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Commentary

Taming platelets with cyclic nucleotides

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Abstract

Cardiovascular diseases are often accompanied and aggravated by pathologic platelet activation. Tight regulation of platelet function is an essential prerequisite for intact vessel physiology or effective cardiovascular therapy. Physiological platelet antagonists as well as various pharmacological vasodilators inhibit platelet function by activating adenylyl and guanylyl cyclases and increasing intracellular cyclic AMP (cAMP) and cyclic GMP (cGMP) levels, respectively. Elevation of platelet cyclic nucleotides interferes with basically all known platelet activatory signaling pathways, and effectively blocks complex intracellular signaling networks, cytoskeletal rearrangements, fibrinogen receptor activation, degranulation, and expression of pro-inflammatory signaling molecules. The major target molecules of cyclic nucleotides in platelets are cyclic nucleotide-dependent protein kinases that mediate their effects through phosphorylation of specific substrates. They directly affect receptor/G-protein activation and interfere with a variety of signal transduction pathways, including the phospholipase C, protein kinase C, and mitogen-activated protein kinase pathways. Regulation of these pathways blocks several steps of cytosolic Ca^{2+} elevation and controls a multitude of cytoskeleton-associated proteins that are directly involved in organization of the platelet cytoskeleton. Due to their multiple sites of action and strong inhibitory potencies, cyclic nucleotides and their regulatory pathways are of particular interest for developing new approaches for the treatment of thrombotic and cardiovascular disorders.

Keywords: Platelets; Cyclic nucleotides; Vasodilators; Protein kinases; Phosphodiesterases

1. Introduction

Platelets, platelet–vessel wall interactions, and platelet-derived factors are essential for the regulation of vascular tone and hemostasis. Platelets are activated by a number of stimuli resulting in platelet shape change, expression and/or activation of surface receptors, secretion of vasoactive substances, adhesion, aggregation, and finally thrombus formation. Vasoconstrictors (TxA_2, vasopressin) and endothelium-dependent vasodilators (ADP, thrombin, serotonin) are potent physiologic platelet activators. The initial event in platelet activation is binding of such agonists to specific membrane-spanning G-protein-linked receptors on the platelet surface, which transmit the signal via heterotrimeric and small GTP-binding proteins and multiple protein kinases into an ordered cascade of intracellular signaling pathways. All these pathways induce remodeling of the platelet cytoskeleton resulting in the appearance of a variety of F-actin structures, including filopodia, lamellipodia, and adhesion plaques. Platelet activation is tightly regulated under physiological conditions; however, it is often impaired in cardiovascular diseases and contributes to the development and aggravation of atherosclerosis, ischemic heart disease, diabetes, and other thrombotic disorders. Physiological platelet antagonists, like the endothelium-derived vasodilators $PG\text{-}I_2$ and EDRF, inhibit platelet function by activation of ACs and GCs, and increase intracellular levels of the cyclic nucleotides cAMP and cGMP, respectively. Also, various pharmacological vasodilators, like $PG\text{-}E_1$, sodium nitroprusside, and organic nitrates, use the potency of cyclic nucleotides to inhibit platelet function.

This review will focus on the regulation of cyclic nucle-

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Abbreviations: ABP, actin binding protein; AC, adenylyl cyclase; cAMP, cyclic AMP; cAMP-PK, cAMP-dependent protein kinase; cGMP, cyclic GMP; cGMP-PK, cGMP-dependent protein kinase; DAG, 1,2-diacylglycerol; EDRF, endothelium-derived relaxing factor; GC, guanylyl cyclase; GP, glycoprotein; Hsp27, heat shock protein 27; IP_3, inositol 1,4,5-trisphosphate; IRAG, IP_3 receptor-associated cGMP-PK substrate; MAPK, mitogen-activated protein kinase; MAPKAPK-2, MAPK-activated protein kinase-2; MARCKS, myristoylated alanine-rich C kinase substrate; MLC, myosin light chain; MLCK, myosin light chain kinase; PDE, phosphodiesterase; $PG\text{-}E_1$, prostaglandin E_1; $PG\text{-}I_2$, prostacyclin; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; TxA_2, thromboxane A_2, and VASP, vasodilator-stimulated phosphoprotein.

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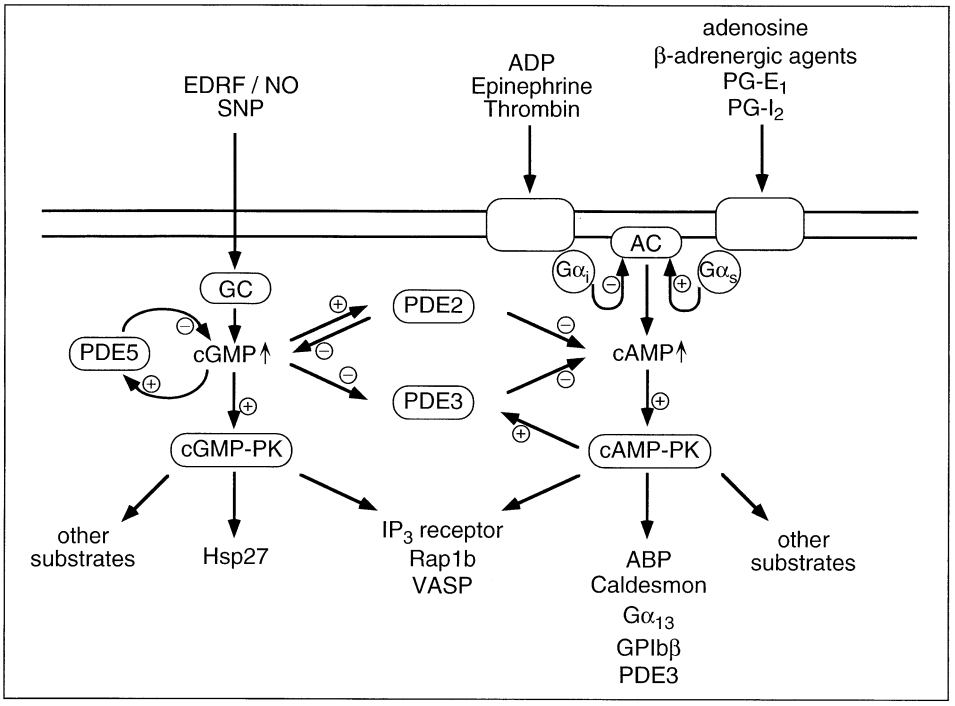

Fig. 1. Regulation and known effector sites of cyclic nucleotides in platelets.

otide levels in human platelets and the known as well as potential target sites within platelet activatory signaling pathways that are inhibited by cyclic nucleotide-regulated signaling molecules.

2. Cyclic nucleotide levels in human platelets

Elevation of intracellular cAMP and cGMP is the most potent endogenous mechanism of platelet inhibition. Cyclic nucleotide levels are up-regulated by synthesis through ACs and GCs and down-regulated by degradation through PDEs. Major target enzymes of cyclic nucleotides involve cAMP-PK and cGMP-PK, which mediate their effects through phosphorylation of specific substrate proteins.

2.1. Regulation of AC and GC activity

ACs are integral membrane glycoproteins that catalyze the synthesis of cAMP from ATP, leading to an increased level of intracellular cAMP. Platelet AC is activated by the α subunit of the stimulatory G-protein ($G\alpha_s$), and strongly inhibited by the α subunit of the pertussis toxin-sensitive inhibitory G-protein ($G\alpha_i$) (Fig. 1) (reviewed in Ref. [1]). Binding of prostaglandins ($PG\text{-}I_2$, $PG\text{-}E_1$, $PG\text{-}E_2$) to their receptor, which is coupled to G_s, therefore leads to stimulation of cAMP formation. Receptors for adenosine and β-adrenergic agents are also coupled to G_s and activate AC. Some platelet activators such as thrombin, epinephrine, or ADP induce the release of $G\alpha_i$, thereby down-regulating cAMP levels. This might contribute to the platelet-activating potency of these agonists; however, a decrease in platelet cAMP levels does not seem to be a prerequisite for platelet aggregation since in platelets with selectively activated G_q, an AC inhibitor did not induce aggregation [2]. Nevertheless, for complete platelet aggregation, activation of both G_q- and G_i-coupled receptors is necessary [3]. Inhibition of only one of these pathways effectively suppresses platelet aggregation, an effect that is utilized by the widely used anti-platelet drugs ticlopidine and clopidogrel, which act on the G_i-coupled ADP receptor ($P2Y_{AC}$, also termed $P2Y_{12}$) and abrogate the ADP-mediated inhibition of AC [4,5].

GCs exist in membrane-bound and soluble forms. To date, in platelets only the soluble form of GC, which is located in the cytoplasm, has been found, and there is no evidence that platelets also contain a membrane-bound GC. Soluble GC contains heme as a prosthetic group and is activated by EDRF (NO), nitrovasodilators, and other NO-generating agents.

2.2. PDEs

PDEs are a large group of enzymes consisting of several isozyme families that hydrolyze the 3′-phosphoester bond on cAMP and/or cGMP, converting them into biologically inactive 5′-nucleotide metabolites. Platelets contain at least three different types of PDEs (Table 1 and Fig. 1): the cGMP-stimulated PDE2, the cGMP-inhibited PDE3, and the cGMP-binding cGMP-specific PDE5 (reviewed in Ref. 6). PDE2 hydrolyzes both cAMP and cGMP, with similar

Table 1
Phosphodiesterases (PDEs) in human platelets

Isozyme family	Substrates	K_m (cAMP) (μM)	K_m (cGMP) (μM)	Inhibitors
PDE2 (cGMP-stimulated PDE)	cAMP, cGMP	50	35	EHNA
PDE3 (cGMP-inhibited PDE)	cAMP >> cGMP[a]	0.2	0.02	cGMP, milrinone, amrinone, anagrelide, cilostazol, lixazinone, NSP-513
PDE5 (cGMP-binding cGMP-specific PDE)	cGMP	150	1	dipyridamol, zaprinast, DMPPO, E4021, sildenafil

[a] Despite a lower K_m for cGMP, PDE3 preferentially hydrolyzes cAMP due to a much higher V_{max} for cAMP degradation.

affinities (K_m values of 50 and 35 μM, respectively), and is stimulated by the binding of cGMP to two allosteric regulatory sites. PDE3 has similar K_m values for cAMP and cGMP (0.2 and 0.02 μM), but due to a V_{max} that is 10-fold higher for cAMP than for cGMP, PDE3 preferentially hydrolyzes cAMP. PDE3 is inhibited by the binding of cGMP. Therefore, cGMP can potentially decrease (via PDE2) or enhance (via PDE3) a cAMP response. PDE3 activity is increased by a direct cAMP-PK-catalyzed phosphorylation [7]. This appears to be a negative feedback loop, which rapidly returns elevated cAMP levels back to basal. PDE5 is highly specific for cGMP hydrolysis (K_m of 1 μM for cGMP and 150 μM for cAMP). It contains a cyclic nucleotide-dependent protein kinase phosphorylation site and two allosteric cGMP binding sites on the regulatory domain of each subunit. Binding of cGMP to the catalytic site facilitates cGMP binding to the allosteric sites, which causes exposure of the phosphorylation site [8]. Recent *in vitro* studies with purified enzymes showed that PDE5 can be phosphorylated by cGMP-PK and by cAMP-PK, leading to increased catalytic activity of PDE5 [9]. This might provide a physiological negative feedback regulation of intracellular cGMP levels. With regard to the overall PDE activity of platelets, the cGMP PDE activity is considerably higher than the total cAMP PDE activity [6].

Inhibitors of PDEs regulate platelet cAMP and cGMP levels via decreased degradation of cyclic nucleotides. The clinical use of specific inhibitors of platelet PDEs, especially in combination with low doses of cyclic nucleotide-elevating vasodilators, could be very useful in situations where specific inhibition of platelet function is desired without side-effects on vascular smooth muscle cells. PDE3 inhibitors have been described as useful anti-platelet and anti-thrombotic tools that also inhibit vascular smooth muscle cell proliferation and intimal thickening in animal models [10,11]. The presence of high concentrations of PDE5 in platelets and vascular smooth muscle cells has led to the development of a variety of selective compounds (reviewed in Ref. 6). One of these, sildenafil (Viagra™), became world-famous for the successful treatment of erectile dysfunction. Other PDE5 inhibitors should prove useful for platelet-specific therapy since they are relatively poor vasodilators [12].

2.3. Effector sites of cAMP and cGMP

Activation of cAMP- and cGMP-dependent protein kinases is the most important mechanism of cAMP and cGMP action in human platelets, as demonstrated by studies with membrane-permeable cyclic nucleotide analogs that selectively activate either cAMP-PK or cGMP-PK and do not affect PDEs. Inhibition of platelet aggregation by these analogs correlated well with activation of cAMP-PK or cGMP-PK in intact platelets [13,14]. Furthermore, in contrast to platelets from healthy persons, cGMP-PK-deficient platelets from patients with chronic myelocytic leukemia showed no inhibition of agonist-induced Ca^{2+} mobilization from intracellular stores by 8-pCPT-cGMP, a specific activator of cGMP-PK [15]. In accordance with this effect is the observation that in cGMP-PK-deficient mice, collagen-induced platelet shape change, aggregation, and serotonin release could not be inhibited by 8-pCPT-cGMP [16].

Comparison of platelet cyclic nucleotide levels and cyclic nucleotide binding sites on the kinases revealed that in human platelets the basal cAMP concentration (4.4 μM) is close to the concentration of cAMP binding sites (6.2 μM) [17]. Therefore, a small increase in the cAMP level is sufficient for activating the majority of cAMP-PKs. In contrast, the basal cGMP concentration in platelets (0.4 μM) is less than one-tenth of the concentration of cGMP binding sites on the cGMP-PKs (14.6 μM), suggesting that even several-fold increases in intracellular cGMP levels are capable of stimulating only a small fraction of cGMP-PK. Incubation with high doses of cAMP- or cGMP-elevating platelet inhibitors increased the intracellular platelet cAMP level to 27 μM and the cGMP level to 3.8 μM, respectively [17]. In certain cell types, high levels of cellular cAMP or cGMP may cross-activate cGMP-PK or cAMP-PK, respectively. However, this effect does not seem to be present in mouse platelets [16]. Other very important targets of cGMP, but not cAMP, are PDEs, which may be stimulated or inhibited by cGMP, as discussed above. In certain cell types, ion channels are directly regulated by cAMP or cGMP without involvement of protein kinases; however, this has not been shown for platelets. In CHO cells and *in vitro*, cAMP directly activates Rap1 guanine-nucleotide-exchange factors (cAMP-GEFs) independently of cAMP-PK [18]. cAMP-GEFs contain one or more cAMP

Table 2
Known and potential platelet cAMP-PK and cGMP-PK substrates

Substrate	Molecular mass (kDa)	cAMP-PK	cGMP-PK	Proposed function of phosphorylation
Known platelet substrates				
ABP (actin binding protein)	240	Platelets		Stabilization of the resting platelet cytoskeleton [38]
Caldesmon	82	Platelets		Stabilization of the resting platelet cytoskeleton [39]
$G\alpha_{13}$ (G-protein α_{13} subunit)	44	Platelets		Inhibition of the RhoA/Rho kinase pathway [71]
GPIbβ (glycoprotein Ib β subunit)	24	Platelets		Inhibition of collagen-induced actin polymerization [40,41]
Hsp27 (heat shock protein 27)	27	*In vitro*	Platelets	Reduced stimulation of actin polymerization *in vitro* [42]
IP_3 receptor	230	Platelets	Platelets	Down-regulation of calcium release from intracellular stores [22,23]
PDE3 (phosphodiesterase 3)	110	Platelets		Enhanced cAMP degradation [7]
Rap1b	22	Platelets	Platelets	Inhibition of Rap1b activation [19,45,55,56]
VASP (vasodilator-stimulated) phosphoprotein	46/50	Platelets	Platelets	Inhibition of integrin $\alpha_{IIb}\beta_3$ activation [30,33,35]
Potential platelet substrates[a]				
IRAG (IP_3 receptor-associated cGMP-PK substrate)	125		Smoth muscle cells	Down-regulation of calcium release from intracellular stores [24]
MLCK (myosin light chain kinase)	105	*In vitro*	*In vitro*	Reduction of MLC phosphorylation [43,44]
PDE5 (phosphodiesterase 5)	92	*In vitro*	*In vitro*	Enhanced cGMP degradation [9]
RhoA	22	Leukocytes	*In vitro*, smooth muscle cells	Reduction of MLC phosphorylation [51–53]
TxA_2 receptor	50	HEK 293 cells	*In vitro*, HEL cells	Inhibition of TxA_2 receptor-associated G protein activation [72,73]

[a] Phosphorylation demonstrated *in vitro* and/or with other cell types.

binding sites as well as a GEF domain. Only after binding of cAMP are these GEFs able to activate Rap1 by promoting the release of GDP and the binding of GTP to Rap1. cAMP-GEFs have been detected in many tissues; however, their presence in platelets has not been reported. In platelets, Rap1 is strongly inhibited during incubation with PG-I_2, an effect that occurs independently of the phosphorylation of Rap1 by cAMP-PK [19].

3. Cyclic nucleotide-dependent protein kinases, their substrates and functional roles in platelets

Cyclic nucleotide-dependent protein kinases are the major effector molecules mediating physiological effects initiated by cyclic nucleotide formation. Compared with other tissues and cell types, human platelets contain particularly high concentrations of both cAMP-PK and cGMP-PK [17], pointing to the functional importance of protein phosphorylation in response to cyclic nucleotide-elevating platelet inhibitors. cAMP-PK types I and II β and cGMP-PK type I β represent the major forms of cyclic-nucleotide-dependent protein kinases in human platelets. Activation of these kinases in intact platelets leads to phosphorylation of a number of proteins. Some of these are targets for both cAMP-PK and cGMP-PK; others may be differentially regulated by the cAMP and cGMP pathways as summarized in Table 2. The following chapters describe the various steps in platelet activation cascades that are inhibited by cyclic nucleotide-regulated pathways.

3.1. Inhibition of intracellular Ca^{2+} elevation

Platelet activation by all stimulatory agonists involves the elevation of cytosolic Ca^{2+} levels. The increase of intracellular free Ca^{2+} plays a key role during platelet activation, since it regulates multiple Ca^{2+}-dependent enzymes, like the Ca^{2+}-dependent PKC isoforms, the Ca^{2+}/calmodulin-dependent MLCK, cytosolic phospholipase A_2 ($cPLA_2$), and the small GTPase Rap1b. Most agonists activate PLC and elevate cytosolic Ca^{2+} by an IP_3-dependent release of Ca^{2+} from intracellular stores, as well as stimulation of the entry of extracellular Ca^{2+}. Furthermore, ADP activates a P2X ADP receptor-operated cation channel, which leads to an immediate influx of extracellular Ca^{2+}. Increased levels of cAMP and/or cGMP antagonize the activator-evoked Ca^{2+} release from intracellular stores and the secondary store-mediated Ca^{2+} influx, but not the ADP receptor-operated cation channel [20]. This effect is probably mediated by several mechanisms. One effect observed after an increase of intracellular cAMP or cGMP is inhibition of PLC activation; however, direct inhibition of PLC by cAMP-PK or cGMP-PK has not been reported. PLC catalyzes the conversion of PIP_2 into IP_3 and DAG. It is likely that cAMP and cGMP inhibit the PLC reaction by reduction of substrate (PIP_2) resynthesis [21]. Additional inhibitory steps of intracellular Ca^{2+} release occur downstream of PLC. IP_3 receptors, which mediate the release of Ca^{2+} from the dense tubular system and probably also the secondary store-related influx, are directly phosphorylated by cAMP-PK and cGMP-PK in human platelets [22,23]. However, the role of IP_3 receptor phosphorylation for inhibition

of intracellular calcium elevation is not clear. In smooth muscle cells, IP_3-mediated Ca^{2+} release is regulated by phosphorylation of an additional IP_3 receptor-associated protein (IRAG) by cGMP-PK, whereas phosphorylation of the IP_3 receptor alone was not sufficient to inhibit Ca^{2+} release [24]. Whether IRAG phosphorylation by cyclic nucleotide-dependent protein kinases also plays a role in the down-regulation of agonist-induced Ca^{2+} elevation in platelets needs to be investigated.

3.2. Inhibition of cytoskeletal reorganization and integrin activation

Binding of soluble fibrinogen to its receptor, integrin $\alpha_{IIb}\beta_3$, is a prerequisite for platelet aggregation and is dependent upon the activation state of the integrin. In resting platelets, the fibrinogen receptor is in an inactive state. Agonist-generated inside-out signals are required to induce the conformational change and clustering that enable integrin $\alpha_{IIb}\beta_3$ to bind soluble fibrinogen. These integrin-activating effects are mediated by actin filament turnover, rearrangement of the platelet cytoskeleton, and integrin regulatory proteins [25,26], some of the latter proteins having potential phosphorylation sites for cyclic nucleotides. For example β_3-endonexin, a well characterized integrin binding protein, which binds specifically to the β_3 subunit of the fibrinogen receptor and induces fibrinogen binding, has potential phosphorylation sites for cAMP-PK [27,28]. However, the exact molecular mechanisms of integrin activation are still mostly unclear. One of the major substrates of cAMP-PK and cGMP-PK is the cytoskeleton-associated VASP that was found in various cell types to be located in focal adhesions, stress fibers, cell–cell contacts, and highly dynamic membrane regions (reviewed in Ref. 29). In platelets, it is present in particularly high concentrations [17]. VASP contains three phosphorylation sites—Ser157, Ser239, and Thr278—that are phosphorylated with different affinities by both kinases [30,31]. Depending upon its phosphorylation status and the system analyzed (purified proteins, listeria, or mammalian cells), VASP is able to affect and regulate actin polymerization and actin filament bundling [29]. *In vitro* experiments with cAMP-PK-phosphorylated VASP diminished VASP binding to F-actin, and suppressed actin polymerization and actin filament bundling [32]. These observations suggest that VASP phosphorylation down-regulates its enhancing functions on actin polymerization; however, the precise molecular mechanism of VASP action in platelets still needs to be characterized. In platelets, phosphorylation at Ser157, which causes a mobility shift in SDS-polyacrylamide gels changing the apparent molecular mass of VASP from 46 to 50 kDa, has been shown to closely correlate with inhibition of the fibrinogen receptor (integrin $\alpha_{IIb}\beta_3$) [33]. In accordance with this observation, inhibition of platelet aggregation by low doses of cyclic nucleotides was impaired in VASP knockout mice [34,35]. Moreover, platelets from VASP-deficient mice also showed reduced aggregation time in response to collagen, increased thrombin-induced activation of integrin $\alpha_{IIb}\beta_{3,}$ and enhanced surface expression of P-selectin [34,35]. These observations indicate that VASP phosphorylation is required to mediate at least part of the inhibitory effects of cyclic nucleotides on integrin $\alpha_{IIb}\beta_3$ activation. Because of the close correlation with fibrinogen receptor inhibition, VASP phosphorylation may serve as a marker for platelet inhibition. The development of a phosphorylation-specific antibody [31] and its use for flow cytometry analysis of platelet VASP phosphorylation provide an excellent diagnostic tool for fast and easy *in vitro* as well as *in vivo* analysis of platelet function and crosstalk between different receptor signaling pathways [36]. However, the role of VASP in cytoskeletal reorganization and integrin activation is not completely understood. In human endothelial cells, VASP phosphorylation disrupted its binding to focal adhesions and induced a progressive reduction of the actin microfilament system, whereas cells transfected with a VASP mutant, where all three known phosphorylation sites were changed into alanine, were resistant to these effects [37].

However, VASP phosphorylation may be only one of the effects of cAMP-PK and cGMP-PK on the platelet cytoskeletal system. Other known substrate proteins that might be involved in microfilament regulation are ABP [38], caldesmon [39], glycoprotein Ibβ (GPIbβ) [40,41], Hsp27 [42], MLCK [43,44], and the small GTPase Rap1b [19,45].

ABP stabilizes actin filaments, and undergoes proteolytic cleavage by calpain during platelet activation, leading to irreversible loss of its ability to cross-link actin filaments into networks. ABP hydrolysis during platelet activation might therefore provide a chance for reorganization of the cytoskeleton. Phosphorylation of ABP by cAMP-PK in human platelets stabilizes the protein against proteolysis, thereby stabilizing the actin filament network and making it resistant to cytoskeletal reorganization [38]. In accordance with this model is the observation that actin filament stabilization in intact platelets leads to inhibition of ADP-induced fibrinogen binding [25].

The platelet protein caldesmon binds actin and myosin simultaneously, forming an actin–caldesmon–myosin complex, and thereby promotes translocation of actin filaments [46]. Caldesmon is phosphorylated by cAMP-PK, and its phosphorylated state may stabilize the cytoskeleton and inhibit platelet activation [39].

GPIbβ is a subunit of the GPIb-V-IX complex, which is the platelet receptor for von Willebrand factor and mediates adhesion to the subendothelium of damaged vessel walls. This complex is also a receptor for thrombin and is linked to underlying actin filaments by ABP [47]. PG-E_1-induced phosphorylation of GPIbβ led to inhibition of collagen-induced actin polymerization, an effect that was absent in platelets from patients with Bernard-Soulier syndrome, which lack the GPIb-V-IX complex [40]. Therefore, cAMP-PK-mediated GPIbβ phosphorylation may also participate in inhibition of the agonist-induced cytoskeletal reorganiza-

tion. Another protein involved in the regulation of actin polymerization is Hsp27. Phosphorylation of this protein by the p38 MAPK-activated MAPKAPK-2 leads to stimulation of actin polymerization. In human platelets, Hsp27 is additionally phosphorylated by cGMP-PK and probably also by cAMP-PK including one site that is different from the MAPKAPK-2 phosphorylation sites [42]. *In vitro* experiments showed that cGMP-PK-phosphorylated Hsp27 reduced the MAPKAPK-2-induced stimulation of actin polymerization [42]. Inhibition of p38 MAP kinase by cyclic nucleotide-regulated pathways will be discussed below.

Another effect of cyclic nucleotides in platelets is reduction of MLC phosphorylation. MLC phosphorylation has been implicated in the regulation of cytoskeletal reorganization during platelet shape change [48]. It promotes myosin filament formation and contractility, resulting in actin stress fiber formation and clustering of integrins into focal adhesions (reviewed in Ref. 49). The Ca^{2+}/calmodulin-dependent MLCK is directly phosphorylated by cAMP-PK and cGMP-PK *in vitro* [43,44], leading to a decreased affinity of this enzyme for calmodulin and thereby decreased MLC phosphorylation. However, additional mechanisms of inhibition of MLC phosphorylation are possible. The involvement of cyclic nucleotide-dependent protein kinases in the regulation of myosin phosphatase has to be considered. In vascular smooth muscle cells, direct interaction of cGMP-PK with myosin phosphatase was essential for MLC dephosphorylation [50]. Another possible mechanism for the regulation of MLC phosphorylation is through phosphorylation of the GTPase RhoA. RhoA stimulates Rho-kinase, which in turn phosphorylates myosin phosphatase, decreasing its activity, thereby leading to increased MLC phosphorylation [49]. In leukocytes, RhoA was phosphorylated by cAMP-PK, resulting in down-regulation of RhoA activity [51,52]. In smooth muscle cells, RhoA was phosphorylated by cGMP-PK, leading to inhibition of RhoA-induced Ca^{2+} sensitization of contraction and actin cytoskeleton organization [53]. In platelets, these mechanisms of regulating MLC phosphorylation may also play a role in cyclic nucleotide-mediated inhibition of cytoskeletal rearrangement.

Rap1b, a small GTPase of the Ras family, is highly expressed in human platelets. The functional role of Rap1 is not yet understood, but it may be involved in cell adhesion, since in leukocytes, activation of Rap1 induced cell adhesion, while its inactivation inhibited cell adhesion. In platelets, Rap1b is subjected to a complex sequential regulation [54]. During platelet activation, Rap1b is activated within seconds by several different mechanisms, one that is PKC-independent and Ca^{2+}-mediated (initial activation) and another that is PKC-dependent (second wave of activation). Rap1b activation is sustained via an integrin $\alpha_{IIb}\beta_3$-dependent mechanism. However, platelet aggregation also induces the inactivation of Rap1b, which correlates with translocation of Rap1b from the plasma membrane to the cytoskeleton. Treatment of platelets with PG-I_2 or NO led to Rap1b phosphorylation and inhibition of agonist-induced Rap1b activation [45,55,56]. However, it is likely that the inhibition of Rap1b activation is mediated by mechanisms other than phosphorylation, e.g. by inhibition of intracellular Ca^{2+} elevation, which is both necessary and sufficient for Rap1b activation [19].

3.3. Inhibition of granule secretion and surface molecule expression

Incubation of human platelets with vasodilators affects dense granule, α-granule, and lysosomal secretion, thereby inhibiting the release of pro-coagulatory factors into the blood, and translocation of adhesive glycoproteins, like integrin $\alpha_{IIb}\beta_3$, P-selectin (CD62P), and CD40 ligand (CD40L), from intracellular stores to the platelet surface membrane [57,58]. The pro-inflammatory molecules P-selectin and CD40L, expressed on activated platelets, interact with leukocytes and endothelial cells, inducing cytokine and chemokine secretion, as well as up-regulation of endothelial adhesion receptors [59,60]. Thus, cyclic nucleotide-elevating platelet antagonists have the potential to inhibit inflammatory responses initiated by platelet P-selectin and CD40L at sites of injured vessel walls. A detailed picture of the molecular mechanisms underlying granule secretion is currently not available. It was shown that both increased cytosolic Ca^{2+} and PKC activation are essential for granule secretion [61]. By inhibiting the PLC reaction, cAMP-PK and cGMP-PK down-regulate PKC activation not only via decreased Ca^{2+} levels but also via reduced DAG production, which, besides Ca^{2+}, is also necessary for PKC activation. The major target of PKC in platelets is the 47-kDa protein pleckstrin (*p*latelet and *le*ukocyte *C k*inase subs*tr*ate). As PKC inhibition leads to decreased pleckstrin phosphorylation and inhibition of serotonin release, an involvement of pleckstrin in platelet secretion has been proposed [62]. In addition to pleckstrin, MARCKS is a platelet PKC substrate, and the phosphorylation of MARCKS was shown to be essential for dense granule secretion [63]. MARCKS can bind actin and cross-link actin filaments, a property that is inhibited by phosphorylation through PKC [64]. This suggests a model in which preventing the phosphorylation of MARCKS would lead to actin filament stabilization and inhibition of granule trafficking to the platelet membrane. Thus, inhibition of platelet secretion by cAMP and cGMP might be mediated by down-regulation of PLC activation and subsequent intracellular Ca^{2+} elevation and the PKC pathway. However, platelet secretion is a very complex process, and it is likely that cAMP-PK and cGMP-PK interfere also at other steps of the secretory process, especially since cytoskeletal changes are involved.

3.4. Inhibition of p38 MAPK and p42 MAPK pathways

MAPKs are expressed in all eukaryotic cells as an essential part of complex signaling networks, leading to cell

proliferation or differentiation. In platelets, both p38 MAPK and p42 MAPK are rapidly activated in response to a variety of agonists [58]; however, little is known about their substrates and functions in these cells. p38 MAPK directly phosphorylates the Ca^{2+}-dependent $cPLA_2$ [65], thereby contributing to $cPLA_2$ activation and subsequent arachidonic acid release. Another substrate of p38 MAPK is MAPKAPK-2, which phosphorylates the small heat shock protein Hsp27, which is involved in the regulation of actin polymerization [42,66]. p42 MAPK phosphorylates ribosomal S6 kinase ($p90^{rsk}$) [67]; however, its function is unclear in platelets. Agonist-induced p38 MAPK and p42 MAPK activation in human platelets is inhibited by both cAMP- or cGMP-elevating agents [58]. The down-regulation of these two enzymes probably occurs at some point upstream in the respective activation cascade. Since p42 MAPK activation seems to be PKC-dependent [68], this MAPK may be inhibited by down-regulation of PLC and subsequent PKC activity through cAMP- and cGMP-PK, as discussed above. The signaling events leading to p38 MAPK activation in platelets have not been intensively investigated; however, p38 MAPK activation was reported to be independent of PKC activation [69]. Thus, another target of cAMP-PK and cGMP-PK seems to be involved in the inhibition of this pathway.

3.5. Inhibition of the thromboxane receptor and G-proteins

The platelet thromboxane receptor has seven transmembrane domains that are coupled to the heterotrimeric GTP-binding proteins (G-proteins) G_q, G_{12}, and G_{13}. These G-proteins link the receptor to intracellular signaling pathways. $G\alpha_q$, which is also associated with the receptors for thrombin, ADP (P2Y1), and serotonin, activates PLC and is necessary for agonist-induced aggregation (reviewed in Ref. 1). G_{12} and/or G_{13} are suggested to be important mediators of platelet shape change [70]. They are probably linked to the RhoA/Rho-kinase pathway leading to inhibition of myosin phosphatase and increased MLC phosphorylation [1,70]. In human platelets, cAMP-PK phosphorylates the $G\alpha_{13}$ subunit associated with the thromboxane receptor [71]. Assuming a link between G_{13} and the RhoA/Rho-kinase pathway, this provides an additional mechanism of inhibiting MLC phosphorylation, as discussed above for inhibition of cytoskeletal reorganization. Recently, it was shown that the thromboxane receptor itself is a substrate of cAMP-PK and cGMP-PK in HEK293 cells, HEL cells, or *in vitro*, and that phosphorylation of its cytoplasmic carboxy-terminal domain prevents the thromboxane receptor from coupling to and activating G-proteins [72,73]. For intact platelets, TxA_2 receptor phosphorylation has not been shown yet; however, it would provide another explanation for the observed inhibition of PLC activation and subsequent intracellular Ca^{2+} elevation and granule secretion in response to cyclic nucleotides, as discussed above.

4. Conclusion

Inhibition of platelet function by many clinically used platelet antagonists blocks only certain functions of platelet activation, leaving others untouched. For example, selective blockade of fibrinogen binding to activated platelets will not prevent expression of pro-inflammatory molecules. The increase of intracellular cyclic nucleotide levels leads to broad inhibition of platelet functions, including adhesion, degranulation, aggregation, and even down-regulation of pro-inflammatory platelet surface molecules, an effect very important for prevention of the development of atherosclerotic lesions. The inhibitory actions induced by cyclic nucleotide-elevating substances are mainly mediated by cyclic-nucleotide dependent protein kinases and interfere at multiple sites of the platelet activation signaling cascades, explaining the high potency of these agents to inhibit platelet activation. Inhibition occurs on the receptor/G-protein level, and includes a variety of signal transduction cascades, like the PLC, PKC, and MAPK pathways. It affects several steps of cytosolic Ca^{2+} elevation as well as a multitude of cytoskeleton-associated proteins (like VASP, ABP, and caldesmon) that are involved in cytoskeletal reorganization. Further clarification of the complex relationships between cyclic nucleotides and the platelet activatory signaling pathways will certainly lead to future diagnostic and therapeutic implications for hemostasis and cardiovascular diseases.

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