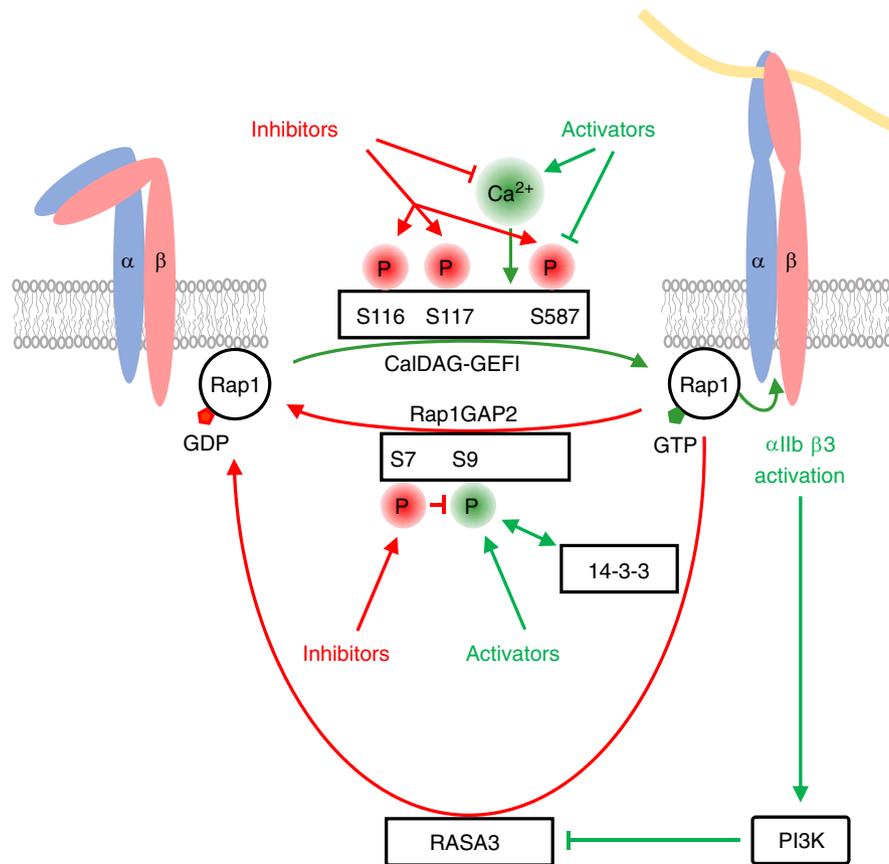


FIGURE 7 Regulation of Rap1 by GEFs and GAPs. Integrin $\alpha\text{IIb}\beta\text{3}$ is a major platelet integrin required for platelet aggregation. The small G-protein Rap1 is a positive regulator of integrin $\alpha\text{IIb}\beta\text{3}$ activation and cycles between an inactive, GDP-bound and an active, GTP-bound state. Formation of Rap1-GTP and integrin activation are enabled by the calcium ion (Ca^{2+}) dependent guanine-nucleotide exchange factor CalDAG-GEFI, whereas hydrolysis of Rap1-GTP to inactive Rap1-GDP requires the GTPase-activating proteins (GAPs) Rap1GAP2 and RASA3. Rap1GAP2 interacts with the phospho-serine/threonine binding protein 14-3-3 through phosphorylated serine 9 on Rap1GAP2. During platelet activation levels of free intracellular Ca^{2+} rise (Figure 4) leading to the activation of CalDAG-GEFI, increased Rap1-GTP formation and integrin activation. In contrast, cyclic nucleotide-dependent inhibitory pathways on one hand suppress intracellular Ca^{2+} levels, thus indirectly inhibit CalDAG-GEFI activation, and on the other hand result in phosphorylation of CalDAG-GEFI on serines 116, 117 and 587 inhibiting its activity. Simultaneously, cyclic nucleotide-dependent inhibitory pathways induce Rap1GAP2 phosphorylation on serine 7 leading to an inhibition of 14-3-3 binding, activation of Rap1GAP2, reduced Rap1-GTP levels and lowered Rap1 activity. In contrast, platelet activation leads to phosphorylation of Rap1GAP2 on serine 9 resulting in enhanced 14-3-3 binding and inhibition of Rap1GAP2. In parallel, $\alpha\text{IIb}\beta\text{3}$ -mediated outside-in signaling leads to phosphatidylinositol 3-kinase (PI3K) activation, which in turn reduces the GAP activity of RASA3. Platelet activators also suppress the phosphorylation of the inhibitory serine 587 site on CalDAG-GEFI. The consequence of Rap1GAP2 and RASA3 inhibition, along with CalDAG-GEFI stimulation is enhanced Rap1 activity. Phosphorylation sites linked to platelet inhibition are highlighted in red, whereas sites linked to platelet activation are marked in green

association of RhoA with ROCK2 and the myosin-binding regulatory subunit of PP1, MYPT1.^{77,96}

5.4 | Arf6

ADP-ribosylation factor 6 (Arf6) belongs to the Arf family of small G-proteins and is involved in receptor trafficking in platelets.⁹⁷ In contrast to Rap1, Rac1, and RhoA, Arf6 is inhibited during platelet activation, whereas cyclic nucleotide pathways elevate Arf6-GTP levels.⁹⁸ Cytohesin-2 is a GEF for Arf6 which plays a role in the constitutive inhibition of dense granule secretion in resting platelets.⁹⁹ Cytohesin-2 is phosphorylated by PKC during platelet activation leading to the dissociation of Arf6 and its GEF⁹⁹ and might be

involved in the opposing regulation of Arf6-GTP levels during platelet activation and inhibition. Phosphoproteome studies indicate that many other GEFs and GAPs might play important roles in PGI_2 - and ADP-mediated platelet regulation.¹⁸

6 | CLINICAL IMPLICATIONS

Inherited disorders affecting genes involved in the positive or negative regulation of platelet cyclic nucleotide levels underscore the importance of this regulatory system to hemostasis and thrombosis in humans. For example, Gs hyperfunction leads to an enhanced tendency for bleeding,¹⁰⁰ enhanced NO signaling correlates with

reduced risk of thrombosis,¹⁰¹ whereas Gs or sGC dysfunction culminates in enhanced susceptibility to thrombosis.^{60,102} In particular, loss of sGC expression, through a combination of mutations in the $\alpha 1$ subunit of sGC and in the sGC-interacting chaperone CCT η , was shown to reduce platelet cGMP levels and to increase the risk of myocardial infarction.⁶⁰ Recently, a meta-analysis identified PDE3A as a novel stroke risk locus.¹⁰³

The clinical relevance of crosstalk between activating and cyclic nucleotide-dependent inhibitory pathways is further highlighted by the success of drugs targeting either the Gi-coupled P2Y₁₂ receptor or PDEs 3A and 5A,^{12,104,105} which prevent the blockade of cyclic nucleotide production or degradation, respectively.¹⁰⁶ Stimulators of the cGMP pathway are commonly used to treat vascular disease due to their ability to lower vascular tone by acting on smooth muscle cells, however, simultaneous platelet inhibition might contribute to their beneficial effects. For example, NO donors are employed in ischaemic heart disease,¹⁰⁷ and the sGC stimulator riociguat has recently been developed as a new treatment for thromboembolic pulmonary hypertension.¹⁰⁸ Besides acting as a vasodilator, riociguat promotes platelet inhibition by acting synergistically with PGI₂.⁷⁵ The state of inhibitory pathways can be monitored by measuring intracellular phosphorylation of the PKA/PKG substrate protein VASP in platelets, which has been used to study the efficiency of P2Y₁₂ inhibition in patients.^{109,110} Pharmacological studies of P2Y₁₂ receptor based anti-platelet therapy show that addition of low concentrations of PGI₂ and NO to platelets from volunteers receiving dual anti-platelet therapy with aspirin and the P2Y₁₂ blocker prasugrel strongly enhances platelet inhibition in the presence of TRAP-6 or collagen.⁸ This synergy between P2Y₁₂ inhibitors and cAMP/cGMP signaling suggests that in the presence of similar levels of platelet blockade thrombosis risk might critically depend on the availability of PGI₂ and NO and thus on endothelial function. Since endothelial dysfunction is commonly observed in patients with cardiovascular disease it could be beneficial to include endothelial function testing in the assessment of anti-platelet therapy.⁸ Combining stimulators of endothelium-dependent cAMP pathways such as the PDE3A inhibitor cilostazol with P2Y₁₂ inhibitors might improve anti-thrombotic protection.¹⁷ Interestingly, cilostazol treatment has been associated with fewer hemorrhagic events compared to aspirin.^{104,111} Although P2Y₁₂ and PDE inhibitors are widely used in anti-platelet therapy, their downstream effects on the platelet signaling network are unappreciated, and likely involve systems-level changes, including altered phosphorylation of many PKA substrate proteins and their functional implications.

7 | CONCLUSIONS

Cyclic nucleotide-mediated inhibitory signaling is emerging to be closely intertwined with activating pathways. On the other hand, effective platelet activation mandates interference with inhibitory pathways at multiple levels, including blocking cyclic nucleotide production and promoting their degradation, and dephosphorylating

PKA substrates. Inhibitors and activators tend to regulate the same signaling nodes in opposite directions (Table 1). Larger protein assemblies such as the RGS18/spinophilin/14-3-3/SHP-1/PP1 or GPIb/IX/V/14-3-3/filaminA/actin/Syk complexes have been revealed which are likely to facilitate spatial and temporal coordination of signaling. Many of the described signaling processes reorganize protein-protein interactions by multiple phosphorylation and de-phosphorylation events often involving binding or detachment of 14-3-3 proteins. GEFs and GAPs are becoming apparent as major targets in the control of small G-proteins. Focused studies of individual proteins, phosphorylation sites and kinases in cells and animal models as well as proteomics approaches combined with computational analyses will be required to gain deeper insights into patterns of platelet regulation. Although knockout mouse studies are essential in our understanding of these patterns, to accurately interpret results it is important to consider possible compensatory changes in gene expression. This issue is particularly relevant when analyzing interactions between positive and negative signals, and it is prudent to verify if the expression of related proteins involved in the same or the opposite pathway are altered which could contribute to the phenotype.¹¹² New information on the topology of platelet signaling networks and the crossover points between cyclic nucleotide inhibition and platelet activation will likely result in either new drug targets or better combinations of existing drugs.

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Authors have nothing to disclose.

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