

## **Novel roles of cAMP/cGMP dependent signaling in platelets**

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## **Summary**

Endothelial prostacyclin and nitric oxide potently inhibit platelet functions. Prostacyclin and nitric oxide actions are mediated by platelet adenylyl and guanylyl cyclases which synthesize cAMP and cGMP, respectively. Cyclic nucleotides stimulate cAMP- and cGMP-dependent protein kinases (PKA and PKG) to phosphorylate a broad panel of substrate proteins. Substrate phosphorylation results in the inactivation of small G-proteins of the Ras and Rho families, inhibition of the release of calcium ions from intracellular stores and a modulation of actin cytoskeleton dynamics. Thus, PKA/PKG substrates translate prostacyclin and nitric oxide signals into a block of platelet adhesion, granule release and aggregation. cAMP and cGMP are degraded by phosphodiesterases which might restrict signaling to specific subcellular compartments. An emerging principle of cyclic nucleotide signaling in platelets is the high degree of interconnection between activatory and cAMP/cGMP-dependent inhibitory signaling pathways at all levels including cAMP/cGMP synthesis and breakdown as well as PKA/PKG mediated substrate phosphorylation. Furthermore, defects in cAMP/cGMP pathways might contribute to platelet hyperreactivity in cardiovascular disease. This article focuses on recent insights into the regulation of the cAMP/cGMP signaling network and on new targets of PKA and PKG in platelets.

## **Keywords**

cAMP, cGMP, kinase, nitric oxide, phosphodiesterase, prostacyclin

## Introduction

Cyclic adenosine monophosphate (cAMP) is known as powerful inhibitor of platelet aggregation since the 1960s. Initial studies showed inhibitory effects of prostaglandin E1 on platelet activation [1], which were soon linked to cAMP [2]. Later endothelium-derived prostacyclin was discovered as the main physiological stimulator of cAMP production in platelets [3]. Nitric oxide (NO) and cyclic guanosine monophosphate (cGMP) were observed to inhibit platelet functions around 1980 [4], and endothelial release of NO was linked to cGMP dependent platelet inhibition [5]. Defects in platelet cyclic nucleotide signaling might play a role in common diseases such as ischemic heart disease, heart failure and diabetes, where a reduced sensitivity of platelets to the inhibitory effects of NO contributes to platelet hyperreactivity [6]. In addition, a number of rare genetic abnormalities in platelet cyclic nucleotide signaling have been described. Defects in prostacyclin signaling reduce cAMP levels resulting in hyperreactive platelets and a prothrombotic state [7]. Hypersensitivity of prostacyclin signaling due to gain of function mutations in a Gs protein results in elevated cAMP levels and induces a bleeding phenotype, presumably via an exaggerated inhibition of platelet functions [7]. cAMP and/or cGMP elevating agents have shown clinical benefit as platelet inhibitors. For example, dipyridamole in combination with low-dose aspirin is an approved therapy for stroke prevention [8]. Dipyridamole elevates cAMP levels in platelets by several mechanisms including an inhibition of phosphodiesterase (PDE) mediated breakdown. Cilostazol is a specific inhibitor of PDE3 which has been FDA-approved for the treatment of intermittent claudication and which appears to be effective in reducing the risk of restenosis after coronary angioplasty [8]. Cilostazol has also been used successfully for the secondary prevention of ischaemic stroke [9]. Recently, a group of compounds that activate cGMP production by soluble guanylyl cyclase have been shown to reduce thrombus formation in animal models [10]. The exact mechanisms involved in cAMP/cGMP mediated platelet inhibition and the wiring of the cyclic nucleotide signaling network are only partly understood. cAMP and cGMP are able to block many aspects of platelet activation including early activatory signals such as release of calcium ( $\text{Ca}^{2+}$ ) ions from intracellular stores and G-protein activation, as well as adhesion, granule release, aggregation and apoptosis [11, 12]. In general, platelet inhibition by cyclic nucleotides does not appear to be

restricted to any particular activatory pathway. cAMP and cGMP block platelet activation mediated by ligands of G-protein-coupled receptors like thrombin, ADP or thromboxane, as well as by collagen, vWF, or fibrinogen. This article will provide an overview over the various components of the cAMP/cGMP signaling network in platelets including novel insights into mechanisms of regulation and new target molecules.

### **Regulation of cAMP synthesis**

Levels of free cytosolic cAMP are controlled by their synthesis through adenylyl cyclases (Figure 1). Platelet proteome analysis suggests that platelets express a number of different membrane-bound adenylyl cyclase (AC) isoforms, including AC3, AC6 and AC7 [13]. ACs are activated by G-protein-coupled receptor signaling. Binding of prostacyclin (PGI<sub>2</sub>) to its receptor on the platelet surface (IP receptor or IP-R) [14] triggers the activation of intracellular receptor-linked stimulatory G protein  $\alpha$ -s (Gs) subunits. Gs is turned into its active GTP-bound form, Gs-GTP then binds to AC and stimulates the synthesis of cAMP from ATP. Gs-mediated activation of AC is turned off by RGS2 (regulator of G-protein signaling 2), a specific GTPase-activating protein that helps to hydrolyze Gs-GTP back to inactive Gs-GDP [15]. Other receptors linked to Gs-mediated AC stimulation in platelets include A2A and A2B receptors for adenosine [16, 17] and VPAC1 receptors for pituitary adenylyl cyclase-activating and vasoactive intestinal peptides [18]. Platelet activators like ADP or thrombin block AC function through inhibitory  $\alpha$ -i (Gi) proteins, resulting in a drop of cAMP levels during platelet activation [19, 20]. The specific roles of individual AC isoforms and their possible regulation by other G-protein-independent factors have not been studied in platelets to date.

### **Regulation of cGMP synthesis**

cGMP production in platelets depends on a single enzyme, the soluble NO-sensitive guanylyl cyclase (sGC or NO-GC) composed of two subunits,  $\alpha$ 1 and  $\beta$ 1 [21]. NO originates from endothelial cells containing endothelial NO synthase, and thorough analyses of mouse and human platelets appear to rule out the presence of any significant NO synthase enzyme within platelets [22-24]. Recent data from eNOS knock-out animals indicate that other as yet unidentified sources

of NO might play a role in platelet inhibition [25]. NO permeates the plasma membrane of platelets and activates cytosolic sGC to generate cGMP (Figure 1) resulting in an approximately 10-fold increase in cGMP levels. A number of recent studies of sGC deficient platelets demonstrated that most effects of NO in platelets are indeed mediated by sGC and cGMP, at least in mice. Absence of sGC abolished cGMP synthesis resulting in an almost complete loss of NO effects both in a constitutive sGC-deficient mouse model as well as in platelet specific sGC-deficient mice [11, 26, 27]. Using very high concentrations of the NO-donor sodium nitroprusside (SNP, above 100  $\mu$ M) one group observed a residual inhibition of collagen induced platelet aggregation and ATP release in isolated sGC-deficient platelets, which was attributed to cGMP-independent NO-mediated protein nitrosylation [27]. However, in a different study 800  $\mu$ M SNP as well as high concentrations of other NO-donors did not affect collagen induced aggregation of isolated sGC-deficient platelets [26]. sGC function can be activated by von Willebrand factor (vWF), thrombin or collagen (Figure 1). A small (mostly about 2-fold) increase in cGMP levels after exposure of platelets to vWF, calcium ionophore,  $H_2O_2$  [22, 28, 29] thrombin or collagen [27, 30, 31] has been described, although these findings are not supported by all studies [23, 32, 33]. It should be noted that current methods for determining intracellular cyclic nucleotide levels in whole cell lysates are limited by the rapid phosphodiesterase mediated breakdown and the potential subcellular compartmentalization of cAMP and cGMP (see below). Alternative pathways for sGC activation might involve phosphorylation. The vWF induced increase in cGMP levels is absent in mice lacking either the serine/threonine kinases Akt1 and Akt2 or the tyrosine kinase Lyn [34, 35]. Tyrosine phosphorylation of  $\alpha 1$  and  $\beta 1$  subunits of sGC has been observed in vWF and collagen treated platelets [22, 36]. The physiological role of these alternative and possibly NO-independent pathways of sGC activation remains to be determined. sGC was recently suggested to be involved in platelet activation in a platelet specific sGC-deficient mouse model. Aggregation and ATP release of isolated sGC-deficient platelets were reduced at low concentrations of collagen and thrombin, and tail bleeding times and thrombus formation were slightly increased in these animals [27]. However, in constitutive sGC-deficient mice strongly reduced bleeding times were described [26]. Thus, a potential role

for sGC in platelet activation will need to be clarified by further studies. Recently, platelet specific sGC-deficient mouse were used to establish a new role for NO and cGMP in the inhibition of platelet apoptosis [11]. Defective platelet sGC function has been described in patients with ischemic heart disease, heart failure and diabetes [6]. Studies of patients with obesity suggest that not only the function of platelet sGC might be disturbed but additional defects in cAMP synthesis as well as in downstream cAMP and cGMP targets might contribute to platelet hyperreactivity in cardiovascular and metabolic disease [37]. In these patients impaired NO mediated inhibition of platelet aggregation has been linked to reduced levels of cGMP synthesis in the presence of oxidative stress [6]. sGC is known to be inactivated by oxidation which might eventually lead to the loss of the NO binding heme group from the enzyme. Oxidized or heme-free sGC has been shown to play an important role in the pathogenesis of cardiovascular disease and new compounds have been developed that are able to activate heme-free sGC [10]. In addition, stimulators of sGC are available that act in synergy with NO to increase sGC activity. The sGC stimulator BAY 41-2272 is a potent new platelet inhibitor [38], although some of its effects might be mediated by blocking of PDE5 mediated degradation of cGMP [39].

### **Phosphodiesterase mediated degradation of cyclic nucleotides**

Platelet cyclic nucleotide levels are controlled by phosphodiesterase mediated degradation providing a negative feedback loop on cyclic nucleotide signaling. Platelets have been shown to express PDEs 2A, 3A and 5A [40]. PDE2 and 3 are able to degrade cAMP and cGMP, however, inhibitor studies suggest that PDE2 and PDE3 mainly regulate cAMP in platelets [41, 42], whereas PDE5 specifically degrades cGMP. cGMP has a regulatory effect on all three platelet PDEs (Figure 1). cGMP stimulates PDE2 and PDE5 activity by binding to specific cyclic nucleotide binding domains, called GAF domains. cGMP inhibits PDE3 activity by competition of cAMP binding at the catalytic site. PDE3A helps to maintain low basal levels of cAMP in platelets [41-43]. PDE3A activity is upregulated by about 50% during thrombin activation involving PKC and possibly also PKB mediated phosphorylation [44, 45]. PKC mediated phosphorylation of PDE3A on S428 results in binding of 14-3-3, although this does not appear to be required for increased activity. PDE3A is also activated by PKA mediated phosphorylation,

suggesting a negative feedback loop on cAMP signaling [44, 46]. cGMP mediated inhibition of PDE3A has been implicated in elevation of cAMP levels leading to cross-activation of PKA, which could play a role in NO induced inhibition of platelet shape change [47]. The main function of PDE5 is to provide a negative feedback on cGMP levels. PDE5 is not only activated by cGMP, but additional phosphorylation of PDE5 by PKGI results in activation of PDE5 catalytic activity and long-term desensitization of an NO-induced cGMP response [48]. PDE5 might be involved in compartmentalization of cGMP signaling in specific subcellular regions (see below) [49].

### **cAMP/cGMP-dependent protein kinases**

cAMP- and cGMP-dependent protein kinases (PKA and PKG) translate cAMP/cGMP levels into protein phosphorylation patterns. Human platelets contain micromolar concentrations of PKA and PKG [50]. PKA is a heterotetramer composed of 2 catalytic subunits and 2 regulatory subunits. When cAMP binds to the regulatory subunits, the catalytic subunits dissociate from the complex and phosphorylate their substrates thereby suppressing platelet activation. The main isoforms of regulatory and catalytic subunits expressed in human platelets are RI $\alpha$ , RI $\beta$ , RII $\beta$  and C $\alpha$ , C $\beta$  resulting in the formation of PKAI and PKAII holoenzymes [13, 24]. The specific role of PKAI versus PKAII in platelets has not been investigated. Recent data suggest that platelets lacking RII $\beta$  regulatory subunit of PKA might be suppressed in their ability to become activated, presumably due to the unregulated, inhibitory activity of the catalytic subunit [51]. Another way of activating PKA involves the release of the catalytic subunit of PKA from an NF $\kappa$ B-I $\kappa$ B complex [32]. Thrombin and collagen are able to trigger the release of the catalytic subunit from I $\kappa$ B resulting in the phosphorylation of the substrate proteins VASP and Rap1GAP2 [32]. This alternative mechanism for PKA activation might represent a negative feedback loop in thrombin- and collagen-induced platelet activation (Figure 1).

In contrast to PKA the regulatory cGMP-binding and catalytic domains of PKG are combined within one molecule that dimerizes via its N-terminal regulatory region [52-54]. The main isoform expressed in human platelets is PKGI $\beta$ . Knockout of the PKGI gene in mice revealed a pro-thrombotic phenotype. Effects of cGMP-analog on platelet shape change, granule release and aggregation were

abolished in PKG deficient platelets, whereas effects of cAMP-analog were maintained [55]. In addition, PKGI-deficient mouse platelets adhered more strongly to injured vascular surfaces [55]. Inhibitory effects of NO-donors on fibrinogen binding were lost in PKGI-deficient platelets, whereas effects of a prostacyclin analog were maintained indicating that most effects of endogenous cGMP are mediated by PKGI. Studies in PKGI-deficient human platelets confirm the role of PKGI for cGMP-mediated inhibition of  $\text{Ca}^{2+}$  release from intracellular stores [56]. In 2003 a paper by Li et al. sparked a series of investigations into a potential role for PKGI in platelet activation. Platelets from PKGI knockout mice were observed to exhibit impaired spreading on vWF and bleeding times of these animals were slightly increased [28]. In experiments using membrane-permeable cGMP analogs low levels of PKGI stimulation were suggested to contribute to platelet activation whereas prolonged stimulation of PKGI would result in platelet inhibition [31]. These data were challenged by other groups showing that short-term treatment of platelets with low concentrations of cGMP-analogs may lead to unspecific effects [33, 57]. Another issue with the proposed biphasic model of PKGI function in platelets is the lack of consistent data on possible targets of cGMP or PKGI that might mediate platelet activation. Initial suggestions that PKGI might activate p38 MAPK signaling [58] could not be confirmed [59, 60]. On the other hand, a recent investigation by an independent group showed small but significant stimulatory effects of low concentrations of NO-donors on  $\text{Ca}^{2+}$  release from intracellular stores induced by low concentrations of thrombin [61]. Taken together a potential role of PKGI during a specific early phase of platelet activation has not yet been established.

Cyclic nucleotide signaling is compartmentalized in many cell types and some evidence for localized cAMP and cGMP function in platelets has been provided [49, 62]. Localized cAMP signaling is often coordinated by A-kinase anchoring proteins (AKAPs) that bind PKA and other signaling components such as PDEs resulting in the formation of subcellular cAMP signaling compartments. Recently, using a mass spectrometry based screening approach seven AKAPs (AKAP 1, 2, 7, 9, 10, 11, MAP2) were identified in platelets [63], although these initial findings need to be verified by independent methods. Transcriptome analysis suggests that platelets might also express AKAPs 5, 8, 8L and 13 [24]. The PKGI substrate



protein IRAG represents the only known G-kinase anchoring protein in platelets [64].

### **Substrates of cAMP/cGMP-dependent protein kinases**

cAMP and cGMP pathways inhibit platelet activation, adhesion, granule release and aggregation. The phosphorylated substrates of PKA and PKGI link cAMP/cGMP signaling to the functional outcomes of blocked platelet functions. Few PKA and PKGI substrate proteins have been identified to date, however, the total number and identity of all phosphorylated substrates remains unknown. PKA and PKGI substrates in platelets can be broadly grouped into two main categories, signaling regulators and actin binding proteins (Figure 1). An interesting point about cyclic nucleotide signaling in platelets is that in many cases, cAMP and cGMP signals appear to converge at the level of substrate proteins, since PKA and PKGI activation tends to result in the phosphorylation of the same proteins (Table 1). An important tool in assessing protein phosphorylation in intact platelets are phosphorylation site specific antibodies. Only a few antibodies against PKA/PKG phosphorylation sites are currently available for platelet studies (Table 1). The substrate proteins included in this review are proteins where phosphorylation in response to cAMP/cGMP activation was shown in intact platelets using either phosphorylation site specific antibodies or by  $^{32}\text{P}$  isotope labeling.

### **G-proteins and G-protein regulators**

One of the first substrates of PKA and PKGI to be identified in platelets was the small G-protein Rap1B [65]. The phosphorylation site was localized to the C-terminus of the protein at serine 179, within a membrane-binding region [66]. Rap1B is a potent regulator of integrin activity and rap1b  $-/-$  mice display impaired platelet aggregation and prolonged tail bleeding times [67]. Little is known about the functional consequences of Rap1B phosphorylation. The phosphorylation does not affect the ability of Rap1 to bind GTP or its GTPase activity [68]. The phosphorylation kinetics are much slower (minutes) compared to the rapid switch between GTP and GDP-bound states (seconds). The only known functional consequence of S179 phosphorylation is a redistribution of Rap1 from the plasma membrane to the cytosol [69]. Live microscopy of DsRed-tagged wt Rap1B in

HeLa cells shows plasma membrane staining, whereas a phosphomimetic S179E mutant Rap1B localises to the cytoplasm (O. Danielewski and A. S., unpublished data). Thus, phosphorylation of Rap1B on S179 appears to impact on the subcellular localization of Rap1B.

Considering the vital role of Rap1 in integrin signaling, the function and regulation of its activity is of central importance. Studies of Rap1-GTP levels in human platelets show that prostacyclin blocks thrombin-induced Rap1-GTP formation [70]. NO-donors and PKG-activating cGMP-analogs block thrombin, collagen, and ADP induced Rap1 activation, and this effect was shown to involve PKGI [71]. It is important to note that human platelets do not appear to express any Epac, which is a cAMP-dependent activator of Rap1 [72]. Thus, cAMP signaling in platelets is mainly inhibitory towards Rap1. To identify the possible target of PKA and PKGI that could regulate Rap1 activity in platelets, Schultess et al. screened platelet mRNA for the expression of specific guanine-nucleotide exchange factors (GEF) and GTPase-activating proteins (GAP) of Rap1. This resulted in the identification of Rap1GAP2 as the only GAP of Rap1 in platelets [72]. Further studies revealed that Rap1GAP2 could be phosphorylated on serine 7 by PKA and PKGI and a phosphorylation site-specific antibody was used to verify phosphorylation in platelets treated with activators of cAMP and cGMP signaling [72, 73]. A 14-3-3 binding site was mapped to the neighboring serine 9 of Rap1GAP2 [73]. 14-3-3 proteins are small, phosphoserine/threonine binding proteins that function as scaffolds and, despite possessing no catalytic activity, may regulate key signaling components [74]. There are seven highly conserved isoforms in humans, six of which are expressed in platelets [75]. Elevation of cyclic nucleotides and therefore activation of PKA and PKGI, results in phosphorylation of serine 7 and dissociation of 14-3-3 from Rap1GAP2. 14-3-3 binding appears to dampen the function of Rap1GAP2, whereas detachment of 14-3-3 triggers increased activity resulting in markedly reduced cell adhesion in transfected cells [73]. Interestingly, platelet activation with agonists like ADP or thrombin enhanced the binding of Rap1GAP2 to 14-3-3, indicating that this interaction is regulated by activatory as well as cyclic nucleotide dependent inhibitory pathways. Targeting of Rap1 via Rap1GAP2 might explain some of the inhibitory effects of cyclic nucleotides on integrin activation, platelet adhesion and aggregation. Furthermore, PKA/PKG block the release of  $\text{Ca}^{2+}$  from intracellular

stores which might contribute to the inhibition of Rap1 activation, since one of the GEFs of Rap1 in platelets, CalDAG-GEFI, is activated by  $\text{Ca}^{2+}$  [76]. The activation of the Rap1B related protein Rap2B is also effectively blocked by cAMP pathways, although the PKA substrates involved have not been determined [77]. Another small G-protein regulated by cAMP and possibly also cGMP is RhoA. RhoA is involved in myosin light chain phosphorylation, actin remodelling, integrin activation and platelet aggregation. Prostacyclin as well as direct PKA activators block the formation of RhoA-GTP, whereas PKG activators have a less pronounced effect [78]. Inhibition of RhoA might be mediated by phosphorylation of the heterotrimeric G $\alpha$ 13 (G $_{13}$ ), which activates Rho-GEFs. G $_{13}$  can be phosphorylated by cAMP pathways in platelets, and phosphorylation of G $_{13}$  on T203 reduces RhoA activation [79, 80]. However, no phosphorylation site specific antibodies against T203 of G $_{13}$  have been described, and phosphorylation by PKGI has not been tested. Yet another small G-protein shown to be regulated by cyclic nucleotides is Rac1. Rac1 is involved in lamellopodia formation and platelet granule release and aggregation. cAMP and cGMP were shown to block the formation of Rac-GTP [78], but the PKA and PKGI substrates mediating Rac inhibition are not known.

### **IP $_3$ -R complex**

Elevation of intracellular  $\text{Ca}^{2+}$  concentrations plays an important role in platelet activation. Activation of phospholipase C beta and gamma isoforms leads to the hydrolysis of phosphatidylinositol 4,5-bisphosphate generating inositol-1,4,5-trisphosphate which in turn triggers the release of  $\text{Ca}^{2+}$  ions from intracellular stores via IP $_3$  receptor channels (IP $_3$ -R). cAMP and cGMP pathways strongly inhibit elevations of cytosolic  $\text{Ca}^{2+}$  concentrations in platelets including all types of  $\text{Ca}^{2+}$  oscillations that have been observed under flow conditions [81]. At least some of these effects are thought to be mediated by direct phosphorylation of IP $_3$ -R [82]. All three isoforms of IP $_3$ -R are expressed in platelets and all can be phosphorylated by PKA and PKGI on as yet unknown sites [83].

A type I IP $_3$ -R associated protein called IRAG (IP $_3$ -R-associated cGKI substrate protein, also MRVI1) has been clearly linked to PKGI mediated inhibition of  $\text{Ca}^{2+}$  release in platelets [64, 84]. The main phosphorylation sites of IRAG are serines 664 and 677 and phosphorylation site specific antibodies have been used to

show phosphorylation in intact platelets in response to activators of cGMP signaling [64]. In a mouse model expressing a mutant form of IRAG that does not bind to IP<sub>3</sub>-R inhibition of platelet collagen- and thrombin-induced aggregation by cGMP analogs or by NO-donors was significantly impaired [64]. The inhibitory effects of NO-donors on thrombus formation in the intact carotid artery were abolished in the IRAG mutant, whereas prostacyclin and cAMP effects were maintained. Complete deletion of IRAG expression in mice resulted in hyperreactive platelets with a significantly enhanced aggregation response towards thrombin, collagen and U46619, a thromboxane mimetic [84]. Studies of these IRAG-deficient platelets confirmed that IRAG is involved in NO/cGMP mediated inhibition of thrombin, collagen and U46619 dependent integrin  $\alpha$ IIb $\beta$ 3 activation, aggregation and ATP release [84]. Effects of cAMP analog on integrin activation were not altered in IRAG-deficient platelets, however, prostacyclin and cAMP effects were not studied extensively [84]. PKGI associates with IP<sub>3</sub>-R/IRAG in platelets [64]. In addition, the type I IP<sub>3</sub>-R was shown to bind PDE5 in platelets thus providing a link between PKGI and PDE5 [49]. Interestingly, only a fraction of platelet PDE5 was reported to associate with the IP<sub>3</sub>-R, and PKGI might preferentially phosphorylate and activate this IP<sub>3</sub>-R associated PDE5 resulting in local control over cGMP-dependent inhibition of Ca<sup>2+</sup> release [49]. These results represent initial evidence for compartmentalized cGMP signaling in platelets, however, other data suggest that most platelet PDE5 is activated and phosphorylated by cGMP and PKGI [48].

Ca<sup>2+</sup> levels are further regulated by the transient receptor potential channel 6 (TRPC6), which was shown to be a substrate of PKA and PKGI in platelets [85]. TRPC6 could play a role in store-operated calcium entry, but the consequences of phosphorylation are not clear. TRPC6 was described to form a complex with type II IP<sub>3</sub>-R in platelets [86].

### **Other signaling proteins**

PDE5A is preferentially phosphorylated by PKGI on serine 92 resulting in long-term activation of PDE activity and degradation of cGMP [48]. Similarly, PDE3A is phosphorylated by PKA on serine 312, which is associated with an increase in catalytic activity resulting in cAMP degradation [44]. Another signaling target of

cyclic nucleotide pathways in platelets is the p38 MAP kinase. p38 MAPK activation is blocked by cAMP and cGMP [59, 60], however, the PKA or PKGI substrates mediating p38 MAPK inhibition are unknown. The GPIb complex is required for platelet adhesion and GPIb $\beta$  was shown to be phosphorylated by PKA on serine 166 [87]. A phosphorylation site-specific antibody against S166 was described [88]. The functional consequences of GPIb $\beta$  S166 phosphorylation are unclear. Inhibition as well as activation of cell adhesion has been observed [88, 89]. S166 was also suggested to play a role in binding of 14-3-3 to the GPIb complex [90, 91], however, other 14-3-3 binding sites have been described and the significance of S166 phosphorylation in 14-3-3 binding has been questioned [75]. The role of cGMP/PKGI in GPIb phosphorylation has not been investigated. Findings from a study using isolated platelets suggest that NO might be less efficient in inhibiting adhesion by the GPIb complex itself but might rather block secondary integrin activation [92]. A potential substrate of PKA and PKGI in platelets that is commonly mentioned is the thromboxane receptor alpha. However, in  $^{32}\text{P}$ -labelled platelets no significant incorporation of phosphate into the thromboxane receptor in prostaglandin or forskolin treated platelets could be detected [93] and no other data on phosphorylation of the receptor in intact platelets has been reported. Mass spectrometry based screening approaches have led to the identification of other putative PKA and PKG substrates in platelets which need to be verified using independent methods [94].

### **Actin-binding proteins**

The regulation of the actin cytoskeleton is a major function of cAMP/cGMP signaling in platelets. One of the first substrates of PKA and PKGI to be identified in platelets was the vasodilator-stimulated phosphoprotein (VASP) [95]. VASP is expressed at a high concentration of about 25  $\mu\text{M}$  in platelets [50] and the main phosphorylation sites are serines 157 and 239 [96]. Analysis of phosphorylation kinetics indicates that S157 might be preferentially phosphorylated by PKA, whereas S239 is preferred by PKGI [97]. Phosphorylation site specific antibodies have been generated against pS157 and pS239 and these antibodies have been used extensively as markers of cyclic nucleotide activity in platelets and other cells [97, 98]. The 16C2 monoclonal antibody against phosphorylated S239 has

been developed into a flow cytometry assay for monitoring Gi function of the P2Y<sub>12</sub> ADP receptor in patients [99]. Mouse models deficient in VASP expression have shown that platelet VASP is involved in platelet activation and that VASP mediates NO-dependent inhibition of platelet adhesion to the vessel wall [100, 101]. Furthermore, VASP appears to be involved in inhibitory effects of PKA and PKGI on fibrinogen binding and platelet aggregation but not in the inhibition of Ca<sup>2+</sup> release or granule secretion [102]. VASP is clearly an important regulator of actin dynamics but the molecular consequences of VASP phosphorylation in platelets are not well defined. Phosphorylation inhibits binding of VASP to F-actin and reduces F-actin bundling in-vitro [103]. VASP phosphorylation has also been shown to be involved in focal adhesion dynamics [104] and in regulating the rigidity of the actin cytoskeleton [105]. PKA mediated phosphorylation of VASP on S157 might be controlled locally by integrin  $\beta$ 3 [106]. Other actin-associated proteins that are phosphorylated in human platelets include LASP (Lim and SH3 domain protein), HSP27, Filamin-A (actin binding protein, ABP-280), and caldesmon. LASP is phosphorylated by PKA and PKGI on serine 146 resulting in reduced binding of LASP to F-actin and to focal adhesions [107]. During thrombin activation LASP gets phosphorylated on tyrosine 171, probably by Src kinase [108]. Phosphorylation of HSP27 by PKA and PKGI on threonine T143 attenuates HSP27 dependent actin polymerization [109]. Upon ADP treatment of platelets HSP27 is phosphorylated on additional serine residues (S15, S78, S82) by p38 MAPK dependent pathways [110], suggesting that, similar to LASP, multiple phosphorylation events contribute to the regulation of HSP27. Another actin-binding substrate of PKA is filamin-A. Phosphorylation of filamin-A on serine 2152 protects filamin-A protein against degradation [111, 112]. The relevance of filamin-A stabilization for platelet function is unclear. Interestingly, filamin-A is required for maintaining platelet membrane stability at high shear levels by binding to the GPIb complex [113]. Caldesmon is an actin-binding protein that has been shown to be phosphorylated by prostacyclin induced signaling in human platelets [114]. The functional consequences of caldesmon phosphorylation for the actin cytoskeleton in platelets have not been studied. A potential substrate of PKA and PKGI that has been mentioned in previous reviews is the myosin light chain kinase (MLCK). However, the only

study of MLCK phosphorylation has shown phosphorylation of purified MLCK by purified PKA in-vitro, but not in intact platelets [115].

## **Conclusions**

Although cAMP and cGMP have been known to play a powerful role in platelet regulation for many years, the molecular patterns and details mediating this effect are only beginning to emerge (Figure 1). Most, if not all, of the inhibitory functions of cAMP and cGMP in platelets can be attributed to phosphorylation of substrate proteins by PKA and PKGI. Groups of different substrate proteins with related functions contribute to the inhibitory actions of PKA and PKGI. Currently known substrates can be broadly classified into regulators of signaling and/or actin dynamics. Signaling regulation involves small G-proteins of the Ras and Rho families like Rap1, RhoA and Rac. Dynamic changes in the architecture of the actin cytoskeleton are involved in many platelet responses including shape change, adhesion, granule release, and aggregation. Effects of PKA and PKGI on actin-binding proteins might complement the effects on small G-proteins resulting in the net outcome of inhibited platelet adhesion and aggregation. Initial evidence for compartmentalized cAMP/cGMP signaling in platelets has emerged from studies of the IP<sub>3</sub>-RI complex involved in the regulation of Ca<sup>2+</sup> release from intracellular stores. Regulation of Ca<sup>2+</sup> levels is likely to have a broad impact on many pathways including the activation of G-proteins and the granule release reaction. Some of the identified PKA and PKGI substrates, e.g. Rap1GAP2, PDE3A, HSP27, appear to harbor additional phosphorylation sites, which are targeted by activatory pathways. Multiple phosphorylation events need to be translated into appropriate functional outcomes. This might at least in some cases be achieved by differential binding of 14-3-3, as shown for Rap1GAP2 and PDE3A [44, 73].

Adequate control of platelet reactivity requires a careful balance between activatory and inhibitory signaling pathways. Platelet activators are known to counteract the cAMP/cGMP system at various levels. For example, Gi-coupled receptor signaling attenuates cAMP synthesis, activation of PDE3A results in the degradation of cAMP [44] and platelet activatory signaling interferes with cyclic nucleotide signaling at the level of PKA/PKG substrate proteins [73]. The platelet activator thrombospondin-1 was suggested to mediate platelet activation by

blocking cyclic nucleotide signaling at the level of sGC, PDE3A, PKA and PKGI [116, 117]. On the other hand, platelet activators may cross-activate inhibitory cyclic nucleotide signaling, as shown for vWF induced activation of sGC [22, 28] and for thrombin and collagen induced activation of PKA [32].

Major open questions in platelet cAMP/cGMP signaling that need to be addressed are (i) mechanisms and significance of new pathways of sGC activation, (ii) compartmentalization of cAMP/cGMP signaling and the role of AC isoforms, AKAPs and GKAPs, (iii) the specific role of PKAI and PKAII isoforms, (iv) the proposed role of cGMP in platelet activation by low concentrations of platelet agonists, (v) the identity of all PKA and PKGI substrates and the coordination of their actions and (vi) the contribution of defects in cAMP/cGMP pathways to platelet hyperreactivity in cardiovascular disease. Further studies of the cAMP/cGMP signaling network in platelets might lead to the identification of novel markers of platelet function and reactivity and possibly new therapeutic targets.

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**Table 1.**

Substrates of PKA/II and PKGI in platelets.

The PKA and PKGI columns indicate if substrates have been shown to be phosphorylated by PKA or by PKGI. Question marks indicate that no data on phosphorylation in intact platelets has been published. P-AB indicates the availability of phosphorylation site specific antibodies against the indicated sites.

Substrate	Site	PKA	PKGI	P-AB	Proposed role of phosphorylation	Ref.
G-proteins and other signaling regulators						
Rap1B	S179	√	√	-	Detachment of Rap1B from plasma membrane	[65, 66, 68]
Rap1GAP2	S7	√	√	√	Disruption of complex with 14-3-3, reduced Rap1 function, reduced cell adhesion	[72, 73]
Galpha13	T203	√	?	-	Inhibition of RhoA activity	[79, 80]
IP <sub>3</sub> -R	?	√	√	-	Inhibition of Ca <sup>2+</sup> release from intracellular stores	[82, 83]
IRAG	S664	?	√	√	Inhibition of Ca <sup>2+</sup> release from intracellular stores	[64, 84]
	S677	?	√	√		
TRPC6	?	√	√	-	Unknown	[85]
PDE5A	S92	-	√	√	Increased cGMP degradation	[48]
PDE3A	S312	√	?	√	Increased cAMP degradation	[44, 46]
GPIIb/IIIa	S166	√	?	√	Regulation of cell adhesion	[87-90]
Actin-binding proteins						
VASP	S157	√	√	√	Regulation of actin dynamics	[95-97, 103, 106]
	S239	√	√	√		
LASP	S146	√	√	-	Reduced F-actin binding	[107]
HSP27	T143	√	√	-	Reduced actin polymerization	[109]
filamin-A	S2152	√	?	-	Protection against proteolysis	[111, 112]
caldesmon	?	√	?	-	Unknown	[114]

## Figure Legends

### Figure 1.

Model of the cAMP/cGMP signaling network in platelets.

The intact endothelium releases prostacyclin ( $\text{PGI}_2$ ) and nitric oxide (NO) which bind to the prostacyclin receptor (IP-R) and the soluble guanylyl cyclase (sGC), respectively. The IP-R stimulates cAMP synthesis by adenylyl cyclase (AC) via heterotrimeric Gs protein. NO activates sGC resulting in the synthesis of cGMP. Von Willebrand Factor (vWF), thrombin and collagen are probably also able to activate sGC, although to a much lesser extent than NO. cGMP activates phosphodiesterases 2A and 5A resulting in the degradation of cAMP and cGMP, whereas cGMP inhibits PDE3A. cAMP stimulates cAMP-dependent protein kinase (PKA) which is expressed in two isoforms composed of regulatory subunits  $\text{RI}\alpha$  or  $\text{RI}\beta$  together with catalytic C subunits, or  $\text{RII}\beta$  and C subunits. C subunit of PKA has also been found in association with an  $\text{NF}\kappa\text{B}$ - $\text{I}\kappa\text{B}$  complex from which it can be released by thrombin and collagen signaling. Only the  $\text{PKGI}\beta$  isoform of cGMP-dependent protein kinase (PKG) is expressed in platelets. C subunits of PKA and  $\text{PKGI}\beta$  phosphorylate common substrates which have been grouped according to function (box, for details see main text and Table 1). Substrate phosphorylation results in an inhibition of platelet activation, granule release, adhesion and aggregation.

