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REVIEW ARTICLE

Diverse functions of protein kinase C isoforms in platelet activation and thrombus formation

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Summary. Platelet activation is a complex balance of positive and negative signaling pathways. The protein kinase C (PKC) family is a major regulator of platelet granule secretion, integrin activation, aggregation, spreading and procoagulant activity. As broad-spectrum PKC inhibitors reduce secretion and aggregation, the PKC family is generally considered to be a positive regulator of platelet activation. However, the individual members of the PKC family that are expressed in platelets are regulated in different ways, and an increasing body of evidence indicates that they have distinct, and often opposing, roles. Many of the recent advances in understanding the contributions of individual PKC isoforms have come from mouse gene knockout studies. PKCα, a classic isoform, is an essential positive regulator of granule secretion and thrombus formation, both *in vitro* and *in vivo*. Mice lacking PKCα show much reduced thrombus formation *in vivo* but do not have a bleeding defect, suggesting that PKCα could be an attractive antithrombotic target. Important, apparently non-redundant, roles, both positive and negative, for the novel PKC isoforms δ, θ and ε in granule secretion have also been proposed, indicating highly complex regulation of this essential process. Similarly, PKCβ, PKCδ and PKCθ have non-redundant roles in platelet spreading, as absence of either PKCβ or PKCθ reduces spreading, whereas PKCδ negatively regulates filopodial formation. This negative signaling by PKCδ may reduce platelet aggregation and so restrict thrombus formation. In this review, we discuss the current understanding of the regulation and functions of individual PKC isoforms in platelet activation and thrombus formation.

Keywords: aggregation, protein kinase C, protein kinase, secretion, thrombosis, thromboxane generation.

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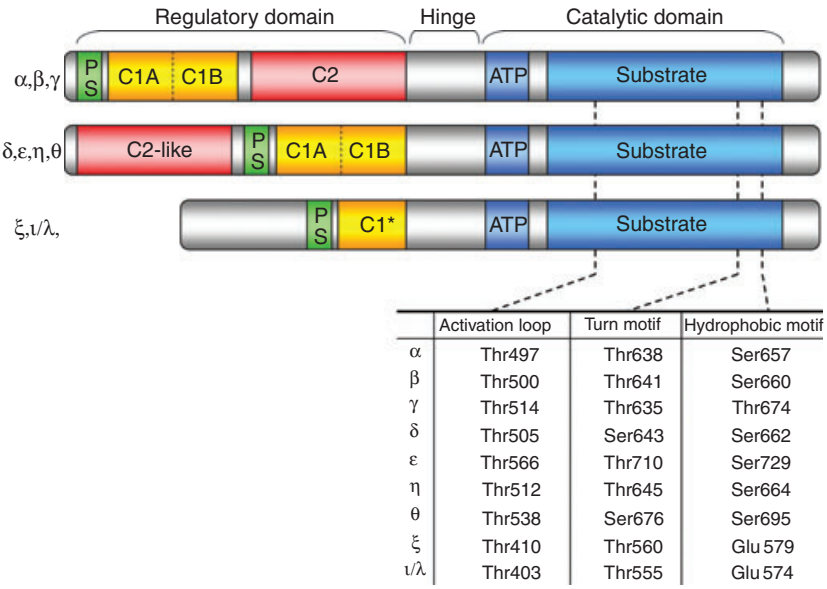
Introduction

Cardiovascular and cerebrovascular diseases are the leading causes of death in the developed world [1,2]. Although platelet plug formation is central to physiologic hemostasis following blood vessel injury, platelet activation at the site of atherosclerotic plaque rupture can lead to occlusive thrombi. Platelets adhere to the site of vascular injury and are activated, whereupon they secrete their granule contents, synthesize thromboxane A_2 (TxA_2), extend filopodia and lamellipodia, and activate surface adhesion molecules, in particular the fibrinogen receptor, integrin $\alpha_{IIb}\beta_3$ [3–5]. Each of these functions is regulated, at least in part, by members of the protein kinase C (PKC) family. Individual PKC isoforms may sometimes have very opposing functions. In this review, we will discuss some of the recent findings concerning the isoform-specific roles in PKC in platelets, focusing in particular on data obtained from PKC-deficient mouse platelets.

The PKC family in platelets

Members of the PKC family of serine/threonine kinases are classified into three subfamilies on the basis of the structure of their regulatory domains (Fig. 1). Conventional (or classic) (cPKC) isoforms (α, βI/II and γ) contain a tandem C1 domain capable of binding diacylglycerol (DAG), and a Ca^{2+}-binding C2 domain. Novel (nPKC) isoforms (δ, ε, η and θ) also contain C1 domains, but lack the ability to bind Ca^{2+} at the C2-like domain. Atypical isoforms (ζ and ι/λ) lack a C2 domain and have an atypical C1 domain. Of these isoforms, human platelets express cPKCα and cPKCβ, and nPKCδ and nPKCθ [6–8]. Although there is some recent evidence for expression of PKCη in human platelets [8,9], other groups could find no evidence for its expression [6,7,10], making further investigation necessary to resolve this discrepancy. Human megakaryocytes express PKCε, but its downregulation during megakaryocyte maturation results in no detectable expression of PKCε in human platelets [7,10,11]. Mouse platelets, in contrast, do express PKCε [10,12].



	Activation loop	Turn motif	Hydrophobic motif
α	Thr497	Thr638	Ser657
β	Thr500	Thr641	Ser660
γ	Thr514	Thr635	Thr674
δ	Thr505	Ser643	Ser662
ε	Thr566	Thr710	Ser729
η	Thr512	Thr645	Ser664
θ	Thr538	Ser676	Ser695
ξ	Thr410	Thr560	Glu 579
ι/λ	Thr403	Thr555	Glu 574

Fig. 1. Protein kinase C (PKC) family structure. In addition to the ATP-binding and substrate-binding sites in the catalytic domain, conventional PKC (top) isoforms contain several regulatory domains, the diacylglycerol (DAG)-binding tandem C1 domains, a Ca^{2+}-binding C2 domain, and a pseudosubstrate (PS) domain. Phosphorylation of the indicated sites in the activation loop, the turn motif and the hydrophobic motif is required for full catalytic activity. Novel PKCs (middle) have a Ca^{2+}-insensitive C2-like domain. Atypical PKCs (bottom) lack a C2 domain, and their C1 domain is insensitive to DAG. In addition, the hydrophobic motif contains a glutamate in place of the conserved serine/threonine.

PKC activation

PKC 'maturation'

Newly synthesized PKCs are catalytically inactive and must be constitutively phosphorylated at three sites. First, phosphoinositide-dependent kinase-1 phosphorylates a conserved threonine near the 'activation loop' (Fig. 1). Although activation loop autophosphorylation has been described for PKCδ and may not be required for activity [13,14], for most isoforms phosphorylation is constitutive and an essential part of PKC maturation [14–16]. Inducible phosphorylation of PKCθ Thr538 has been described in some cells [17,18] (but see also [19]), including platelets [20]. However, many reports suggest that PKCθ is constitutively phosphorylated in the activation loop [19,21–23] and that this is necessary for PKCθ activity. Activation loop phosphorylation may therefore not be a useful indicator of agonist-induced PKCθ activation and autophosphorylation. Following activation loop phosphorylation, PKC autophosphorylates the 'hydrophobic motif' and the 'turn motif', yielding a phosphatase-resistant and protease-resistant kinase that can respond to second messengers, but with the substrate-binding pocket occupied by the pseudosubstrate motif [15,16].

PKC activation by second messengers

Many platelet activators are coupled through their receptors to phospholipase C (PLC). Collagen, or the glycoprotein (GP)VI-specific agonist, collagen-related peptide, induces tyrosine kinase-dependent activation of PLCγ2 [24]. Thrombin, ADP and TxA_2 activate PLCβ through Gαq. PLC catalyzes the synthesis of DAG and inositol 1,4,5-trisphosphate (IP_3). IP_3-mediated Ca^{2+} release from intracellular Ca^{2+} stores results in an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The C2 domain tethers cPKCs to membrane phosphatidylserine in a Ca^{2+}-dependent manner. [25–27]. Once tethered, the cPKC C1 domain binds DAG, inducing a conformational change that expels the pseudosubstrate domain from the substrate-binding pocket, allowing phosphorylation of nearby substrates [14,15]. nPKCs are insensitive to Ca^{2+} and activated only by DAG. PKC regulation by second messengers may result in both positive and negative feedback loops, as elevations in the levels of these messengers may be regulated by PKC isoforms. Agonist-induced increases in $[Ca^{2+}]_i$, for example, are enhanced by broad-spectrum PKC inhibitors [28]. In contrast, Ca^{2+} entry may be positively regulated by PKC [29].

Differential localization of specific PKC isoforms by scaffolding proteins

Scaffolding proteins selectively localize isoforms to different signaling complexes or cellular compartments. The best characterized PKC scaffolds are the membrane-associated receptors for activated C-kinase (RACKs) [30,31]. These proteins bind PKCs in their active, secondary messenger-bound conformation and, although not themselves substrates for PKC, enhance PKC-dependent phosphorylation of other targets. Mochly-Rosen and colleagues [32,33] have proposed that there are unique RACKs for each PKC isoform, and that distinct cellular localizations of these RACKs would bring each PKC isoform into proximity with a distinct set of substrates.

Peptides based on the RACK-binding site, which thereby act as competitive inhibitors of RACK–PKC interactions, have been developed as isoform-specific inhibitors for many cell types, including platelets [9,20,34].

Tyrosine phosphorylation of PKC isoforms

PKC activity or localization may be regulated by tyrosine phosphorylation. Tyrosine phosphorylation of PKCα, PKCδ and PKCθ has been described in platelets, with each isoform being regulated by different tyrosine kinases [10,35–37]. In human platelets, collagen or thrombin induce PKCδ Tyr311 and Tyr565 phosphorylation by Src-family kinases [38,39], which increases PKCδ activity but does not affect plasma membrane translocation [39]. Interestingly, PKCδ tyrosine phosphorylation appears to be regulated differently in mouse platelets, with little phosphorylation being detected after stimulation with collagen-related peptide [10]. Phosphorylation of PKCθ Tyr90 is important in T-cell function and can be induced by collagen in platelets [40]. This may enhance PKCθ affinity for lipid membranes [41].

PKCs in granule secretion

Platelets secrete a vast array of soluble small molecules and proteins from their dense granules, α-granules and lysosomes. Platelet activators stored in dense granules, in particular ADP, amplify and sustain initial platelet activation [5]. α-Granules contain adhesion molecules, coagulation factors, growth factors and chemokines, in addition to multiple adhesive receptors that are inserted into the plasma membrane during secretion. The (patho)physiologic functions of these proteins and receptors extends beyond thrombus formation, as chemokines such as RANTES and interleukin-8, and adhesion receptors such as P-selectin, promote inflammation, recruiting monocytes and neutrophils [42,43].

PKC activity is essential for granule secretion. Broad-spectrum PKC inhibitors abolish granule secretion [35,44,45]. Multiple proteins involved in secretion are phosphorylated in a PKC-dependent manner, including SNARE complex proteins, SNAP-23, syntaxin 4, Munc18c [46–48], the cytoskeletal-associated proteins pleckstrin and MARCKS [49–53], and type II phosphatidylinositol-5-phosphate-4-kinase [54]. However, the contribution of individual PKC isoforms to granule secretion cannot be studied using broad-spectrum PKC inhibitors. Furthermore, many reportedly isoform-specific inhibitors, such as rottlerin for PKCδ, have actions on other kinases or cellular processes [55]. Therefore, to study the roles of individual PKC isoforms in platelet function, several groups have turned to gene deletion strategies in mouse platelets.

PKCα positively regulates granule secretion

An essential role for PKCα in Ca^{2+}-induced secretion was first suggested by Yoshioka *et al.* [56], as a necessary cytosolic factor that supported secretion in permeabilized platelets. Using PKCα$^{-/-}$ mice, we were able to directly test this hypothesis in intact platelets. Collagen-related peptide-induced or thrombin-induced secretion of dense granules and α-granules was almost completely abolished in PKCα$^{-/-}$ platelets. Consistent with this, PKCα$^{-/-}$ platelets showed significantly reduced SNAP-23 phosphorylation on Ser95 [57]. The role of this phosphorylation site is not entirely understood, although it may play a role in SNARE complex formation or in SNARE recycling [47]. This direct demonstration of PKCα in granule secretion is complicated by another role for PKCα, in dense granule biogenesis. PKCα$^{-/-}$ platelets have fewer dense granules [57], and so reduced secretion may be, in part, secondary to reduced granule number. However, α-granule number is not affected by loss of PKCα, but α-granule secretion is also mostly ablated. These data suggest that although reduced granule biogenesis may partially account for reduced dense granule secretion, it cannot account for the importance of PKCα in α-granule secretion, and so PKCα is likely to be an essential positive regulator of granule secretion.

Novel PKC isoforms in secretion

PKCε. Human platelets do not express detectable levels of PKCε, although there is significant expression of this isoform in mouse platelets. PKCε$^{-/-}$ mouse platelets show minor defects in thrombin-induced dense granule secretion, and a much more marked reduction in dense granule secretion in response to low or intermediate concentrations of collagen or collagen-related peptide [10]. This was consistent with reduced tyrosine phosphorylation of proteins downstream of GPVI, such as the FcR γ-chain, Syk and PLCγ_2, likely to be associated with reduced PLCγ_2 activation, and may lead to reduced IP_3-dependent Ca^{2+} release and DAG synthesis. This would, in turn, reduce activation of cPKCs such as PKCα, and so reduce granule secretion. Intriguingly, the authors reported that a broad-spectrum PKC inhibitor had similar effects on FcR γ-chain and Syk phosphorylation in mouse platelets, but not in human platelets, suggesting that species-specific differences in isoform content may result in differences in intracellular regulation.

PKCδ. That PKCδ regulates granule secretion was first proposed on the basis of the reported effects of rottlerin (also known as mallotoxin). Rottlerin enhances GPVI-dependent granule secretion, but inhibits granule release following activation of protease-activated receptor (PAR) 1 or PAR4, suggesting that PKCδ regulates dense granule secretion in an agonist-dependent manner [8]. However, many concerns have been raised regarding the specificity and mechanism of action of rottlerin [55]. Studies with PKCδ$^{-/-}$ platelets have not fully resolved the role of PKCδ. We found no difference in GPVI-dependent dense granule secretion in PKCδ$^{-/-}$ platelets as compared with the wild type. Significantly, rottlerin still enhanced granule secretion in PKCδ$^{-/-}$ platelets, underscoring its likely off-target effects, and demonstrating that,

under these conditions, it was possible to observe a potentiation of secretion [58]. In contrast, Chari *et al.* [34] found enhanced GPVI-dependent dense granule secretion in PKCδ$^{-/-}$ platelets, an effect replicated in human platelets by δ(V1-1)-TAT, a cell-permeant peptide designed to block the interaction between PKCδ and its RACK. The reasons for this apparent difference are not clear. Although differences in methodology may possibly account for the discrepancy, both studies used washed platelets treated with indomethacin, to exclude possible effects of PKCδ on TxA_2 synthesis, and similar concentrations of collagen were used.

Chari *et al.* [34] also found that PKCδ$^{-/-}$ platelets showed partially reduced granule secretion in response to stimulation of PAR4, and dense granule secretion was also inhibited by δ(V1-1)-TAT. Chari *et al.* [34] indicate that reduced secretion was only seen at submaximal concentrations of AYPGKF, and lost at higher concentrations, suggesting that other mechanisms may compensate for the loss of PKCδ signaling at higher agonist concentrations. This contrasts with the situation with PKCα, where gene deletion leads to almost complete loss of dense granule and α-granule secretion even at high agonist concentrations [57].

The mechanisms that might underlie differential regulation of granule secretion by PKCδ by GPVI and G-protein-coupled receptor stimulation are still unclear. It has been shown recently that phosphorylation of PKCδ by the tyrosine kinase Lyn occurs downstream of GPVI but not PARs, and that Lyn and PKCδ together regulate SHIP1 phosphorylation in an agonist-dependent manner [59]. This is consistent with negative regulation of $\alpha_{IIb}\beta_3$ signaling by a Lyn–SHIP1 complex that has been previously proposed [60], and this may begin to explain the differential roles for PKCδ in signaling downstream of collagen and thrombin.

PKCθ. The role of PKCθ, which is closely related to PKCδ, in GPVI-dependent granule secretion is also the subject of debate. This may again reflect important differences in platelet preparation, leading to significant differences in platelet responsiveness to collagen-related peptide [12,20,61]. Possible reasons for these differences have been discussed at length in recent correspondence [61]. We have found that PKCθ negatively regulates GPVI-dependent granule secretion, especially at low levels of stimulation [12,61]. However, reduced granule secretion in PKCθ$^{-/-}$ platelets has been reported at higher collagen-related peptide concentrations, suggesting a positive signaling role at these higher concentrations [20]. It is therefore possible that the role of PKCθ may vary with the extent of platelet stimulation. Experiments using more physiologic conditions, such as in whole blood or under arterial shear conditions, may help to clarify the role of PKCθ in GPVI-dependent signaling. In response to thrombin, PKCθ$^{-/-}$ platelets show reduced secretion, indicating a positive regulatory role [7,20]. This differential role downstream of GPVI or thrombin receptors is reminiscent of the differential signaling roles of PKCδ discussed above. This would lead to a complex contribution of novel PKCs to thrombus formation and hemostasis. The two-fold to four-fold increase in tail bleeding time observed in PKCθ$^{-/-}$ mice, for example [7], may reflect the role of PKCθ in thrombin-induced platelet activation. This would be consistent with one study in which complete loss of GPVI-dependent signaling (in FcRγ$^{-/-}$ mice) had little effect on tail bleeding time, whereas hirudin (as anticoagulant) prolonged the tail bleeding time almost three-fold in wild-type mice, and nearly seven-fold in FcRγ$^{-/-}$ mice [62]. Similarly, the role of PKCθ in thrombus formation *in vivo* will depend on the relative importance of collagen-induced and thrombin-induced platelet activation at the site of vascular injury.

PKCs in thromboxane synthesis

Multiple platelet agonists induce release of the TxA_2 precursor arachidonic acid from membrane phospholipids by activating cytosolic phospholipase A_2 (PLA_2). This is dependent upon increased $[Ca^{2+}]_i$ and is regulated by phosphorylation by stress kinase p38 [63] and possibly also extracellular signal-related kinase-2 (ERK2) [64]. Broad-spectrum PKC inhibitors do not prevent ADP-induced TxA_2 synthesis, or collagen-induced or thrombin-induced PLA_2 phosphorylation [64,65]. However, more recently, it has been suggested that several nPKCs regulate TxA_2 synthesis.

PKCδ

Rottlerin, the reported PKCδ inhibitor, was used to first study a role for PKCδ in TxA_2 synthesis [8]. This has been further investigated by Chari *et al.* [34], using PKCδ$^{-/-}$ mouse platelets, in which AYPGKF-induced TxA_2 synthesis was reduced, and GPVI-dependent TxA_2 synthesis was enhanced. How this differential regulation may occur requires further investigation.

PKCθ

Nagy *et al.* [20] reported that in PKCθ$^{-/-}$ mouse platelets, agonist-induced TxA_2 synthesis was reduced to about half that seen in wild-type mice, and that this correlated with reduced ERK2 phosphorylation, suggesting that PKCθ indirectly regulates TxA_2 synthesis via regulation of ERK2.

PKCη

Bynagari *et al.* [9] have shown that ADP stimulates PKCη activation through the $P2Y_1$ receptor, but not $P2Y_{12}$, and that PKCη is necessary for ADP-induced TxA_2 synthesis. The contribution of PKCη to ADP-induced TxA_2 was assessed with a η-RACK inhibitory peptide. The mechanism by which PKCη regulates TxA_2 synthesis is unclear, because although ADP-induced TxA_2 synthesis is dependent on ERK2 [64], the η-RACK inhibitor peptide had no effect on ERK2 phosphorylation. The situation is clearly complex, because PKC inhibition has been shown to enhance ADP-induced TxA_2

synthesis [64], and further work is therefore required to clarify the role of individual isoforms in TxA_2 synthesis.

PKCs in platelet spreading

Positive regulation of spreading by PKCβ and PKCθ

$PKC\beta^{-/-}$ or $PKC\theta^{-/-}$ platelets do not fully spread on fibrinogen [6,12,66], suggesting essential and non-redundant roles for these two isoforms. Spreading on fibrinogen requires signaling from ligand-engaged $\alpha_{IIb}\beta_3$ (known as 'outside-in signaling'), suggesting that PKCβ and PKCθ are involved in this signaling pathway. PKCβ is recruited to $\alpha_{IIb}\beta_3$ via RACK1, following adhesion of platelets to fibrinogen or integrin activation by Mn^{2+} and soluble fibrinogen [6]. However, the formation of an $\alpha_{IIb}\beta_3$–PKCβ signaling complex appears to be entirely dependent on released TxA_2, requiring recruitment of $PLA_2\alpha$ to the integrin and a TxA_2-dependent increase in $[Ca^{2+}]_i$ [67]. This suggests that factors in addition to outside-in signaling are important in the control of platelet spreading. Interestingly, although $PLA_2\alpha^{-/-}$ platelets spread less well than wild-type platelets in fibrinogen, even when costimulated with ADP, collagen-related peptide or PAR4-activating peptide (PAR4-AP) [67], the reduced spreading in $PKC\beta^{-/-}$ platelets could be overcome by costimulation with ADP or PAR4-AP [6], suggesting that TxA_2 release/PKCβ recruitment might not be the only function of $PLA_2\alpha$ in spreading.

$PKC\theta^{-/-}$ platelets also display reduced spreading on fibrinogen, although the extent of the defect differs between studies [12,66]. The reasons for the quantitative difference may lie in part in the method used to analyze spreading [12], but may also depend on the conditions of platelet preparation. Whereas in our study [12] TxA_2 synthesis was blocked with indomethacin, Soriani *et al.* [66] found a more marked defect in platelets with functional TxA_2 synthesis and signaling. As noted above, TxA_2 synthesis is clearly an important signaling intermediate in platelet spreading, and it has been suggested that PKCθ positively regulates TxA_2 synthesis under some conditions [20], and therefore this technical difference in approach may be very important in explaining differences between studies of platelet spreading and integrin activation.

Negative regulation of filopodia by PKCδ

When activated on contact with surfaces, platelets rapidly and transiently extend filopodia, which are quickly superseded by lamellipodia to form the fully spread platelet. In PKCδ-deficient platelets, however, although both filopodia and lamellipodia do form sequentially, as in wild-type platelets, the filopodia do not subsequently disappear, unlike in the wild type, where filopodia are not apparent in the fully spread platelet [58]. This sustained appearance of filopodia in $PKC\delta^{-/-}$ platelets also results in more extensive platelet aggregation. The molecular mechanism underlying this functional change involves a physical interaction between

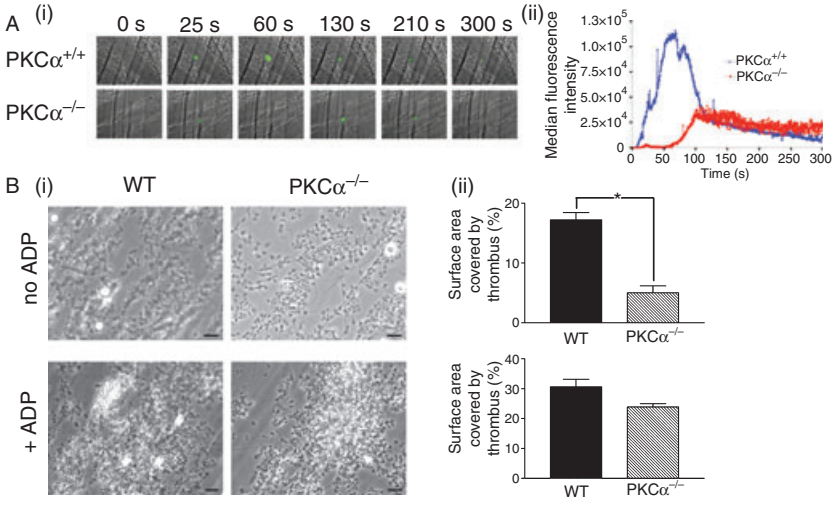


Fig. 2. Protein kinase C (PKC)α regulates thrombus formation *in vivo* and *in vitro*. (A) Thrombus formation in mice *in vivo*. Mice were either $PKC\alpha^{-/-}$ or littermate-matched wild-type (WT) controls. Platelets were labeled *in vivo* with Alexa 488-conjugated anti-CD41 antibodies. (i) Platelets (green) composited with brightfield images (black/white) of the cremaster arteriole were viewed at × 40 magnification. Platelet accumulation may be seen after laser damage to endothelium. (ii) Traces shown are median integrated platelet fluorescence of 15 thrombi induced in three or more mice for each group. Absence of PKCα causes A marked reduction in thrombus formation in this model. (B) Thrombus formation *in vitro*. Heparin/PPACK-anticoagulated blood from WT or $PKC\alpha^{-/-}$ mice was passed over collagen (shear rate of 1000 s^{-1}). For the lower panels, ADP solution was coinfused at a 10% flow rate (20 μM ADP, final concentration). (i) Rrepresentative phase-contrast images after 4 min of blood flow. Images were taken under × 63 oil immersion, and the bars represent 20 μm. (ii) Surface area coverage with thrombi (mean ± standard error of the mean, $n \geq 3$, $^*P < 0.0001$). Absence of PKCα here also causes a marked reduction in thrombus formation, although initial adhesion of platelets to collagen is unaffected. Thrombus formation was rescued by addition of ADP to the blood, suggesting that the major functional defect in PKCα-deficient platelets is their ability to secrete ADP. Figure taken from Konopatskaya *et al.* (2009) [57], reproduced with permission from the *Journal of Clinical Investigation*.

PKCδ and the actin regulator vasodilator-stimulated phosphoprotein (VASP). PKCδ was shown to negatively regulate phosphorylation of VASP at Ser157, reducing filopodial extension or promoting filopodial retraction, and thereby negatively controlling platelet–platelet interaction to limit aggregation.

PKCs in integrin activation, aggregation and thrombus formation

Activation of $\alpha_{IIb}\beta_3$ enables platelets to aggregate via fibrinogen, and is partially dependent on the small GTPase, Rap1b [68]. Two signaling pathways from agonist-induced platelet stimulation to Rap1b activation have been described. In the first, DAG and elevated $[Ca^{2+}]_i$ activate CalDAG-GEFI, a guanine nucleotide exchange factor. CalDAG-GEFI$^{-/-}$ platelets show reduced aggregation in response to all agonists [69]. The required Ca^{2+}/DAG signal may be enhanced by secreted ADP, or newly synthesized TxA_2. The second pathway is dependent on PKC, as broad-spectrum PKC inhibitors reduce aggregation and completely inhibit aggregation in CalDAG-GEFI$^{-/-}$ platelets [70]. However, whether PKC directly regulates $\alpha_{IIb}\beta_3$ is unclear, as these PKC inhibitors also abolish dense granule secretion and subsequent P2Y signaling, which is also important in $\alpha_{IIb}\beta_3$ activation. In particular, $P2Y_{12}$ signaling is essential for maintaining Rap1b in its active, GTP-bound form [71], and continuous $P2Y_{12}$ signaling is essential for the maintenance of integrin activation and thrombus stability [72]. Therefore, any apparent effect of PKC inhibitors (or PKC-deficient platelets) on $\alpha_{IIb}\beta_3$ activation must be interpreted cautiously, and the possibility that the effect is indirect, through altered granule secretion or TxA_2 synthesis, should be considered.

PKCs in aggregation

PKCα$^{-/-}$ platelets show reduced collagen-related peptide-induced $\alpha_{IIb}\beta_3$ activation, which is paralleled by reduced aggregation in response to submaximal agonist concentrations [57]. However, as discussed above, PKCα$^{-/-}$ platelets also have a large reduction in dense granule secretion, and addition of exogenous ADP can restore aggregation back to wild-type levels [57], indicating that it is the reduced capacity to secrete ADP that leads to the reduced aggregation seen in PKCα$^{-/-}$ platelets.

In contrast, although PKCδ$^{-/-}$ platelets show enhanced collagen/collagen-related peptide-induced aggregation [58] and, under some conditions, may show slightly enhanced GPVI-dependent dense granule secretion (see above), the two are possibly unrelated. Inhibition of $P2Y_1$ and $P2Y_{12}$ did not prevent enhanced aggregation in PKCδ$^{-/-}$ platelets [58]. Furthermore, aggregation of PKCδ$^{-/-}$ platelets could not be

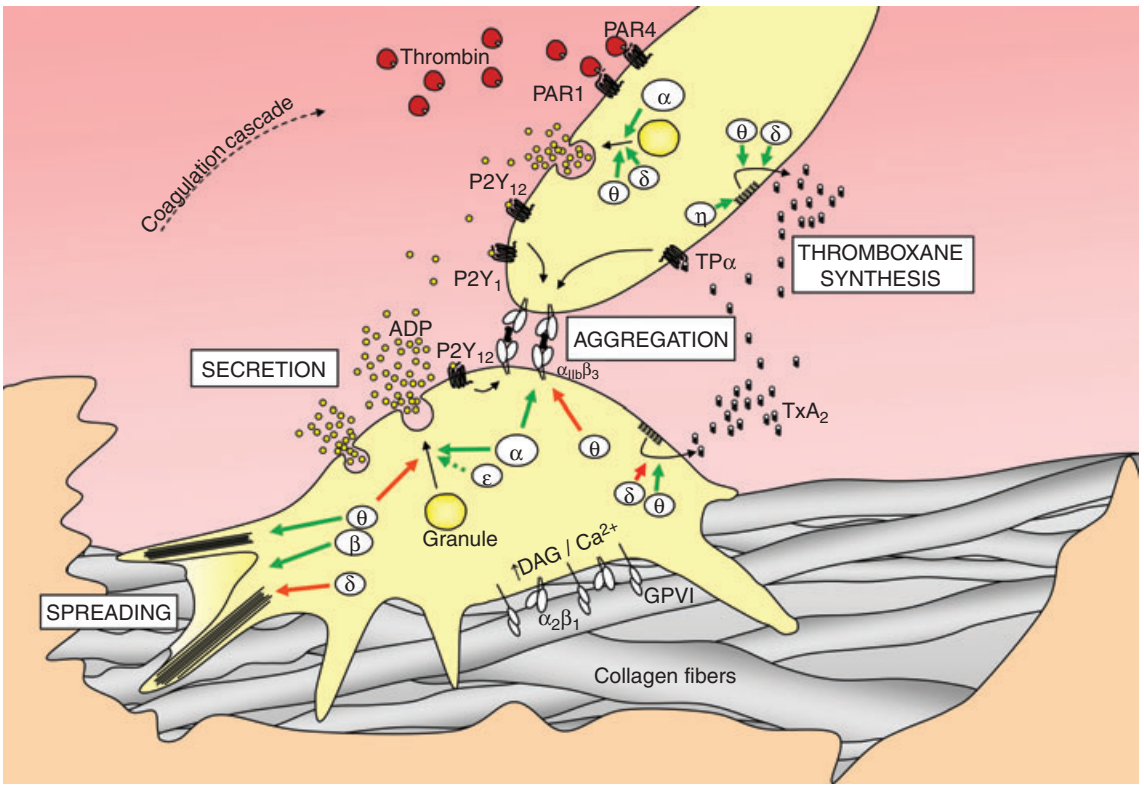


Fig. 3. Currently proposed roles for individual protein kinase C (PKC) isoforms in platelet activation. Platelets are activated by thrombin, through protease-activated receptor (PAR) 1 and PAR4, and by collagen, through glycoprotein VI and $\alpha_2\beta_1$. Secretion of ADP and synthesis of thromboxane A_2 enhances platelet activation. PKC isoforms have been implicated in many platelet responses, including granule secretion, thromboxane synthesis, $\alpha_{IIb}\beta_3$ activation, and spreading. Positive roles are indicated by green arrows, and negative roles by red arrows. The role of PKCθ and PKCδ may depend on whether the platelet is stimulated by thrombin (upper platelet) or collagen (lower platelet). See main text for further details. DAG, diacylglycerol.

further enhanced by rottlerin, whereas dense granule secretion was further potentiated. Instead, we have proposed that the enhanced aggregation results from increased filopodia formation in PKC$\delta^{-/-}$ platelets [58].

Effect of PKC inhibitors on thrombosis in vitro

Broad-spectrum PKC inhibitors substantially reduce thrombus formation on collagen under relatively high physiologic shear conditions in anticoagulated blood [28]. This is likely to be secondary to reduced granule secretion or TxA_2 synthesis, at least in part. G$\alpha q^{-/-}$ platelets form much smaller aggregates under flow, showing the importance of secreted mediators released from platelets, such as ADP or TxA_2, in enhancing thrombus formation. Reduced granule secretion, as seen with PKC inhibition, would therefore be expected to affect thrombus formation. However, even in G$\alpha q^{-/-}$ platelets, broad-spectrum PKC inhibition further reduced aggregate formation on collagen under flow [28]. This may suggest that one or more PKC isoforms promote thrombus growth independently of ADP and TxA_2. Alternatively, this Gαq-independent role for PKC may reflect $P2Y_{12}$ signaling downstream of ADP secretion that is necessary for thrombus stability [72].

PKCα in thrombosis in vitro *and* in vivo

Because we found that PKCα is a major regulator of platelet secretion (see above, and [57]), we tested whether blood from PKC$\alpha^{-/-}$ mice would be able to form thrombi on collagen under flow conditions *in vitro*. Although adhesion of individual platelets to collagen fibers was not affected, large multiplatelet aggregates were not present. This is despite apparently normal aggregation to high concentrations of collagen-related peptide, indicating that *in vitro* thrombus formation models may be more sensitive indicators of platelet function than aggregation. The defect in thrombus formation could be largely attributed to reduced dense granule secretion, as coinfusion with ADP restored the ability to generate large thrombi ([57], and reproduced in Fig. 2). Importantly, a similar effect was seen *in vivo*, with the use of intravital microscopy to follow thrombus formation following laser-induced injury of the cremaster muscle vasculature (Fig. 2). Thrombus formation was delayed, and fewer platelets accumulated in mice lacking PKCα. In contrast, hemostasis, assessed by tail bleeding time, appears to be unaffected in PKC$\alpha^{-/-}$ mice, and no occult fecal blood, indicative of gastrointestinal bleeding, was observed [57]. Therefore, pharmacologic inhibition of PKCα may also reduce thrombus formation without disrupting hemostasis.

Conclusions

Individual PKC isoforms are differentially regulated and functionally distinct during platelet activation. The different PKC isoforms have specific functions in granule secretion, TxA_2 synthesis and platelet spreading, ultimately leading to very different effects in thrombosis *in vitro* and *in vivo* (Fig. 3). Isoform-specific drugs could potentially provide novel antithrombotic agents that might be safer than broad-spectrum inhibitors. To understand how the roles of the isoforms differ in human platelets, however, we need selective inhibitors. Until then, mouse knockout models have provided, and will continue to provide, a wealth of valuable data.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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