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Protein kinase C mechanisms that contribute to cardiac remodelling

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Abstract

Protein phosphorylation is a highly-regulated and reversible process that is precisely controlled by the actions of protein kinases and protein phosphatases. Factors that tip the balance of protein phosphorylation lead to changes in a wide range of cellular responses, including cell proliferation, differentiation and survival. The protein kinase C (PKC) family of serine/threonine kinases sits at nodal points in many signal transduction pathways; PKC enzymes have been the focus of considerable attention since they contribute to both normal physiological responses as well as maladaptive pathological responses that drive a wide range of clinical disorders. This review provides a background on the mechanisms that regulate individual PKC isoenzymes followed by a discussion of recent insights into their role in the pathogenesis of diseases such as cancer. We then provide an overview on the role of individual PKC isoenzymes in the regulation of cardiac contractility and pathophysiological growth responses, with a focus on the PKC-dependent mechanisms that regulate pump function and/or contribute to the pathogenesis of heart failure.

Keywords

myocardial remodelling; post translational modification; protein kinase C

INTRODUCTION

Protein kinase C (PKC) isoenzymes transduce the myriad of signals resulting from receptor-mediated hydrolysis of phospholipids, playing critical roles in diverse cellular functions [1–5]. The discovery in the 1980s that PKCs are the receptors for the potent tumour-promoting phorbol esters, coupled with the discovery that they mediate signalling by the lipid second messenger diacylglycerol (DAG), secured a centre-stage position for this family of enzymes in cellular regulation [6,7]. The results of early studies showing that PKC activation by phorbol esters (or PKC overexpression) leads to cellular proliferation and enhanced cell survival is the basis of the prevailing dogma that PKCs contribute to the pathogenesis of certain cancers. In fact, a recent analysis of human cancer-associated mutations in PKC

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family enzymes reveals just the opposite: PKC isoenzymes function as tumour suppressors and their loss confers a survival advantage to cells [8]. This raises the question as to whether PKC inhibitors, which have failed in clinical trials for cancer may be better suited for treating heart failure or ischaemia-reperfusion injury, conditions where increased PKC isoenzyme expression or activity may adversely affect clinical outcome [9–14]. This review summarizes the mechanisms that regulate various PKC isoenzymes and then discusses current concepts regarding the role of PKC enzymes in the pathogenesis and treatment of heart disease.

PROTEIN KINASE C PRIMER

There are nine PKC genes that are classified into three subfamilies based on their domain composition and consequently the cofactor dependence of the proteins they encode (Figure 1). Conventional PKCs (cPKCs) (α, β, γ) are activated by DAG, which binds one of the tandem repeat C1 domains, and Ca^{2+}, which binds the Ca^{2+}-sensitive plasma membrane-targeting C2 domain. Second messengers stabilize cPKCs in an active conformation at membranes where they phosphorylate primarily membrane-delimited substrates. Novel PKCs (nPKCs) (δ,ε, θ, η) are activated by DAG; although they possess a C2 domain, nPKC C2 domains lack the key residues that coordinate Ca^{2+}. Some nPKC C2 domains bind lipids in a Ca^{2+}-independent manner, whereas the PKCδ C2 domain functions in a unique manner as a protein–protein interaction motif; the PKCδ C2 domain contains a phosphotyrosine (pY) binding motif that binds the consensus sequence (Y/F)-(S/A)-(V/I)-pY-(Q/R)-X-(Y/F) [15]. Atypical PKCs (ζ, I/λ) are not regulated by second messengers; rather their function is regulated as a result of precise positioning on protein scaffolds [16]. Indeed, the lack of regulation of atypical PKCs by second messengers suggests they should belong to their own distinct class of enzymes. Here we focus on the second messenger-regulated conventional and novel PKC isoenzymes – and particularly the ubiquitously expressed cPKC (α and β) and nPKC (δ and ε) isoenzymes that have been implicated as pathophysiologically important regulators in the heart.

Protein kinase C is primed by phosphorylation

Newly-synthesized PKCs are processed by a series of ordered conformational transitions and phosphorylations that result in a constitutively-phosphorylated species that is catalytically competent and ready to respond to second messengers (Figure 2). This species is autoinhibited but in a conformation that is relatively stable, resulting in a half-life of days for the conventional PKCs [17]. PKC that cannot be phosphorylated is rapidly degraded. Several molecules are necessary for the phosphorylation and thus stabilization of PKC; inhibition or loss of any one of them prevents PKC processing. First, the molecular chaperones Hsp90 and Cdc34 must bind to a molecular clamp between a conserved PXXP motif on the kinase domain and the C-terminal tail to permit PKC processing phosphorylations [18]. Second, the kinase complex mTORC2 performs an as yet unidentified function that is also required to permit PKC processing by phosphorylation [19–21]. Conventional PKCs cannot be processed by phosphorylation if either Hsp90 binding is prevented or if mTORC2 is absent or inhibited. The first phosphorylation is catalysed by the phosphoinositide-dependent kinase (PDK-1) on a segment near the entrance to the active site

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called the activation loop [22,23]. This triggers two phosphorylations on the C-terminal tail, at the turn motif and the hydrophobic motif. The turn motif has a highly conserved LTP motif and the hydrophobic motif is FXFSF [24]. At least *in vitro*, the hydrophobic motif site is a target for an intramolecular autophosphorylation [25]. Once cPKC isoenzymes have been processed by phosphorylation at all three sites, phosphate on the activation loop becomes dispensable [24,26]. The sites on the C-terminal tail are constitutively phosphorylated and are not a measure of the activation state of the enzyme.

Novel PKCs are likely processed by similar mechanisms with two notable exceptions. (1) PKCδ and PKCη do not have an LTP at their turn motif and this coincides with mTORC2-independent processing [20]. (2) PKCδ is fully processed and catalytically active without activation loop phosphorylation [27,28]. PKCδ activation loop phosphorylation is dynamically regulated through an autocatalytic mechanism that does not require PDK-1 [29]. Studies with a PKCδ mutant harbouring an activation loop non-phosphorylatable substitution indicates that activation loop phosphorylation plays a specific role to influence PKCδ's substrate specificity [30]. PKCδ functions as a Ser kinase (phosphorylates substrates with Ser residues at the phosphoacceptor site) without activation loop phosphorylation, but is converted into a serine/threonine kinase following activation loop phosphorylation [30].

Ligand affinity is tuned by intramolecular conformational changes

Optimal tuning of PKC activity is essential for maintaining cellular homoeostasis. Following its maturation by phosphorylation, PKC assumes an autoinhibited conformation that ensures minimal signalling in the absence of agonist. In this conformation, not only is the active site occluded by binding the pseudosubstrate, but the ligand binding modules are masked. Early work with PKCγ revealed that its C1 domains are masked in the full-length protein and become exposed upon Ca^{2+}-dependent membrane translocation [31]. Subsequent studies by Larsson and colleagues showed that the membrane translocation of unprocessed, kinase-dead PKCα is much more sensitive to DAG than wild-type enzyme [32] because of intramolecular interactions between the C-terminal tail and the C2 domain that masks the C1A domain [33]. Surface plasmon resonance analysis and phospholipid monolayer analyses on PKCα suggested that engagement of the C2 domain in the membrane induces yet another intramolecular conformational change [34] that allows the C1A and C1B domains to become more exposed and thus able to bind DAG [35]. Additional experiments visualizing conformational transitions in cells supported a model in which newly-synthesized PKC is in an open conformation in which its membrane targeting modules are fully exposed and thus can readily bind DAG. Subsequent processing phosphorylations induce a series of intramolecular conformational transitions that mask the ligand binding domains to optimally tune their affinity for second messengers and thus optimize PKC's dynamic range of signalling [36]. Recent re-interpretation of the crystal lattice packing of PKCβII reported by Hurley and co-workers [37] reveals the C2 domain interfaces with the kinase domain and C-terminal tail to clamp PKC in an autoinhibited conformation [38]. This closed, but catalytically competent conformation reduces the affinity of the C1 domains for DAG, thus tuning the ability of PKC to become activated in such a way that it is inactive in the absence of DAG, but quickly becomes activated upon its production.

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Down-regulation of PKC

Because PKC activity has to be exquisitely controlled in order to maintain cellular homoeostasis, diverse mechanisms ensure that PKC signalling is rapidly terminated following its induction. First, removal of DAG via its phosphorylation by DAG kinase ensures inactivation of PKC, with the decay in PKC activity following agonist stimulation tracking with the loss of DAG [39]. Second, sustained activation of PKC as occurs with phorbol esters, which lock PKC on the membrane, results in the disappearance of the enzyme, a process referred to as down-regulation. Specifically, in the ligand-engaged membrane-bound conformation, PKC adopts an open conformation that is readily dephosphorylated [40] at the hydrophobic motif by the PH domain leucine-rich repeat protein phosphatase (PHLPP) [41], which shunts it to the detergent-insoluble fraction where okadaic acid-sensitive phosphatases such as protein phosphatase 2A further dephosphorylate PKC at the activation loop and turn motif [42]. The isomerization of the turn motif of conventional PKCs by the peptidyl-prolyl isomerase Pin1 is necessary for conventional PKCs to become dephosphoryled [43]. This dephosphorylated species is then degraded [44] via ubiquitin-mediated degradation mediated in part by the RING-finger protein that interacts with C kinase (RINCK) E3 ligase [45]. Because the stability of PKC depends on its phosphorylation state, the steady-state levels of PKC are dictated by its phosphorylation. Binding to either the natural ligand DAG or to the potent phorbol esters induces the ubiquitination and degradation of PKC, suggesting that this is a physiological response that terminates PKC signalling [46]. PKCα has recently been shown to be sumoylated [47], a modification that prevents its ubiquitination and down-regulation [48]. Phosphorylated PKCα has also been reported be degraded in a proteasomal-independent manner by trafficking to the endosomal compartment where it can be degraded via lysosomes [49] or via a caveolae-mediated process [50].

Spatiotemporal dynamics of protein kinase C signalling

A number of distinct fluorescence resonance energy transfer (FRET)-based reporters have been developed to visualize the spatial and temporal dynamics of PKC signalling in real-time and with sub-cellular compartment resolution. For example, the C kinase activity reporter (CKAR), which monitors activity of all PKC isoenzymes, was developed based on an optimal, *de novo*, PKC substrate sequence [51]. Upon phosphorylation of this substrate by PKC, the phospho-peptide binding domain of the reporter binds the phosphorylated substrate sequence, leading to a conformational change and thus a change in FRET that is used as a read-out for PKC activity. Inhibition of PKC leads to dephosphorylation of the reporter by phosphatases, thus reversing the FRET change; this enables the reporter to monitor the antagonistic dynamics between PKC and phosphatases. Another reporter, KCP-1, is based on the PKC substrate pleckstrin and measures the activity of novel PKC isoenzymes [52]. The Eevee-PKCβ FRET biosensor was designed on an Eevee backbone that renders the FRET signal mostly distance dependent and excludes the orientation-dependent FRET signal because of a long flexible linker [53]. The presence of the C1 domain of PKCβ fused at the N-terminus of this biosensor allows simultaneous measurement of PKC activity and PKC translocation with one reporter. Isoenzyme-specific reporters such as δCKAR, a PKCδ-specific reporter, were later developed [54]. Considering that one of the limitations on PKC research is the lack of isoenzyme-specific modulators,

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such reporters provide a powerful approach to discerning the contribution of specific PKC isoenzymes and their redundancy.

Because FRET-based reporters are genetically encoded, they can be targeted to diverse intracellular compartments to specifically measure PKC activity at these sites. For example, pm-CKAR and Eevee-PKC-pm were targeted to the plasma membrane 51,53]. pmCKAR revealed that PKC activity oscillates on and off at the plasma membrane and that this is phase locked with oscillations in Ca^{2+} [51]. Studies with CKAR targeted to different subcellular regions revealed that PKC activity is more sustained at the Golgi compared with the plasma membrane or the cytosol and that this is attributable to the persistence of DAG at this membrane [55]. A nuclear-localized CKAR reporter identified PKC activity in the nucleus, although the levels of PKC activity in the nucleus are relatively low due to suppression by a high local level of phosphatase activity. A PKCδ-specific reporter targeted to the nucleus was used to establish that PKCδ translocates to the nucleus in a Src dependent, and a phorbol ester-independent, manner [54]. This isoenzyme was also shown to be active at the outer membrane of the mitochondria, where its catalytic activity is required for this interaction [56]. PKC activity reporters can also be targeted or fused to scaffolds in order to measure PKC activity at these sites. For example, CKAR fused to an A-kinase anchoring protein revealed that binding to a scaffold changes the pharmacological profile of PKC in that PKC becomes refractory to active site inhibitors while engaged on a scaffold [57]. In conclusion, these studies using targeted and/or isoenzyme-specific PKC reporters not only revealed that PKC is active at diverse subcellular compartments, but that the temporal dynamics of its signalling differ among these compartments based on the presence of second messengers and of phosphatases.

Tipping the balance: protein kinase C in disease

As described above, the signalling output of PKC is exquisitely controlled. Binding to molecular chaperones, peptidyl-prolyl isomerization, and post translational modifications such as phosphorylation, sumoylation and ubiquitination are among the mechanisms that control the steady-state levels of the enzyme. PKC activity is then dynamically controlled through conformational transitions that result from second messenger binding. Deregulation of any of the mechanisms that control PKC can contribute to pathophysiological phenotypes. Insufficient PKC activity tips the balance towards cancer, with mutations in human cancer thus being loss-of-function [8]. On the other hand, increased PKC activity has been identified in neurodegenerative diseases such as spinocerebellar ataxia 14 [58,59], Alzheimer’s disease [60,61], ischaemic neurodegeneration [62] and heart disease [11–13]. However, studies of PKC in various cardiac models emphasize that PKC actions are isoenzyme specific and that there are yet additional mechanisms such as cleavage and enzyme phosphorylation that ‘fine-tune’ PKC activity and contribute to certain pathological phenotypes. The following section elaborates on PKC isoenzyme regulation and function that have been identified in heart disease.

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PROTEIN KINASE C IN HEART DISEASE

Early studies established that cardiomyocytes co-express multiple PKC isoenzymes that display distinct subcellular localization patterns [5]. However, progress towards defining a precise functional role for any individual PKC isoenzyme has been slow, in large part because PKC enzymes phosphorylate a large number of cellular substrates, sit at nodal points in a large number of signal transduction pathways (including those activated by G protein-coupled receptors, receptor tyrosine kinases, integrin mediated cell–matrix adhesion pathways, mechanosensing pathways and oxidative stress) and play pleiotropic roles in a wide range of cellular responses. Progress has also been slow because many of the studies designed to interrogate PKC isoenzyme-specific function in the heart have relied on peptide inhibitors of enzyme translocation to membranes [63]. This approach is based upon the assumption that individual PKCs are active only when docked to their cognate receptor for activated C kinase (RACK), the specific scaffolding protein that functions to anchor an activated PKC isoenzyme to its specific membrane subdomain in close proximity to its target substrates. However, recent studies in cardiac models expose non-canonical mechanisms for PKC activation involving proteolytic cleavage or tyrosine phosphorylation (Figure 3). These mechanisms are triggered by oxidative/pro-apoptotic stresses and generate catalytically active 'rogue' kinases that can phosphorylate substrates throughout the cell, not just in membrane compartments. Studies that relied on translocation inhibitor peptides never considered possible alternative modes for PKC activation that would be refractory to this type of inhibition and might drive certain pathological cardiac phenotypes. This section interprets the results of previous studies that have attempted to deconvolute PKC isoenzyme-specific functions in the heart in the context of these newer concepts regarding PKC isoenzyme localization and activation.

cPKC isoenzymes (PKCα and PKCβ)

Early studies from the Molkentin laboratory showed that adenoviral-mediated PKCα overexpression induces hypertrophy in cultured neonatal rat ventricular cardiomyocytes [64]. Although these results were taken as evidence that PKCα acts as a direct inducer of the cardiomyocyte growth response, subsequent studies in more physiologically relevant mouse models indicated PKCα's primary/direct role is to regulate cardiac contractility; any links between PKCα and cardiac hypertrophy are the secondary consequence of changes in cardiac contractility. Specifically, Braz et al. [65] showed that the hearts of PKCα knockout mice are hypercontractile, but these mice hypertrophy in response to transverse aortic coarctation (TAC) induced pressure overload in a manner that is indistinguishable from the hypertrophic response displayed by wild-type mice. In fact, the enhanced cardiac contractility observed in PKCα knockout mice confers protection against TAC-induced heart failure. Conversely, mice with modest levels of PKCα overexpression develop contractile dysfunction; signs of decreased contractile performance are evident by ~4 months of age when the hearts are still grossly normal. The animals develop overt signs of cardiac hypertrophy only at 6–8 months, when contractile function has further deteriorated. These investigators then went on to show that PKCα regulates cardiac contraction by phosphorylating inhibitor-1, a negative regulator of protein phosphatase-1 (PP-1); PP-1 regulates cardiac contractility by dephosphorylating phospholamban (PLB), the negative

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regulator of the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA-2) pump in the sarcoplasmic reticulum (SR). The decrease in inhibitor-1 phosphorylation in PKCα knockout mice (and the resultant decrease in PP-1 activity and increased PLB phosphorylation) leads to enhanced SERCA-2 activity, increased calcium transients and enhanced cardiac contractility. Conversely, PKCα overexpression increases inhibitor-1 phosphorylation; the resultant increase in PP-1 activity leads to a decrease in PLB phosphorylation. Since hypophosphorylated PLB is an effective inhibitor of SERCA-2, this leads to a decrease in SR Ca^{2+} loading, SR Ca^{2+} release, and cardiac contractility.

Although studies in genetic models of PKCα gene deletion or PKCα overexpression in mice have focused on inhibitor-1 as the PKCα substrate that mediates changes in cardiac contractility, it is important to note that PKCα phosphorylates a large number of other cellular proteins that are predicted to also either directly or indirectly regulate cardiac contraction, including the α_{1C} subunit of the L-type calcium channel [66], various sarcomeric proteins that influence cardiomyocyte contraction and myocardial stiffness (including cardiac troponin I, cardiac troponin T, myosin binding protein C and titin [67–69]) and G protein-coupled receptor kinase 2 (GRK2); PKCα-dependent phosphorylation of GRK2 leads to enhanced enzyme activity and β-adrenergic receptor desensitization [70]. Studies to date have not formally excluded possible alternate roles for these PKCα substrates in the cardiac phenotypes that develop in these models.

Irrespective of mechanism, the observation that PKCα knockout mice develop a cardioprotective phenotype provided the rationale to examine whether pharmacological inhibitors of PKCα (the most abundant cPKC isoenzyme in cardiomyocytes [71,72]) could provide protection in various animal models of heart failure. Studies showing that cPKC-selective bisindolylmaleimide compounds such as Ro-32-0432 or ruboxistaurin (LY333531) enhance contractility and left ventricular developed pressure in wild-type and PKCβ/γ knockout, but not PKCα knockout mice, have been interpreted as evidence that these compounds exert their pharmacological effects by inhibiting PKCα (and not other PKC isoenzymes or related enzymes) and that PKCα is a primary negative regulator of cardiac contractility [71,73]. Subsequent studies showed that Ro-32-0432 enhances contractility in certain heart failure models [muscle LIM protein (MLP) gene deletion or Gαq overexpression] and that Ro-31-8220 (a related bisindolylmaleimide compound that can be produced more economically and therefore is better suited for long-term studies) rescues cardiac contractile function in MLP knockout^{-} mice [71]. Finally, the observations that the inotropic support that develops in response to PKCα inhibitors is well tolerated and that ruboxistaurin confers protection in a pig model of myocardial infarction-induced heart failure [74] have been touted as evidence that PKCα inhibitory compounds may be useful as novel therapeutics for some clinical models of heart failure in humans, particularly since ruboxistaurin has been used safely in large scale clinical trials in humans [75].

Efforts to develop PKCα-targeted compounds as novel therapeutics for cardiac indications will have to consider possible alternative modes for PKCα activation. In particular, there is evidence that PKCα can be activated via a non-canonical mechanism that does not involve translocation to membranes, but rather involves a proteolytic cleavage by calpains (calcium-activated cysteine proteases) in the calcium-overloaded ischaemic heart [76,77] (Figure 3,

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left). Calpain cleaves PKCα at a site in the V3 hinge region of the enzyme, liberating a C-terminal catalytic domain fragment (termed PKMα). PKMα acts as an unregulated/mislocalized 'rogue' kinase; it partitions to the nucleus where it phosphorylates histone deacetylase 5 (HDAC5, a signal-responsive repressor of myocyte enhancer factor 2 [MEF2]-dependent pathological gene programmes and cardiac remodelling). This neutralizes the anti-hypertensive actions of HDAC5, leading to de-repression of MEF2-dependent gene expression and bi-ventricular dilation and contractile dysfunction. A PKCα-targeted therapeutic must exhibit activity toward the PKCα catalytic domain fragment.

The cardiac actions of PKCβ – and particularly the notion that PKCβ also contributes to cardiac growth and/or pathological cardiac remodelling – are less convincing for several reasons. First, although some early studies identified PKCβ immunoreactivity in a number of cardiomyocyte preparations, this result was only variably reproduced by other investigators [78–80]. Issues related to the sensitivity/specificity of the antibodies used in the various studies never adequately resolved these discrepancies, which remain unresolved in the literature. In this context, there is general consensus that PKCα is the predominant PKC isoenzyme in cardiomyocytes [71,72]; it is expressed at levels that are considerably higher than the nPKCs (PKCε and PKCδ) or any PKCβ that might be co-expressed in these preparations. Second, although there is evidence that transgenic mice that overexpress high levels of PKCβ from birth through adulthood develop severe cardiomyopathic changes (including depressed contractility, muli-focal cardiac fibrosis and dystrophic calcification [81,82]), the physiological significance of these findings remains uncertain. This is because (a) studies in a binary transgenic mouse model (that allow for spatial and temporal control of PKCβ expression) implicate PKCβ in calcium cycling abnormalities and sudden death in the perinatal period, but only a relatively mild/progressive form of ventricular hypertrophy with impaired diastolic function but no gross histological evidence of pathological changes in the adult [83], and (b) studies in PKCβ knockout mice provide convincing evidence that PKCβ is not required for the induction of cardiac hypertrophy [73,84]. In fact, although there is literature showing that PKCβ is selectively activated in the setting of uncontrolled diabetes (due to either glucose-induced *de novo* synthesis of DAG from glycolytic intermediates or hyperglycaemia-induced generation of reactive oxygen species) [85,86], there is only limited evidence that PKCβ contributes to the pathogenesis of diabetes-associated cardiac dysfunction in certain experimental models of diabetes in rats [87,88]. Rather, ruboxistaurin (the PKCβ inhibitor compound developed by Eli Lilly and Company) has been evaluated as a therapy for diabetic retinopathy based upon the rationale that PKCβ is the predominant PKC isoenzyme in the retina and it is activated at an early stage in diabetes, before any clinical evidence of retinopathy [89,90]. Although early clinical trials with ruboxistaurin as a therapy for diabetic retinopathy were promising, Eli Lilly and Company dropped this compound from its development pipeline in 2006 when the FDA announced that it was unwilling to give approval without an additional 3 year, phase III, clinical trial that provides further evidence of efficacy. In the interim, there continues to be some interest in ruboxistaurin (a drug that actually also inhibits other cPKCs and PIM1 [73,91]) for the therapy of diabetic peripheral neuropathy or diabetic nephropathy, but attempts to develop ruboxistaurin for cardiac indications have been conspicuously absent.

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nPKC isoenzymes (PKCδ and PKCε)

Our current concepts regarding the cardiac actions of PKCε and PKCδ derive in large part from studies that rely on peptide translocation inhibitors to identify nPKC isoenzyme-specific actions or studies that characterize the phenotype of nPKC knockout mice. Although nPKCs exert pleiotropic effects on a range of structural and functional properties of the cardiomyocyte, studies to date have focused almost exclusively on nPKC-dependent events in mitochondria that mediate ischaemic preconditioning (IPC), a mechanism whereby transient ischaemic episodes protect against a subsequent severe ischaemia/reperfusion injury. Studies that rely on peptide translocation inhibitors to identify PKC isoenzyme-specific actions suggest antithetical roles for PKCε and PKCδ in cardioprotective and pro-apoptotic responses. These studies show that an activated form of PKCε localizes to mitochondria where it phosphorylates components of the mitochondrial IPC signalling machinery that contribute to cardioprotection [92] and that PKCδ-activated events in mitochondria contribute to ischaemia-reperfusion injury by decreasing intracellular pH and ATP production, increasing mitochondrial ROS accumulation, inhibiting the cytoprotective AKT-BAD phosphorylation pathway, and promoting the release of cytochrome *c*, the activation of caspase 3 and the induction of apoptosis [93–96]. However, it is worth emphasizing that experiments that rely exclusively on peptide translocation activators or inhibitors to identify PKC isoenzyme-specific function assume a single allosteric model for PKC enzyme activation by lipid cofactors (i.e. a lipid cofactor-dependent translocation mechanism that delivers the enzyme in an active conformation to DAG-enriched plasma membranes); this ignores a considerable literature showing that certain PKC isoenzymes (most notably PKCδ) can be recovered as lipid-independent enzymes in the soluble fraction of cardiomyocytes subjected to various forms of oxidative stress [97,98]. It also ignores evidence that PKCδ can contribute to receptor-dependent pathways that activate protein kinase D and thereby influence HDAC5- and/or CREB-dependent transcriptional programmes that promote cardiomyocyte growth/survival and induce cardioprotection [99–102]. In this context, it is worth noting that PKCδ and PKCε knockout mice have been available for ~15 years [103–105] but these mice have never been used to validate the assumption that the cardiac phenotypes that develop in response to treatment with nPKC translocation modifier peptides are due exclusively to the specific inhibition of nPKCs rather than some off-target actions.

Until quite recently, the studies that have attempted to define the cardiac actions of PKCδ and PKCε in knockout mouse models also have been inconclusive. For example, Mayr et al. reported that PKCδ knockout mice display proteomic and metabolomic evidence of altered energy metabolism, with a decrease in certain glycolytic enzymes and glycolytic end-products and an increase in several enzymes involved in lipid metabolism and end products of lipid metabolism [106] and that PKCδ knockout mice show a defect in their metabolic adaptation to ischaemia preconditioning [99]. However, mice lacking either PKCδ or PKCε alone do not develop any gross baseline cardiac phenotype. In fact, although some studies in PKCε knockout mice have been interpreted as evidence that PKCε plays a role in IPC [107], the results obtained in the PKCε knockout mouse model are undermined by the fact that these mice display a compensatory increase in PKCδ protein and activity [107,108].

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Recognizing that conventional gene ablation methods produce only very modest and/or uninterpretable phenotypes, Song et al. recently generated mice with combined postnatal cardiac-specific genetic ablation of PKCδ and germline deletion of PKCε as a strategy to deconvolute the cardiac actions of PKCδ and PKCε. These mice have no gross baseline cardiac phenotype by histology and or measurements of contractile performance. However, they display a markedly abnormal cardiac transcriptome, with changes in gene expression that are reminiscent of pathological cardiac hypertrophy. They also display an accelerated/exaggerated response to TAC-induced pressure overload; this is quite distinct from the responses to TAC-induced pressure overload in single PKCε or PKCδ knockout mice, which are indistinguishable from wild-type mice [109]. These investigators also showed that the growth limiting effects of PKCδ and PKCε are not restricted to the adult heart, since combined embryonic PKCδ/PKCε double knockout (but not single PKCδ or PKCε knockout) results in enhanced cardiomyocyte growth, pathological ventricular stiffening and early lethality. Collectively, these newer studies – in the context of previous experiments in cardiomyocyte cultures [29] – emphasize that the interpretation of any cardiac phenotype in single PKCδ or PKCε knockout models is confounded by the high level of signalling cross-talk and functional redundancy between nPKC isoenzymes. In stark contrast with the literature (derived largely from studies with translocation modifying peptide) which ascribes antithetical roles to PKCδ and PKCε in cardiac growth and ischaemia/reperfusion injury responses, these studies provide compelling evidence that PKCδ and PKCε play similar roles to limit cardiomyocyte growth and ventricular fibrosis/remodelling, and that the presence of either PKCδ or PKCε alone is sufficient to restrict the cardiac stress response to haemodynamic overload. These tonic effects of nPKCs to restrain cardiomyocyte growth responses might limit the efficacy of any nPKC-targeted inhibitor that displays less than optimal nPKC isoenzyme specificity.

Finally, any efforts to develop PKCδ-targeted therapeutics must consider an alternate mode for PKCδ activation during oxidative stress. Oxidative stress (which contributes to the pathogenesis of ischaemic injury) releases PKCδ from membranes and activates Src, which then phosphorylates PKCδ at Tyr^{311} [110]. (Note: Tyr^{311} in rodent PKCδ corresponds to Tyr^{313} in human PKCδ.) Studies that used molecular and biochemical approaches implicated Tyr^{311} phosphorylation as the mechanism underlying the redox-dependent increase in PKCδ catalytic activity, showing that PKCδ acts as a serine kinase (it shows a clear preference for substrates with a serine residue at the phosphoacceptor site) when allosterically-activated by lipid-cofactors and that PKCδ becomes a lipid-independent kinase that phosphorylates substrates with either serine or threonine residues at the phosphoacceptor site (i.e. it becomes a serine/threonine kinase) when Tyr^{311} is phosphorylated by Src [30]. PKCδ's unique C2 domain (which has been characterized as a phosphotyrosine binding module [15]) functions as an additional structural determinant of the Tyr^{311} phosphorylation-dependent change in PKCδ's enzymology (Figure 3, right). Tyr^{311} in PKCδ's V3 hinge region is flanked by an optimal C2 domain consensus binding motif. Hence, Src-induced phosphorylation of PKCδ at Tyr^{311} generates a binding site for the C2 domain. The redox-induced C2 domain–pTyr^{311} interaction controls PKCδ catalytic activity indirectly by facilitating PKCδ dephosphorylation at a novel phosphorylation site in the highly conserved Gly-rich ATP-positioning loop (G-loop) sequence at Ser^{357} (Ser^{357} in the rodent sequence

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corresponds to Ser359 in human PKCδ [111]). PKCδ displays a high level of Ser357 phosphorylation in resting cells; oxidative stress promotes the docking interaction between the C2 domain and the Tyr311 phosphorylated hinge region of the enzyme and leads to decreased G-loop Ser357 phosphorylation. Mutagenesis studies implicate Ser357 dephosphorylation as the mechanism underlying the redox-dependent change in PKCδ's enzymology, showing that PKCδ-S357A is a constitutively active lipid-independent enzyme and that the non-phosphorylatable S357A substitution facilitates (and a phosphomimetic S357E substitution completely abrogates) PKCδ's Thr kinase activity [111]. These results identify G-loop Ser357 phosphorylation as a novel dynamically-regulated mechanism that controls PKCδ's lipid-requirement for activation and its phosphoacceptor site specificity. Importantly, the redox-dependent G-loop Ser357 dephosphorylation mechanism accounts for the cellular actions of PKCδ in cells subjected to oxidative stress, where PKCδ phosphorylates substrates throughout the cell (not just on lipid membranes).

SUMMARY

This review summarizes our current understanding of the various maturational and stimulus-induced mechanisms that regulate signalling by individual PKCs and the role of individual PKCs in the pathogenesis of cardiac disorders. It is important to note that this narrative is based upon an experimental literature that considers mechanisms for individual PKCs separately, but these enzymes are part of elaborate and complex signalling networks in cells. Understanding the ensemble effects of the many individual PKC-driven events that contribute to the pathogenesis of heart disease and other clinical disorders presents both challenges and opportunities for the development of novel PKC-targeted therapeutics.

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Abbreviations

AGC kinases	protein kinases A, G and C
DAG	diacylglycerol
GRK2	G protein-coupled receptor kinase 2
HDAC	histone deacetylase
MLP	muscle LIM protein
PDK-1	phosphoinositide-dependent kinase-1
PH	pleckstrin homology
PHLPP	PH domain leucine-rich repeat protein phosphatase

PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLB	phospholamban
PP-1	protein phosphatase-1
SERCA-2	sarcoplasmic reticulum Ca^{2+} ATPase
SR	sarcoplasmic reticulum
TAC	transverse aortic coarctation
TORC2	target of rapamycin complex 2

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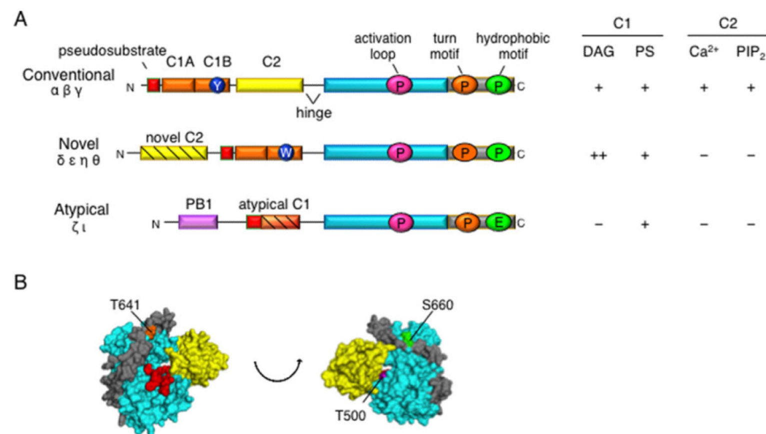

Figure 1. The domain structure of PKC family enzymes

(**A**) Schematic showing pseudosubstrate (red rectangle), C1 domain (orange rectangle; atypical C1 domain is hatched to indicate it is not a DAG sensor), C2 domain (yellow rectangle; novel C2 domain is hatched to indicate it is not a Ca^{2+}-driven plasma membrane sensor), connecting hinge segment, kinase domain (cyan) and C-terminal tail (CT, grey rectangle). Also indicated are the Y/W switch (purple circle) in the C1B domain that dictates affinity for DAG-containing membranes and the three priming phosphorylations in the kinase domain and CT (note atypical PKC isoenzymes have Glu at phospho-acceptor position of hydrophobic motif). Atypical PKCs have a PBI domain that mediates their interaction with protein scaffolds. Table on right shows dependence of PKC family members on C1 domain cofactors, DAG and phosphatidylserine (PS) and C2 domain cofactors, Ca^{2+} and PIP_2. Adapted from [112]: Antal, C.E. and Newton, A.C. (2014) Tuning the signaling output of protein kinase C. Biochem. Soc. Trans. **42**, 1477–1483. (**B**) Reinterpreted structure of PKC*β*II [38] from lattice packing [37] showing intramolecular autoinhibition by the C2 domain (yellow), which clamps over the kinase domain (cyan), interfacing also with the C-terminal tail (grey). Also shown is the pseudosubstrate (red), which was modelled into the substrate-binding cavity. Adapted from [38]: Antal, C.E., Callender, J.A., Kornov, A.P., Taylor, S.S. and Newton, A.C. (2015) Intramolecular C2 domain-mediated autoinhibition of protein kinase C*β*II. Cell Rep. **12**, 1252–1260.

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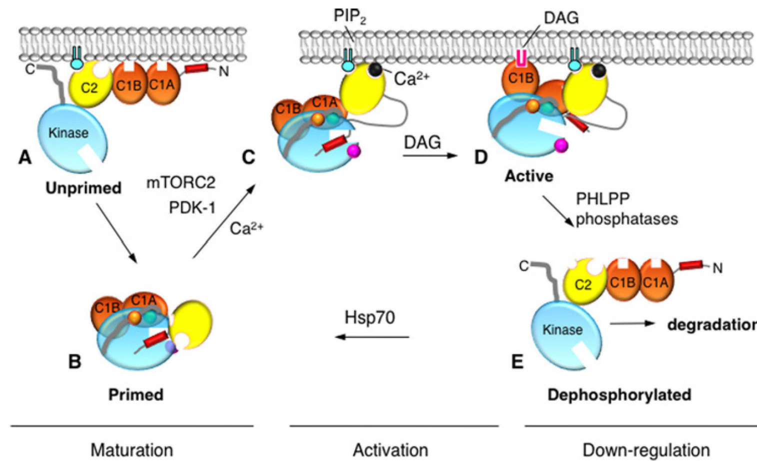

Figure 2. Model for regulation of conventional PKC by phosphorylation and second messengers

(**A**) Unprimed PKCβII is in a membrane-associated, open conformation in which its C1A, C1B and C2 domains are fully exposed and the pseudosubstrate and C-terminal tail are unmasked. (**B**) Upon priming phosphorylation at its activation loop (Thr^{500}, magenta) by PDK-1, followed by autophosphorylation at the turn motif (Thr^{641}, orange) and the hydrophobic motif (Ser^{660}, green), PKCβII adopts a closed conformation in which the C2 domain interfaces with the kinase domain and traps the pseudosubstrate into the substrate-binding site, both C1 domains become masked, and the primed enzyme localizes to the cytosol. This autoinhibition and masking of second messenger sensors ensures efficient suppression of activity in the absence of appropriate stimuli. (**C**) In response to agonists that promote PIP_2 hydrolysis, Ca^{2+} binds the C2 domain of cytosolic PKCβII via a low affinity interaction such that upon the next diffusion-controlled membrane encounter, the Ca^{2+}-bound C2 domain is retained at the plasma membrane via Ca^{2+}-bridging to anionic lipids and binding to PIP_2. This rearrangement of the C2 domain is accompanied by unmasking of the hinge connecting the C2 domain and the kinase domain. (**D**) Membrane-targeted PKC binds the membrane-embedded ligand, DAG, predominantly via the C1B domain, resulting in release of the pseudosubstrate from the substrate-binding cavity, thereby activating PKC. Only one of the C1 domains binds DAG in the membrane at a time. (**E**) Activated PKC is in a conformation in which the phosphorylation sites are exposed, resulting in dephosphorylation at the hydrophobic motif by PHLPP and at the turn motif and activation loop by okadaic-sensitive phosphatases such as PP2A. The dephosphorylated enzyme is shunted to degradation. However, binding of Hsp70 to the dephosphorylated turn motif permits PKC to become rephosphorylated and re-enter the pool of signalling-competent PKC.

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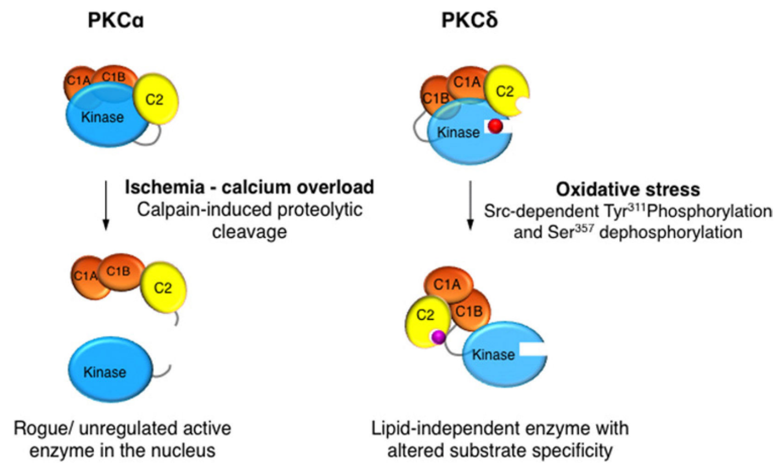

Figure 3. Non-canonical PKC activation mechanisms: proteolytic cleavage of PKCα and tyrosine phosphorylation of PKCδ

Left: Calpain cleavage of PKCα at a site in the hinge region results in the release of a catalytically active kinase domain fragment that targets to the nucleus and induces pathological cardiac remodelling. *Right:* PKCδ is recovered from resting cardiomyocytes as a Ser^{357}-phosphorylated/lipid-dependent serine kinase; the pink circle denotes phosphorylation at Ser^{357} in the ATP-positioning G-loop in the kinase domain. Oxidative stress leads to the activation of Src family kinases and the phosphorylation of PKCδ at Tyr^{311} (denoted by the purple circle). This generates a docking site for the phosphotyrosine binding C2 domain, results in a C2 domain–$pTyr^{311}$ interaction that induces long-range conformational changes that culminate in Ser^{357} dephosphorylation. PKCδ is converted into a lipid-independent serine/threonine kinase as a result of the decrease in G-loop Ser^{357} phosphorylation.