Isoform-specific functions of protein kinase C: the platelet paradigm

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Abstract
Platelets are central to haemostasis and thrombosis. Many key steps in platelet activation and aggregation are regulated by members of the PKC (protein kinase C) family. Multiple isoforms of PKC are expressed in platelets, and evidence is emerging that different isoforms play distinct roles in the platelet activation process. This may, in part, be regulated by isoform-specific interactions between PKC family members and other intracellular signalling molecules, such as tyrosine kinases, or the actin cytoskeleton regulator, VASP (vasodilator-stimulated phosphoprotein). The contributions of individual PKC isoforms can be addressed directly in platelets from knockout mouse models, which are providing key insights into the physiological function of PKC isoform diversity and can be a valuable complimentary approach to more commonly used pharmacological analyses. Using knockout mouse models, recent reports have demonstrated the importance of PKCβ2 and PKCδ in integrin-dependent platelet spreading, and also a novel role for PKCα in regulating filopodial formation, highlighting the utility of such models to investigate the functions of specific PKC isoforms in a physiological process that is significant to our understanding of cardiovascular disease.

Introduction
Platelets play a central role in physiological blood clotting (haemostasis) after vascular damage. Reduced platelet function can lead to prolonged bleeding times, as is seen in Bernard–Soulier syndrome or von Willebrand’s disease. However, inappropriate or excessive activation of platelets also has important pathological implications. A sufficiently large thrombus (intravascular clot) can occlude blood vessels, restricting blood flow. Fragmentation of a thrombus by shear stress or the fibrinolytic system can release circulating emboli that may become lodged in small vessels elsewhere in the body. Occlusion of vessels in the cerebral, coronary or pulmonary vascular beds may result in stroke, myocardial infarction or pulmonary oedema respectively, the consequences of which are often fatal; cardiovascular and cerebrovascular diseases are the leading cause of death in the developed world [1]. A central aim of platelet research is to identify the pathways that are crucial for haemostasis, and those that tend to lead to thrombosis.

Platelet activation
Many intracellular signalling pathways are involved in platelet activation, to which a rise in [Ca2+]i (intracellular Ca2+ concentration) and activation of PKC (protein kinase C) isoforms are central. Damage to the vessel wall exposes components of the extracellular matrix, in particular subendothelial collagen, to flowing blood. Platelets adhere to these sites via GP (glycoprotein) receptors, whose stimulation activates PLCγ (phospholipase Cγ), resulting in generation of IP3 (inositol trisphosphate) and DAG (diacylglycerol) [2]. IP3 induces release of Ca2+ from intracellular stores, leading to an increase in [Ca2+]i, followed by Ca2+ entry across the plasma membrane, which is required for a sustained elevation in [Ca2+]i. The initial platelet responses are amplified by agonists released from the dense granules, such as ADP and ATP, and by TxA2 (thromboxane A2) synthesized from membrane arachidonate. α-Granule contents are also secreted, which include proteins that are important in aggregation, such as vWF (von Willebrand factor), fibrinogen, and also proteins that are important in thrombin generation. Activation of the fibrinogen receptor, integrin αIIbβ3, represents a final common pathway for both soluble and subendothelial agonists. This is known as ‘inside-out’ signalling [3,4], allowing the activated platelet to bind to vWF or fibrinogen via αIIbβ3. Circulating platelets can bind to this vWF/fibrinogen surface if they also have activated αIIbβ3. Ligand binding to αIIbβ3 initiates ‘outside-in signalling’, which is required for platelet responses such as spreading [3]. Outside-in signalling is also involved in presentation of a procoagulant surface in a Ca2+-dependent manner that forms an efficient surface for the assembly of coagulation complexes and results in a large increase in thrombin generation. All of these responses are tightly regulated by PKC.

PKC in platelet activation
PKCs form a family of related serine/threonine kinases that are part of the AGC-type kinase (protein kinase A/protein

Key words: conventional protein kinase C (cPKC), filopodium, mouse model, platelet, rottlerin, vasodilator-stimulated phosphoprotein (VASP).

Abbreviations used: BTK, Bruton’s tyrosine kinase; [Ca2+]i, intracellular Ca2+ concentration; DAG, diacylglycerol; GP, glycoprotein; IP3, inositol trisphosphate; PKA, protein kinase A; PKC, protein kinase C; PKCβ2, atypical PKCβ2; PKCγ, conventional PKC-γ; PKCβ, novel PKC; PLC, phospholipase C; RACK1, receptor for activated C-kinase-1; TxA2, thromboxane A2; VASP, vasodilator-stimulated phosphoprotein; vWF, von Willebrand factor.

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PKC is a key regulator of platelet activation

Adhesion to collagen, or stimulation by soluble agonists such as thrombin, ADP or TXA2, activates numerous intracellular signalling molecules, especially PLC, resulting in a rise in [Ca2+]i, and activation of PKC. PKC regulates many platelet responses to stimulation, such as granule secretion, aggregation and spreading.

PKC isoforms can be split into three groups: conventional, novel and atypical [14]. cPKC (conventional PKC) isoforms (α, βI, βII and γ) are activated by DAGs and Ca2+. Second messenger-dependent translocation is required for removal of the inhibitory pseudosubstrate from the active site. DAG binds to the C1 domain, often present as a tandem repeat, whereas Ca2+ binds the C2 domain [15]. C1 is involved in targeting the kinase to the plasma membrane in addition to activation. This domain can also be activated by phorbolesters, which bind PKC with two orders of magnitude greater affinity than DAGs and are resistant to cellular metabolism. This results in constitutive PKC activation [16]. nPKC (novel PKC) isoforms (δ, ε, η and θ) are sensitive to DAGs but insensitive to Ca2+ because the C2 domain lacks the essential aspartate residues for binding Ca2+ [15]. aPKC (atypical PKC) isoforms (ζ, τ, λ and μ) are insensitive to DAGs and Ca2+. The function of the C1 domain in aPKCs is unclear.

There is also now a significant body of evidence that strongly suggests that some PKC isoforms play highly specific roles in platelet activation. In part, these conclusions are based on the use of isoform-selective inhibitors, such as G66976 (an inhibitor of cPKCs [17]) and rottlerin (commonly used as a selective inhibitor of PKCd [18]). However, studies that rely exclusively on pharmacological approaches are necessarily heavily reliant on the specificity of the agents used, which is sometimes in question. For example, although widely used to demonstrate the contribution of PKCd, rottlerin has also been reported to inhibit PKCθ [19] and act as a mitochondrial uncoupler [20].

Lacking a nucleus, platelets are not directly amenable to genetic manipulation, such as by siRNA (small interfering RNA). An alternative approach to studying isoform-specific functions in platelets is to use mice that are deficient in one isoform, which provide a useful means to bypass the questions that sometimes surround pharmacological strategies. Studies using platelets deficient in PKCβ [9], PKCd [13] or PKCθ [10] have been reported. Combining both pharmacological and genetic approaches is perhaps the best way to counter the drawbacks of either on its own.

Isoform-specific functions of PKC discovered using mouse models

PKCβ and PKCθ regulate integrin αIIbβ3 outside-in signalling

The binding of fibrinogen to activated αIIbβ3 initiates further signals within the platelet. In particular, these ‘outside-in’ signals promote the cytoskeletal rearrangements required for full aggregation and platelet spreading. Despite the importance of outside-in signalling, the intermediates between fibrinogen binding and platelet spreading have not been extensively characterized. Broad-spectrum kinase inhibition, for example the PKC family, inhibits integrin activation. However, using more specifically targeted approaches, such as through knockout mouse models, the roles of individual isoforms are gradually being defined. Platelets deficient in PKCβ do not spread on a fibrinogen-coated surface, although they do not show any change in agonist-induced fibrinogen binding [9]. These results suggest that PKCβ is not required for agonist-induced αIIbβ3 activation but is necessary for subsequent outside-in signalling to the actin cytoskeleton. Interestingly, PKCβ is not associated with αIIbβ3 recruited to the cytoplasmic β3 tail following binding of fibrinogen. Furthermore, PKCθ does not interact directly with the integrin but instead requires an adaptor, RACK1 (receptor for activated C-kinase 1) [9]. It may be this interaction, bringing PKCθ to its substrates, that confers isoform specificity on outside-in signalling.

PKCθ is also required for outside-in signalling and, as with PKCβ, platelets deficient in PKCθ do not fully spread on a fibrinogen-coated surface [10]. In contrast with PKCβ, PKCθ is constitutively associated with αIIbβ3 [9,10]. Fibrinogen binding did however induce association of PKCθ with BTK (Bruton’s tyrosine kinase). A similar interaction is also seen in platelets stimulated with alboaggregin A, a GPV1 and GPLb-IX-V agonist [21], where PKCθ and BTK appear to regulate one another’s activity, with PKCθ...
positively regulating BTK but BTK negatively regulating PKCδ. The precise roles of BTK and PKCδ in outside-in signalling remain to be elucidated, and it may be that these two kinases may be part of a larger signalling complex that controls signalling from αIibβ3 to the actin cytoskeleton. It is interesting to note that lack of either PKCβ or PKCδ in the presence of the other caused a major defect in spreading, which may suggest that the two isoforms play distinct but essential roles in outside-in signalling.

PKCδ negatively regulates collagen-induced aggregation
PKCδ is one of the major nPKC isoforms expressed in platelets. Many studies of PKCδ rely on rottlerin, putatively a specific and selective inhibitor of this isoform. Based on such pharmacological experiments, several roles for PKCδ have been proposed in regulation of platelet function, such as collagen-induced activation of ERK (extracellular-signal-regulated kinase) and subsequent TxA2 synthesis, integrin αIibβ3 activation and platelet aggregation [8]. Similarly, a study using rottlerin suggested that PKCδ is required for dense granule secretion following stimulation by thrombin, and plays a negative regulatory role in dense granule secretion when platelets are stimulated by convulxin [22]. However, it is important to note that PKCδ knockout mice do not show increased dense granule secretion in response to collagen stimulation. Furthermore, rottlerin was still able to increase collagen-evoked dense granule secretion in these mice [13]. These results strongly suggest that rottlerin is not sufficiently selective for PKCδ, and highlight the need for parallel approaches to complement and confirm the pharmacological data.

Although PKC activity is necessary for platelet activation, it has long been known that PKCs also negatively regulate platelets. For example, PKC increases Ca2+ extrusion from the cytosol [12] and desensitizes some G-protein-coupled receptors [11]. Recently, a novel role for PKCδ in inhibiting collagen-induced aggregation has been described [13]. Platelets from PKCδ-deficient mice show enhanced aggregation following stimulation by collagen. Rottlerin also enhances aggregation in human platelets and wild-type mouse platelets, but has no further effect on platelets from PKCδ-deficient mice, suggesting that, in this case, the effect of rottlerin is in fact caused by PKCδ inhibition. However, neither dense granule secretion nor integrin αIibβ3 activation was affected. Rather, PKCδ appears to negatively regulate filopodial dynamics.

When activated, platelets rapidly and transiently extend filopodia. These dynamic structures are quickly superseded by lamellipodia, resulting in a spread platelet that can more easily resist the shear forces acting on it and so remain attached to the damaged vessel wall. In PKCδ-deficient platelets, filopodia are observed but they are not superseded by lamellipodia [13]. Thus PKCδ appears to regulate the dynamic changes in cell shape during a platelet’s spreading on collagen. These results also suggest that filopodia may play an important role in enhancing platelet aggregation.

Figure 2 | Regulation of filopodia by PKCs
Phosphorylation of Ser157 regulates the ability of VASP to promote actin uncapping and so filopodial extension. This phosphorylation is negatively regulated by PKCδ, which is constitutively associated with VASP in platelets. Regulation of filopodia is important in platelet aggregation and spreading.

VASP (vasodilator-stimulated phosphoprotein) is a key regulator of filopodial dynamics. VASP was first isolated from platelets as a 50 kDa protein that was phosphorylated in response to vasodilators such as nitric oxide and prostaglandin I2, agents that are also potent inhibitors of platelet activation and play an important role in preventing platelet aggregation in undamaged blood vessels [23]. VASP has been shown to be phosphorylated in platelets by PKA (protein kinase A) and PKG (protein kinase G) at Ser157 and Ser239 [24] and plays a role by negatively regulating platelet activation through these kinases [25,26].

Although VASP Ser157 is usually considered inhibitory due to its phosphorylation by PKA, collagen and thrombin, both potent platelet agonists, also stimulate phosphorylation of Ser157. This phosphorylation is partially PKC-dependent [27]. The isoform involved is likely to be a cPKC, since the phosphorylation can be prevented by Go6976 [27]. It is, however, negatively regulated by PKCδ, and is enhanced in PKCδ-deficient platelets or after treatment with rottlerin [13]. PKCδ appears to inhibit platelet aggregation by inhibiting VASP phosphorylation at Ser157, reducing filopodial extension (Figure 2).

Thus the evidence from knockout mouse models suggests that different PKC isoforms play specific roles in platelet function, with PKCβ and PKCδ being important in...
signalling between integrin αIIbβ3 and the actin cytoskeleton during platelet spreading, and PKCδ being a key regulator of filopodial formation. In these processes, there is little apparent evidence for functional redundancy between different isoforms since the loss of just one is sufficient to cause observable disruption. Interestingly, in none of these three PKC isoform-deficient strains was αIIbβ3 activation affected, despite the significant inhibitory effect of broad-spectrum PKC inhibitors on aggregation (see, e.g., [7]), perhaps suggesting some redundancy in integrin activation. Alternatively another isoform, such as PKCα, may be the major regulator of integrin activation, as has been suggested by a study in permeabilized platelets [28].

Conclusions
Platelets are an exciting system in which to study the roles of specific isoforms in a physiologically relevant setting that has immediate implications for human disease. The availability of mice deficient in each of the major PKC isoforms present in platelets makes it possible to assess directly their different functions in platelet activation. The sometimes-questionable selectivity of commonly used pharmacological inhibitors of PKC makes a genetic approach an invaluable complement to understanding how the different isoforms contribute to haemostasis and thrombosis.

References

Received 7 June 2007
doi:10.1042/BST0351005