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Review

# Protein kinase C-theta in platelet activation

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ABSTRACT

**Members of the protein kinase C (PKC) family of serine/threonine kinases have been implicated in several physiological processes regulating the activation response of platelets. They are involved in processes leading to granule secretion, integrin activation, platelet aggregation and spreading, and procoagulation. The protein kinase C θ (PKCθ) isoform, which was originally identified in T lymphocytes, is also expressed at relatively high levels in platelets, wherein it is involved in the regulation of hemostasis and thrombosis. Recent studies suggest a role for PKCθ in protease-activated receptor (PAR)-, glycoprotein VI (GPVI) receptor- and glycoprotein $\alpha_{IIb}\beta_3$ integrin receptor-linked signal transduction pathways. The present review focuses on the latest observations relevant to the role of PKCθ in platelet activation.**



## 1. Introduction

Platelets, also termed thrombocytes, are small, anucleated cells, which are formed in the bone marrow by pinching off cytoplasmic fragments of megakaryocytes. Their average lifespan is 5–9 days, they circulate in the blood, and are critical for hemostasis and clot formation in response to vascular injury. They are also involved in restenosis and inflammatory reactions [1–3]. Upon activation, platelets secrete a multitude of soluble effector molecules that stimulate the deposition of extracellular matrix and promote healing of damaged tissue [1–3]. A decreased number or reduced activity of platelets may result in prolonged bleeding times and increased blood loss following injury, while an increased number or excessive activity of platelets can lead to intravascular clot formation, circulating emboli and occlusion of blood vessels [1–3].

Platelet interaction with soluble blood constituents or vascular wall components is mediated by specific surface receptors, which promote adhesion to adjacent platelets, leukocytes, endothelial cells and extracellular matrices. This can result in platelet activation and amplification of thrombo-inflammatory reactions [4]. Adherence of platelets to injured vasculature induces granule secretion, synthesis of thromboxane A2 ($TxA_2$), extension of filopodia and lamellipodia and activation of surface adhesion molecules [4]. Many of these functions are regulated, at least in part, by members of the protein kinase C (PKC) family [5]. In this review, we will discuss some of the recent findings concerning the isoform-specific roles of PKCθ in platelets, focusing on results obtained using platelets of PKC-deficient mice.

## 2. Platelet activation

Platelet activation plays an important role in hemostasis and thrombus formation. Under normal physiological conditions, platelets circulate in the blood as resting cells, where they continuously encounter inhibitory signals provided by endothelial cells that line the inner surface of the blood vessels [6]. Vascular endothelial cells produce nitric oxide that inhibits platelet activation, and soluble ADPase, which degrades the platelet activator, ADP. Endothelial cells also produce the eicosanoid prostacyclin ($PGI_2$) that can associate with the Gs protein-coupled prostacyclin receptor on the surface of platelets. $PGI_2$ binding to its cognate receptor, signals

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adenylyl cyclase to produce cAMP that counteract the effects of the platelet activator, $TXA_2$, thereby inhibiting platelet activation [1,2].

Vascular endothelial cells adhere to the blood vessel basement membrane by physical binding to the collagen. They also associate with the von Willebrand factor (vWF) that functions as a cell adhesion ligand and increases the binding affinity of the endothelial cells to the extracellular matrix [7,8]. Under physiological conditions, the collagen is not exposed to the bloodstream. However, a trauma to the vascular endothelial layer exposes collagen and vWF from the subendothelium to the bloodstream, making them accessible to platelets that respond by a rapid turn-on of several biochemical cascades [7,8]. For example, binding of the platelet receptor glycoprotein VI (GPVI) to collagen initiates a signaling cascade leading to platelet activation, which is characterized by the release of granule-stored mediators and synthesis of thrombin and $TXA_2$ [9]. Secreted mediators, such as adenosine diphosphate (ADP), serotonin, platelet-activating factor (PAF), vWF, and $TXA_2$ activate additional platelets and further amplify the intensity of the entire response. In addition, stimulation of platelets results in the expression of proteins that enable them to adhere to specific receptors on the surface of leukocytes and endothelial cells [9]. This mechanism is mediated by proteins such as P selectins (P stands for platelet; also termed CD62P), which are normally stored in α-granules within the cytoplasm of resting platelets, but move to the platelet's outer surface upon their activation. They can then interact with P-selectin glycoprotein ligand-1 (PSGL1; also termed Selectin P ligand (SELPLG) or CD162) and other ligands on the surface of the leukocytes or endothelial cells [10].

Molecules that belong to a different type of a receptor family, termed PARs (protease activated receptors) are G-protein coupled receptors that undergo activation by the action of serine proteases that cleave an extracellular portion of the receptor. The cleaved N-terminal peptide acts as a tethered ligand or agonist, which activates the PAR receptor and initializes a physiological response.

Both GPVI and PARs receptors can stimulate phospholipase C (PLC), leading to the generation of second messengers, which activate PKC and stimulate the release of calcium ions from intracellular stores, respectively [11]. Activation of this signaling pathway is an early step in biochemical processes regulating many of the functional responses of platelets.

Platelet activation also results in scramblase-mediated transport of negatively charged phospholipids to the platelet surface. These phospholipids provide a catalytic surface (with the charge provided by phosphatidylserine and phosphatidylethanolamine) for the tenase complex (formed by the activated forms of the blood coagulation factor VIII and factor I).

Platelet aggregation involves an additional type of receptor that specifically associates with fibrinogen, the glycoprotein $\alpha_{IIb}\beta_3$ integrin receptor. Fibrinogen binding to platelet $\alpha_{IIb}\beta_3$ triggers 'outside-in' signals that promote actin polymerization and cell spreading [12]. In contrast, $\alpha_{IIb}\beta_3$ inhibitors prevent platelet aggregation and thrombus formation and are therefore useful for treating patients with acute coronary syndromes.

Platelets contain two types of morphological distinct storage granules, the α-granules and the dense granules. Release of soluble mediators from the α-granules amplify the coagulation cascade at the site of the vascular injury and increase the platelets' procoagulation activity. Release of soluble mediators from the dense granules, predominantly the ADP, promotes the recruitment of additional platelets to the site of injury.

## 3. Protein kinase C (PKC)

PKC is a ubiquitous family of structurally related serine/threonine kinases that are part of the AGC-type kinase (PKC/PKG/PKC) superfamily [13]. The PKC family consists of at least 10 distinct isoforms that are grouped into three classes: the classical (cPKC), novel (nPKC) and atypical (aPKC) enzymes. Members of the cPKC (α, βI, βII, and γ) utilize both $Ca^{2+}$ ions and diacylglycerol (DAG) as cofactors for activation, the nPKC (δ, ε, η, and θ) utilize DAG, but are $Ca^{2+}$-independent, and the aPKC (ζ and ι/λ) that are active independent of $Ca^{2+}$ or DAG. Different PKC isoforms exhibit some overlapping functions as well as distinct, non-redundant functions, which depending on the experimental system used, can exhibit specific biochemical properties, expression profiles, and physiological functions. They can differ also in subcellular localization, and although the majority of PKC enzymes reside in the cytoplasm of resting cells, they translocate to the plasma membrane [14,15], nuclear membrane [16], or other subcellular compartments [17–19], following stimulation of cells with a large variety of physiological agonists. The redistribution of distinct PKC isoforms to specific subcellular compartments is critical for the induction of PKC catalytic activity, since the specific location determines the enzyme's ability to interact with selected cofactors and has access to specific substrates. The differential subcellular distribution of PKC is, at least partially, regulated by PKC-binding proteins, such as RACKs (receptor for activated C kinase) [20], scaffold proteins, including AKAP (a kinase-anchoring protein) [21], and cytoskeletal elements, including actin [18,22]. PKC enzymes play a major role in the signaling networks that translate environmental signals into cellular behavior. By phosphorylating specific substrates and altering their conformation and/or biological activity PKC can regulate multiple cellular functions.

## 4. PKCθ

The human PKCθ gene (termed *PRKCQ*) was first discovered in 1993, in a search for new PKC isoforms that are potentially involved in the regulation of T cell responses [23]. Relatively high expression levels of PKCθ were found in T lymphocytes and lymphoid organs, skeletal muscle, lung and kidney [23–26]. Among the hematopoietic cell lineages tested, high levels of PKCθ were observed in T but not B lymphocytes and in representatives of the megakaryoid cell lineage, including megakaryocytes and platelets [24,27,28].

PKCθ is unique among the PKC isoforms in its ability to translocate from the cytosol to the center of the immunological synapse of activated T cells where it colocalizes with the TCR [29,30]. It is also involved in cytoskeletal remodeling and contributes to the formation of the rearranged synapse [31]. Translocation of PKCθ was found to be regulated by the Lck protein tyrosine kinase and correlated with the catalytic activation of PKCθ by inducible cofactors that are produced along the PI3K- and Vav-dependent pathway [32].

Inherited deficiency of PKCθ in mice has no effect on thymocyte development but it impairs TCR-induced activation response in mature T cells [33]. As a result, proliferative responses of antigen-triggered PKCθ-deficient mature T cells are inhibited, predominantly because of reduced production of IL-2 and decreased expression of CD25, the high affinity subunit of the IL-2 receptor [33]. It is suggested therefore that PKCθ plays a critical role in transduction of essential signals downstream of activated T cell antigen receptors [34]. This suggestion is in agreement with the fact that TCR-stimulated PKCθ-deficient mature T cells fail to respond by upregulation of the AP-1, NF-AT and NF-kB transcription factors [33,35]. These effects may possibly account for the defect in IL-2 production, since the *IL-2* gene promoter possesses critical binding sites for AP-1, NF-kB and NF-AT transcription factors, in addition to the Oct-1 [36].

More recent studies with PKCθ-deficient mice demonstrated the need for PKCθ during the differentiation of T cells into specific cell lineages. For example, PKCθ was found to be essential for the

induction of efficient Th2-mediated responses against the helminth *Nippostrongylus brasiliensis,* or the parasite *Leishmania major*, and during allergic lung inflammation induced by an inhaled allergen [37–39]. Th1-mediated responses in these three models were almost unaffected [37,38]. Furthermore, studies in primary PKCθ$^{-/-}$ CD8$^{+}$ T cells activated by peptide-MHC complexes revealed severe defects in ERK and JNK (but not p38) activation [40], suggesting the involvement of PKCθ in the induction of optimal Tc-mediated immune responses.

Whereas the in vitro and in vivo responses of PKCθ-deficient T cells have been thoroughly investigated, understanding the role of PKCθ in platelets functions is only at its early phase.

## 5. PKC in platelet activation

Initial studies suggesting a role of PKC in platelet activation were based on the utilization of broad-spectrum PKC inhibitors that suppressed or abolished a wide range of platelet functions, including granule secretion and aggregation [41–43]. Likewise, PKC activators were found to enhance platelet responses [44–46].

These studies were supported by PKC isoform expression analyses showing that both human and mouse platelets express relatively high levels of the PKCα, βI, βII, δ, and θ isoforms, and low or undetectable levels of PKCη, ε, and ζ [28,47–51].

The development of inhibitors with preferential activity towards specific PKC isoforms has led to the understanding that different receptor-linked signaling pathways can utilize distinct PKC isoforms, which in turn, can phosphorylate and regulate the activity of different effector molecules. However, in vivo studies with such inhibitors were not satisfactory due to the partial cross-reactivity of these drugs, and their partial inhibition of other PKC isoforms.

Genetic models in which the expression of a single PKC gene is modified represented an alternative approach. However, platelets are anucleated cells and therefore not amenable to genetic manipulation by gene overexpression or silencing. As a result, the utilization of platelets from genetically modified mice that lack one or more PKC isoform is the preferred approach for the study of PKC isoform-specific functions.

Accumulated information based on the utilization of platelets from PKC 'KO mice', in addition to the PKC isoform selective inhibitors, suggests that PKC enzymes contribute to the regulation of an array of platelet functions, including $Ca^{2+}$ entry [52,53], 'outside-in' signaling [54,55], exocytosis and secretion of storage granules [47,49,56], $\alpha_{IIb}\beta_3$ integrin activation [55], actin-mediated filopodia formation [57] and platelet adherence to extracellular matrix or cells [58]. This information and additional data indicated that PKC plays a role in multiple biochemical processes leading to platelet aggregation, coagulation and thrombus formation.

Platelet agonists interact with several different surface receptors, which are linked to unique signaling pathways, and some of these pathways converge on the activation of PKC. Furthermore, many receptors that regulate platelet activation are G-protein-coupled receptors. Upon agonist stimulation, they activate PLC, which catalyzes the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate ($PIP_2$) into two types of second messengers, inositol 1,4,5-trisphosphtae ($IP_3$) and 1,2-diacylglycerol (DAG). The hydrophobic DAG remains within the membrane environment where it serves as a cofactor for activation of different cPKC and nPKC isoforms. The hydrophilic $IP_3$ binds to and opens ligand gated ion channels, which allows the release of $Ca^{2+}$ ions into the cytosol where they activate multiple $Ca^{2+}$-dependent enzymes, including cPKC [59,60]. Among other functions, PKC and $Ca^{2+}$ ions synergize in the induction of dense and α-granule secretion in platelets stimulated with agonists, such as thrombin, $TXA_2$, and ADP [61].

## 6. PKCθ in platelet activation

PKCθ is involved in signal transduction downstream of several different receptors, and some of its more important functions during the platelet activation response are schematically presented in Fig. 1. A more detailed list of potential contributions of PKCθ to different platelet functions is presented in Table 1.

### 6.1. The $\alpha_{IIb}\beta_3$ integrin receptor and PKCθ-regulated 'outside-in' and 'inside-out' signaling

The integrin cell adhesion molecule is regulated by both extracellular and intracellular mechanisms that are essential for platelet functions, including cytoskeletal organization, motility, aggregation, and anchorage-dependent cellular responses [62,63]. Their involvement in platelet aggregation is regulated by an 'inside-out' signals that promote conformational changes in the $\alpha_{IIb}\beta_3$ integrin receptor, thereby boost its affinity for cognate ligands, such as fibrinogen. Tethering of fibrinogen to $\alpha_{IIb}\beta_3$ receptor bridges platelets and promotes their activation and adhesion to sites of vascular damage. In turn, ligand binding to the extracellular portion of the $\alpha_{IIb}\beta_3$ receptor triggers 'outside-in' signals that promote actin polymerization and additional cytoskeletal changes necessary to insure efficient platelet aggregation and spreading [12,64,65].

Fibrinogen is a soluble plasma glycoprotein, which functions as a major blood-clotting component. It is synthesized in the liver and converted by thrombin into fibrin during blood coagulation [66]. While monomers of fibrin polymerize into fibers that further associate in forming a fibrin gel, fibrinogen molecules can directly associate with platelets $\alpha_{IIb}\beta_3$ surface receptors, cross-link the receptor by bridging adjacent platelets, and deliver activation signals [67].

Fibrinogen binding to platelets triggers 'outside-in' signals that promote actin polymerization, cytoskeletal rearrangements and cell spreading [12]. The signaling pathway downstream of the $\alpha_{IIb}\beta_3$ receptor (also termed glycoprotein IIb/IIIa, or gpIIb/IIIa) is known to involve PKC, since PKC activators such as phorbol myristate acetate (PMA) activate the platelets $\alpha_{IIb}\beta_3$ receptor and promote platelet spreading [12]. In addition, PKCβ was found to be essential for platelet spreading on fibrinogen but not for ADP- or thrombin-induced activation of $\alpha_{IIb}\beta_3$ [55].

A recent study that analyzed the role of PKCθ in $\alpha_{IIb}\beta_3$-linked signaling pathways revealed that PKCθ-deficient platelets are impaired in their ability to spread on fibrinogen-coated coverslips, suggesting a positive role for PKCθ in 'outside-in' signal transduction downstream of the $\alpha_{IIb}\beta_3$ receptor [54]. These results were further substantiated by Nagy et al. [68], who also utilized platelets from PKCθ$^{-/-}$ and PKCθ$^{+/+}$ mice. PKCθ was found to physically and constitutively associate with the $\alpha_{IIb}\beta_3$ receptor, both in human and mouse platelets. Stimulation of the $\alpha_{IIb}\beta_3$ receptor with fibrinogen promoted the association of PKCθ also with the Btk and Syk PTKs, and promoted tyrosine phosphorylation of PKCθ, Btk and the Wiskott–Aldrich syndrome protein (WASP). A potential functional linkage between PKCθ and WASP/WASP-interacting protein (WIP) was demonstrated in activated T cells in which PKCθ-dependent phosphorylation of WIP disengaged WASP from the WIP-WASP complex, thereby releasing WASP from inhibition by WIP [31]. The PKCθ phosphorylation motif includes the $Ser^{488}$ at the C-terminal end of the WASP binding region of WIP. The resulting uncoupled WASP could then activate Cdc42 that in turn activates the Arp2/3 complex and rearrange F-actin polymerization [69]. The above findings and additional data [70–73] suggest that PKCθ relay signals from the activated $\alpha_{IIb}\beta_3$ receptor by regulating the uncoupling of WASP and WIP, thereby permitting the regulation of actin filament nucleation and branching activity of the Arp2/3 complex.

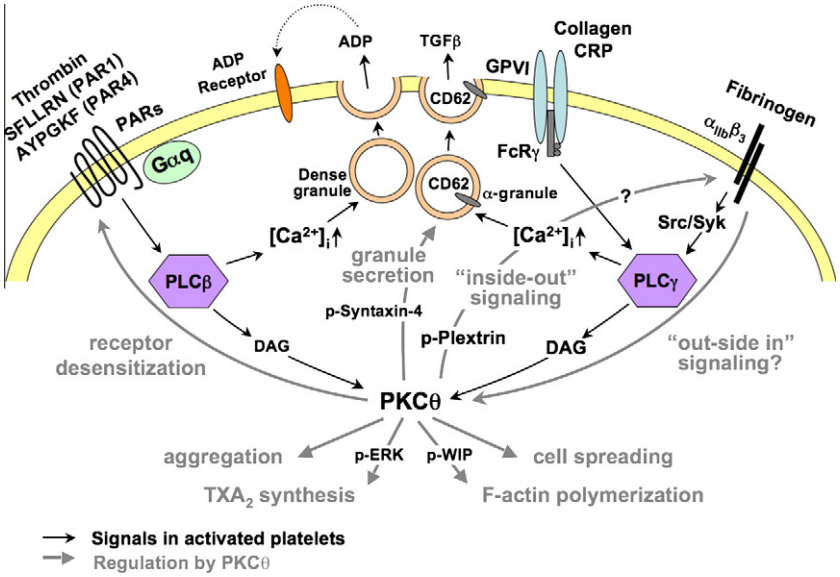


**Fig. 1.** PKCθ-mediated functions in activated platelet. A schematic model depicting PKCθ and other key molecules involved in the regulation of platelet activation. Binding of the indicated agonists to the GPVI and/or PAR receptors trigger receptor-linked signaling pathways that result in the activation of phospholipase C (PLC). PLC, in turn, generates two messengers, 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphtae ($IP_3$), which activates distinct PKC isoforms and mobilizes calcium ions from intracellular stores, respectively. Thrombin-induced DAG formation in platelets promotes the translocation of PKCθ to the membrane, where it can undergo posttranslational modifications (which may vary according to the agonist-induced signal), including phosphorylation of $Thr^{538}$, $Tyr^{90}$, and other residues. PKCθ physically and constitutively associate with the $\alpha_{IIb}\beta_3$ receptor, and upon fibrinogen binding to $\alpha_{IIb}\beta_3$, PKCθ associates with the Btk and Syk protein tyrosine kinases, and promote the phosphorylation of the Wiskott–Aldrich syndrome protein (WASP)-interacting protein (WIP). PKCθ also phosphorylates the tSNARE protein, syntaxin-4, which may influence granule secretion, activates the ERK protein and thereby promotes the generation of thromboxane A2, contributes to the regulation of $\alpha_{IIb}\beta_3$-linked 'outside-in' signaling, and potentially affect the 'inside-out' signaling pathway. All PKCθ-dependent phosphorylation events are either directly mediated by PKCθ, or indirectly, by PKCθ-regulated kinases. Altogether, PKCθ appears to be involved in the regulation of α-granule secretion, platelet adhesion, spreading, and aggregation. Most findings described herein were reported in recently published manuscripts, and obtained using a variety of experimental systems. The effects of PKCθ on selected cellular processes are still controversial and require further substantiation.

**Table 1**
Summary of reported PKC-theta-regulated functions in platelets.

(1) *Platelet functions that are positively regulated by PKCθ*
Platelet adhesion to fibrinogen-coated cover slips, spreading, and increased generation of filopodia (m*) [54,76]
'Outside-in' signaling through $\alpha_{IIb}\beta_3$ integrin receptor (m) [54,76]
'Outside-in' signaling in response to fibrinogen (m) [76]
Signal transduction from PAR4 (m) [49]
Thrombin-induced platelet activation (P-selectin expression) and α-granule secretion (m) [49]
PAR1-, PAR4-, CRP-, collagen- and thrombin-induced platelet aggregation and dense granule secretion (m+h*) [49,68]
PAR1-, PAR4- and CRP-induced $\alpha_{IIb}\beta_3$-receptor activation ('inside-out' signaling) (m+h) [68]
PAR1-, PAR4- and CRP-induced platelet activation (P-selectin expression) and α-granule secretion (m+h) [68]
PAR4- and CRP-induced threonine phosphorylation of syntaxin-4 (h) [68]
PAR4-, CRP- and collagen-induced release of $TXA_2$, and activation of ERK (m) [68]
Hemostasis and termination of bleeding after vascular injury (m) [49]
In vivo thrombus formation after arterial injury (m) [68]

(2) *Platelet functions that are negatively regulated by PKCθ*
CRP-induced GPVI-mediated α-granule secretion, and activation of $\alpha_{IIb}\beta_3$ (m) [76]
Thrombus formation under flow over a collagen-coated surface (m) [76]
Platelet adhesion to collagen or collagen-related peptide (CRP) (m) [76]
GPVI-dependent 'inside-out' signaling (m) [76]

(3) *PKCθ-related functions in platelets*
PKCθ constitutively associates with the $\alpha_{IIb}\beta_3$ integrin receptor (m+h) [54]
GP Ib-V-IX- and GPVI-receptor activation (by Alboaggregin A) and vWF/ristocetin-mediated activation of the GP Ib-V-IX receptor induces PKCθ association with Btk, and PKCθ-dependent threonine phosphorylation of Btk (h) [75]
Fibrinogen-induced $\alpha_{IIb}\beta_3$ integrin receptor activation stimulates PKCθ binding to Btk and Syk, and Btk- and/or Syk-mediated tyrosine phosphorylation of PKCθ (h) [54]
Collagen induces Btk- and/or Syk-mediated tyrosine phosphorylation of PKCθ (h) [54]
Fibrinogen-induced $\alpha_{IIb}\beta_3$ integrin receptor activation results in PKCθ-induced phosphorylation of WIP and activation of WASP (h) [54]
PAR1-induced activation by SFLLRN, PAR4-induced activation by AYPGKF, and GPVI-induced activation by CRP stimulate PKCθ phosphorylation on Thr538 (h) [68]

(4) *Platelet functions that are independent of PKCθ*
Extent of the $\alpha_{IIb}\beta_3$ integrin receptor expression (m) [54]
Fibrinogen binding to the $\alpha_{IIb}\beta_3$ integrin receptor following platelet activation by ADP, epinephrine, or PAR4 agonist (m) [54]
Agonist-induced 'inside-out' signaling (h) [54]
Fibrinogen-induced secretion of dense granules (m) [76]
Collagen- or CRP-induced platelet aggregation (m) [76]

* m, mouse; h, human.

In studies by Soriani et al. [54], PKCθ was not required for transduction of agonist-induced 'inside-out' signals, neither for fibrinogen binding to $\alpha_{IIb}\beta_3$ receptor. In contrast, Nagy et al. [68] have demonstrated that PKCθ-deficient platelets are impaired in GPVI- and PAR4-induced activation of the $\alpha_{IIb}\beta_3$ receptor, suggesting that PKCθ plays a positive role in the 'inside-out' signaling pathway that regulate the activation of the $\alpha_{IIb}\beta_3$ receptor. The latter study is indirectly supported by the results of Sun et al., demonstrating that decreased expression levels of PKCθ, that are secondary to a mutation in RUNX1, are in correlation with an impaired 'inside-out' signal transduction-dependent activation of the $\alpha_{IIb}\beta_3$ receptor [74]. The reason for the discrepancy between these studies is not clear, although it may reflect usage of different assay systems and/or experimental conditions.

### 6.2. PKCθ-mediated signaling via the receptors for collagen (glycoprotein VI) and von Willebrand factor (GPIb-V-IX)

Studies in human and mouse platelets supported a role for PKCθ in signaling from the receptors for collagen (glycoprotein VI; GPVI) and von Willebrand factor (vWF) (GPIb-V-IX) [68,75,76], although the exact role of PKCθ in these signaling pathways is still under debate. Stimulation of GPVI by CRP or collagen is known to induce α-granule and dense granule secretion from platelets, and Poole and co-workers reported that granule secretion in CRP-treated PKCθ-deficient platelets was increased compared to the response of wild type platelets [76,77]. In contrast, Kunapuli and co-workers reported that CRP-induced granule secretion by murine PKCθ-deficient platelets was decreased when compared with their normal counterparts [68,78]. The latter findings were further substantiated in a study utilizing human platelets, wherein a PKCθ antagonistic peptide (that blocks the specific receptor for activated C kinase; RACK) inhibited CRP- and collagen-induced granule secretion [68]. In this study, inhibition of PKCθ correlated with reduced threonine phosphorylation of the tSNARE protein, syntaxin-4, following GPVI or PAR4 activation. Because tSNARE proteins play a positive role in processes of vesicle exocytosis, it is suggested that PKCθ regulates granule secretion through syntaxin-4 phosphorylation [68,78]. While some of the discrepancy in the results could be explained by usage of different concentrations of CRP to stimulate platelets, additional studies are required in order to determine which of the suggested mechanisms are physiological and describes the actual effect of PKCθ on the in vivo response of GPVI-stimulated platelets.

In a different line of studies, Crosby and Poole [75] have used alboaggregin A (a snake venom-derived C-type lectin that is capable of activating GPVI and GPIb-V-IX in combination), or vWF/ristocetin (an *Amycolatopsis lurida*-derived agglutinating antibiotic) to stimulate human platelets. They found that both types of treatments have led to a rapid tyrosine phosphorylation of Btk. Phosphorylation of Btk was likely to be mediated by a Src-family PTK since it was sensitive to inhibition by PP1. Platelet stimulation with the above agonists also resulted in Btk phosphorylation on threonine residues, apparently by a PKC enzyme, and coimmunoprecipitation of Btk with PKCθ, but not with eight other PKC isoforms tested. Association with PKCθ was mediated by the Btk N-terminal PH domain, and at least in Mast cells, a similar association involved the C1 domain of PKC [79]. Platelet activation by alboaggregin A or vWF/ristocetin also induced Src- or Btk-dependent phosphorylation of PKCθ on tyrosine residues. While the exact phosphorylation site on PKCθ has not been identified in this work, $Tyr^{90}$ in the PKCθ regulatory domain was a preferred candidate, because it was identified as a major target of phosphorylation by Lck in TCR activated T cells [80]. In a more recent study, $Tyr^{90}$ was found to be a major phosphorylation site in collagen activated human platelets [54]. However, fibrinogen binding to the $\alpha_{IIb}\beta_3$ integrin receptor induced tyrosine phosphorylation of PKCθ, which did not react with phospho-$Tyr^{90}$-specific Abs. This suggests that platelet activation via different cell surface receptors may involve the activation of distinct PTKs and phosphorylation of PKCθ on tyrosine residues located at different sites. Additional work is required in order to identify the phosphorylation sites on PKCθ, and determine the PTKs involved in the phosphorylation of the individual residues. Nevertheless, our understanding of the mechanisms by which PKCθ regulate platelet functions will be incomplete before clarifying how these posttranslational effects impact on PKCθ ability to interact with other effector molecules, alter its catalytic activity, and/or modulate its subcellular location.

### 6.3. PKCθ-mediated signaling from PAR receptors

The protease-activated receptor (PAR) family includes four known isoforms of heterotrimeric G protein-coupled receptors [81,82], among which the PAR1 and PAR4 are expressed on human platelets [81], whereas mouse platelets express the PAR3 and PAR4 [83,84]. PARs are activated by serine proteases, such as thrombin (acts on PARs 1, 3 and 4) and trypsin (acts on PAR2) that cleave part of the N-terminal extracellular domain of the receptor [85]. The cleaved portion of the receptor acts as the agonist, causing receptor activation and promoting a physiological response – a secretion of intracellular granules and release of their content into the surroundings. Thrombin-induced activation of PAR receptors initiates a signaling cascade involving the activation of PLC and the rapid translocation of PKCθ from the cytosol to the membrane fraction [49]. These data indicate a positive role for PKCθ in signal transduction downstream of the PAR receptors.

To further analyze the involvement of PKCθ in thrombin-induced PAR receptor-linked signal transduction pathways, platelets from $PKC\theta^{-/-}$ mice and wild type mice were stimulated with thrombin, and their responses were monitored and compared. PKCθ-deficient platelets responded less efficiently in aggregation and redistribution of the P-selectin, CD62P, to the plasma membrane [49]. CD62P is a constitutively expressed integral glycoprotein of the α-granule membrane [86]. It is rapidly redistributed from the α-granule's membrane to the outer cell surface following platelet activation [87]. The plasma membrane expressed CD62P serves as an adhesion receptor that can interact with P-selectin glycoprotein ligand-1 (PSGL-1) on the surface of leukocytes, thereby promoting platelet-leukocyte adhesion, leukocyte activation and adhesion to endothelial cells, and trans-migration to sites of inflammation [88,89]. Expression of CD62P on the outer surface of platelets directly correlates with platelet degranulation and secretion, and therefore serves as a marker of platelet activation [90–92].

A positive regulatory role for PKCθ in PAR-linked signaling pathways was demonstrated by Nagy et al. [68] in human and mouse platelets. In their studies, human platelets were pretreated with a control peptide or a PKCθ-selective antagonistic peptide for RACK (receptor for activated C kinase), and stimulated by the PAR1-specific agonist, SFLLRN (single letter amino acid code), or the PAR4-specific agonist, AYPGKF. In their studies, inhibition of PKCθ resulted in impaired PAR-induced α-granule and dense granule secretion and platelet aggregation. Similar results were obtained in PKCθ-deficient murine platelets that poorly responded in granule secretion and aggregation following stimulation with the PAR4 agonist, SFLLRN.

It is interesting to note that PAR1-, PAR2- and PAR3-induced activation of Jurkat T cells promoted tyrosine phosphorylation of VAV1, ZAP-70 and SLP-76, key effector molecules in signal transduction downstream of the TCR [93]. Whether these PAR-induced signaling pathways in T cells are dependent or independent of PKCθ is not clear at present, and this requires the analysis of PAR receptor activation mechanisms in T lymphocytes from PKCθ-deficient mice.

## 7. Effect of PKCθ on hemostasis and extent of bleeding time following blood vessel injury

Platelet activation is crucial for normal hemostasis and prevention of excessive bleeding. Upon vascular injury, platelets are rapidly recruited to the damaged site where they form a hemostatic plug that arrests bleeding. The potential involvement of PKCθ in the regulation of platelet functions, as outlined above, suggests that PKCθ may play a role in processes that regulate hemostasis. In a study by Cohen et at., the effect of PKCθ on bleeding time was monitored in PKCθ$^{-/-}$ and PKCθ$^{+/+}$ mice following tail tip amputation. The bleeding time was found to be significantly longer in PKCθ$^{-/-}$ mice compared to that of their wild type counterparts [49]. Because the defect was observed in both C57BL/6J-PKCθ$^{-/-}$ and BALB/C-PKCθ$^{-/-}$ mice, it is assumed that the impaired hemostasis is PKCθ-dependent rather than a genetically inherited mouse strain specific trait.

Nagy et al. [68] have also analyzed the in vivo function of murine platelets from a PKCθ$^{-/-}$ mouse origin. Utilizing the $FeCl_3$ carotid artery-injury model, they found that PKCθ$^{-/-}$ mice exhibited a prolonged time of occlusion and failed to form a stable thrombus in response to injury. Their findings supported the idea that PKCθ is essential for stabilizing thrombus formation after arterial injury.

The bleeding time of PKCθ$^{-/-}$ mice was also tested by Soriani et al. [54], who reported no obvious defects due to PKCθ-deficiency. However, their actual data were unpublished and it is not clear what type of assay they have utilized in their work.

## 8. RUNX1 regulation of PKCθ gene transcription and PKCθ protein expression in platelets

Characterization of different genetically engineered and knockout mouse models, as well as human diseases revealed that hematopoietic transcription factors might be associated with thrombocytopenia and platelet dysfunctions [94–96]. A human inherited deficiency of the RUNX1 (also termed core-binding factor A2; CBFA2) transcription factor, characterized by thrombocytopenia and platelet dysfunction [96], was found to be associated with reduced expression of PKCθ in platelets [74]. Furthermore, platelets from this patient were impaired in their ability to respond via the $\alpha_{IIb}\beta_3$-receptor, aggregate, and induce the phosphorylation of pleckstrin, the major PKC substrate that undergoes serine/threonine phosphorylation in response of human platelets to thrombin stimulation [97,98]. The pleckstrin protein associates with inositol polyphosphate 5-phosphatase I (5-phosphatase I), and upon its phosphorylation by PKC [99], it turns on the 5-phosphatase I activity [100]. This enzyme in turn hydrolyzes the 5-phosphate from inositol 1,4,5-trisphosphate (Ins(1,4,5)$P_3$) and inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)$P_4$), thereby terminating the inositol phospholipid-stimulated calcium mobilization [101].

The studies by Jalagadugula and co-workers demonstrated that RUNX1 directly associates with a specific site in the PKCθ gene (*PRKCQ*) promoter in megakaryocytic cells, and further showed that siRNA-induced silencing of RUNX1 inhibited *PRKCQ* promoter activity and PKCθ protein expression [102]. The results indicate that *PRKCQ* is regulated at the transcriptional level by RUNX1, and explains the reduction in PKCθ protein levels, and PKCθ-regulated functional activities in platelets of the patient with the mutated RUNX1. Additional studies are required to determine whether RUNX1 is a cell lineage-specific transcription factor that regulates gene expression in megakaryocytes, but not T cells. Should the RUNX1 also regulate gene transcription in T cells, it would be of interest to test the effect of the mutated RUNX1 on *PRKCQ* gene transcription and analyze whether it affects PKCθ-regulated T cell functions.

## 9. Conclusions

PKCθ appears to be an important regulator of signal transduction downstream of the GPVI-, $\alpha_{IIb}\beta_3$ integrin- and PAR-receptors in mouse and human platelets. Its absence results in impaired hemostasis, platelet aggregation, and thrombus formation. However, additional studies indicate that platelet functions are regulated by several distinct PKC isoforms, which can synergize or antagonize the effects of PKCθ, or affect platelet functions independently of PKCθ [47,48,53,55–57,103,104]. Distinct PKC isoforms can also be involved in signal transduction from the same receptor or from different surface receptors. Furthermore, some PKC isoforms can regulate specific functions, such as granule secretion, while others may be involved in platelet adhesion to cells or extracellular matrices. Understanding the role of individual PKC isoforms in the regulation of distinct platelet functions, in combination with isoform-specific drugs, could potentially provide improved means for the treatment of selected platelet-mediated pathologies. While highly specific inhibitors of distinct PKC isoforms are not yet available, studies utilizing the different mouse PKC knockout models can provide a plethora of valuable information.

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