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Protein Kinase C as Regulator of Vascular Smooth Muscle Function and Potential Target in Vascular Disorders

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Abstract

Vascular smooth muscle (VSM) plays an important role in maintaining vascular tone. In addition to Ca^{2+}-dependent myosin light chain (MLC) phosphorylation, protein kinase C (PKC) is a major regulator of VSM function. PKC is a family of conventional Ca^{2+}-dependent α, β, and γ, novel Ca^{2+}-independent δ, ε, θ, and η, and atypical ξ, and ι/λ isoforms. Inactive PKC is mainly cytosolic, and upon activation it undergoes phosphorylation, maturation and translocation to the surface membrane, the nucleus, endoplasmic reticulum, and other cell organelles; a process facilitated by scaffold proteins such as RACKs. Activated PKC phosphorylates different substrates including ion channels, pumps and nuclear proteins. PKC also phosphorylates CPI-17 leading to inhibition of MLC phosphatase, increased MLC phosphorylation and enhanced VSM contraction. PKC could also initiate a cascade of protein kinases leading to phosphorylation of the actin-binding proteins calponin and caldesmon, increased actin-myosin interaction and VSM contraction. Increased PKC activity has been associated with vascular disorders including ischemia-reperfusion injury, coronary artery disease, hypertension, and diabetic vasculopathy. PKC inhibitors could test the role of PKC in different systems, and could reduce PKC hyperactivity in vascular disorders. First generation PKC inhibitors such as staurosporine and chelerythrine are not very specific. Isoform-specific PKC inhibitors such as ruboxistaurin have been tested in clinical trials. Target-delivery of PKC pseudosubstrate inhibitory peptides and PKC siRNA may be useful in localized vascular disease. Further studies of PKC and its role in VSM should help design isoform-specific PKC modulators that are experimentally potent and clinically safe to target PKC in vascular disease.

Keywords

blood vessels; signaling; calcium; protein kinases; contraction; hypertension

1. INTRODUCTION

Protein Kinase C (PKC) is a ubiquitous enzyme found in almost all cell types including the endothelium, VSM and fibroblasts of blood vessels. PKC phosphorylates serine and

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CONFLICT OF INTEREST
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threonine residues in a large number of protein substrates and regulates many cellular processes. PKC exhibits significant and sometimes opposite effects in different tissues, and is widely implicated in multiple physiological and pathological processes. The versatility of the effects of PKC is best illustrated in the observation that it could induce both vascular contraction and relaxation. For instance, PKC may mediate the release of endothelium-derived contracting factors such as endothelin-1 (ET-1) and promote vasoconstriction, but could also mediate endothelial nitric oxide (NO) synthesis and promote vasodilation (Wang et al., 2015). PKC could also affect vascular fibroblasts causing increases in transforming growth factor-β, and extracellular matrix (ECM) production, thus promoting vascular remodeling (Geraldes and King, 2010; Ding et al., 2011a). In addition to its effects on the endothelium and ECM, PKC plays a major role in the regulation of VSM function.

Several excellent reviews have discussed many of the biochemical aspects of PKC and its substrates (Nishizuka, 1992; Kanashiro and Khalil, 1998; Newton, 2010; Mochly-Rosen et al., 2012; Khalil, 2013; Mukherjee et al., 2016). Also, the multiple effects of PKC in different cellular processes have made it an important target in many diseases. Understanding the basic biochemical properties of PKC and its effects in the vascular system should help to provide the basis for targeting PKC in different vascular disorders. The purpose of this chapter is to highlight the role of PKC as a major regulator of VSM function with emphasis on recent discoveries and their relevance to vascular disease. We used data published in PubMed and other databases, as well as data from our laboratory to first provide a brief background on PKC biochemistry, its different isoforms, tissue distribution, substrates, and different activators and inhibitors. We will discuss some findings that challenged the concept that PKC translocation is necessary for its activation, and other theories on how to modulate PKC activity by targeting different sites in its regulatory and catalytic domains. We will then discuss the potential role of PKC in vascular disorders and the potential benefits of PKC inhibitors in the management of vascular disease. While the focus of the chapter is on PKC in VSM, in the instances that there is little information available in VSM, the effects of PKC on other systems will be described.

2. PKC STRUCTURE AND ISOFORMS

In 1977, Nishizuka and colleagues discovered PKC in rat brain extract (Takai et al., 1977). PKC was initially defined as a kinase that is activated by proteolysis, but was soon found to be activated by diacyglycerol (DAG) (Takai et al., 1979), and later by phorbol ester, a tumor promoter (Castagna et al., 1982). The PKC molecule comprises a N-terminal regulatory domain and a C-terminal catalytic domain between which lies the V3 hinge region (Kishimoto et al., 1989) (Fig. 1). The conventional PKC isoforms α, βI, βII, and γ have four conserved regions (C1, C2, C3 and C4) and five variable regions (V1, V2, V3, V4, and V5). The regulatory domain is comprised of the two conserved C1 and C2 regions. The C1 region contains cysteine-rich zinc finger-like motifs as well as lipid-binding sites surrounded by a band of hydrophobic residues that penetrate the lipid bilayer and provide a strong driving force to anchor PKC to DAG-containing membranes. In addition to the membrane-docking interactions caused by binding of the C1 region to DAG, full-length PKC may stably associate with membranes by a second membrane-targeting interaction involving the C2 region (Steinberg, 2008). Furthermore, the C1 region binds the PKC cofactor

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phosphatidylserine (PS) (Kim et al., 2013), and PS may also bind to the C2 region (Poli et al., 2014). An autoinhibitory pseudosubstrate sequence, common to all protein kinases, is embedded in the N-terminal regulatory domain immediately preceding the C1 region and has an amino acid sequence between residues 19 and 36 that resembles the PKC substrate phosphorylation site (Newton, 2001). The catalytic or kinase activity domain contains a conserved C3 region, an ATP/Mg-binding site in a narrow hydrophobic pocket, and a binding site for the phospho-acceptor sequence in the substrate (Mochly-Rosen et al., 2012). The C4 region constitutes the substrate-binding part of the PKC molecule (Newton, 1995). The catalytic domain also contains three key phosphorylation and autophosphorylation sites in the C-terminal activation loop, turn-motif and hydrophobic-motif.

PKC belongs to the AGC family of serine/threonine protein kinases that are related at their primary sequence and are named after the first identified 'founding members' cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA), cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG) and PKC (Linch et al., 2014). PKC is the largest serine/threonine kinase family, comprising ~2% of the human kinome (Mellor and Parker, 1998). The PKC family is encoded by nine different genes and consists of 10 isoforms (Hoque et al., 2014). The N-terminal regulatory domain contains a highly 60–80% homologous C1 region among different PKC isoforms (Mochly-Rosen et al., 2012). Based on the structure of the N-terminal domain, PKC isoforms are classified into conventional cPKCs α, βI, βII, and γ; novel nPKCs δ, ε, η and θ; and atypical aPKCs ζ and ι/λ isoforms (Salamanca and Khalil, 2005) (Fig. 1). The cPKCs consist of four conserved regions (C1–C4) and five variable regions (V1–V5), and are activated by calcium (Ca^{2+}), DAG, and PS. The C1 region contains the recognition site for PS, DAG, and phorbol esters while the C2 region is rich in acidic residues and contains the binding site for Ca^{2+} (Newton, 1995). In cPKCs, the C2 region comprises ~130 residue eight-stranded anti-parallel β-sandwich structures with three inter-strand Ca^{2+}-binding loops responsible for Ca^{2+}-dependent anionic phospholipid binding (Steinberg, 2015). Both cPKCs and nPKCs have twin C1 regions (C1A and C1B) and a C2 region, but the ordering of the C1 and C2 regions is switched in nPKCs compared to cPKCs (Steinberg, 2015). Also, nPKCs have a variant C2 region that lacks the critical Ca^{2+}-coordinating aspartic acid residues that are highly conserved in cPKCs, making it insensitive to Ca^{2+} (Newton, 2001). As a result, the nPKC C2 region does not bind Ca^{2+}, and nPKCs are activated by DAG and PS, but not Ca^{2+}. The C1 region of nPKCs has a higher affinity for DAG than the C1 region of cPKCs, and functions as a lipid-binding membrane-targeting module in a Ca^{2+}-independent manner (Dries et al., 2007). The C2 region of PKCδ does not bind lipids, but has a protein–protein interaction domain that binds phospho-tyrosine residues flanked by the consensus sequence (Y/F)-(S/A)-(V/I)-pY-(Q/R)-X-(Y/F). PKCδ contains several tyrosine phosphorylation sites throughout its structure, including in the highly conserved regulatory and kinase domains and the intervening more flexible variable hinge region, most of which are unique to PKCδ (Benes et al., 2005). The aPKCs do not have a C2 region but have a variant form of C1 and are therefore activated by PS but not Ca^{2+} or DAG (Newton, 2001). However, aPKCs do retain lipid-binding activity, and the C1 region confers DAG binding that is not duplicated, unlike the C1A–C1B tandem repeat found in cPKCs and nPKCs (Linch et al., 2014). The aPKCs also uniquely encode the protein–protein-interacting Phox and Bem 1 (PB1) region

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in the N-terminus domain, which binds ZIP/p62, Par6, or MEK5 through a PB1-PB1 domain interaction that controls the localization of aPKCs (Hirano et al., 2004).

PKCμ and PKCν are often considered a fourth class of PKC isoforms or members of a different family called protein kinase D (PKD) (Rozengurt, 2011; Poli et al., 2014). Other PKC-related-kinases (PRKs) include PRK1–3 and are also considered a fourth group of the PKC family (Mellor and Parker, 1998). PRKs are similar in structure to PKCs except for the C1 region, but do not bind Ca^{2+}, DAG, or phorbol esters, and have homology region 1 (HR1) motifs responsible for RhoA binding (Hage-Sleiman et al., 2015).

The greatest homology between PKC isoforms is in the highly conserved catalytic domain (~70%). Also, similar to other Ser/Thr protein kinases, PKC isoforms have a highly conserved ATP-binding site. The exception to the catalytic domain homology is the variable V5 region, consisting of 60–70 different amino acids. PKC isoforms also differ in the intervening V3 hinge region and the C2 region of the regulatory domain (Mochly-Rosen et al., 2012). PKCβI and βII are generated by alternative splicing from a single gene, but differ at their C-terminal 50 (βI) or 52 (βII) residues (Newton, 1995). The amino acid for each phosphorylation site also varies in different PKCs. For example, the activation loop contains a phosphorylatable Thr497 in PKCα, T500 in PKCβII, T505 in PKCδ and T538 in PKCθ, and the turn motif contains a T638 in PKCα, T641 in PKCβI and PKCβII, S643 in PKCδ and S676 and S685 in PKCθ, while the hydrophobic motif contains a Ser657 in PKCα, S660 in PKCβII, S662 in PKCδ and S695 flanked by bulky hydrophobic residues in PKCθ (Xu et al., 2004; Steinberg, 2008).

3. PKC DISTRIBUTION AND TRANSLOCATION

PKCs are found in varying amounts in different tissues and cells, including various vascular beds. PKCα, δ and ζ are universally expressed in almost all blood vessels examined, while other PKCs show specific distribution in specific vascular beds (Kanashiro and Khalil, 1998; Khalil, 2013) (Table 1). In human VSMCs, the expression of PKCα, β, δ and ε, but not PKCζ, is relatively high (Grange et al., 1998). In endothelial cells, however, the levels of PKCδ are lower than PKCζ, demonstrating how PKC distribution varies depending on the vascular cell type (Mattila et al., 1994; Magid and Davies, 2005). In resting cells, PKCα, β and γ, are localized mainly in the cytosolic fraction, and activated PKC undergoes translocation from the cytosolic to the particulate and membrane fraction (Kraft and Anderson, 1983) (Fig. 2); although other PKCs may show redistribution in specific cell membranes (Khalil, 2013).

The mechanisms of PKC movement from the cytosol to the membrane are not fully understood. Simple diffusion and other physico-chemical forces may drive the movement of PKC inside the cell, while specific targeting mechanisms would allow its translocation to different cell membranes and tight binding to its target location. Targeting mechanisms include conformation changes, altered hydrophobicity, lipid modification, protein-protein interaction, targeting sequences, and phosphorylation (Saito et al., 2003; Khalil, 2013).

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Binding of Ca^{2+} or DAG may cause conformational changes that unfold the PKC molecule and result in exposure of the substrate region, increased PKC hydrophobicity and binding to membrane lipids (Newton, 1995). Changes in the plasma membrane lipid domains could also influence the subcellular distribution of PKC. The VSM plasma membrane is composed of several domains of focal adhesions alternating with zones rich in caveolae, and both harbor a subset of membrane-associated proteins. Caveolae appear to be a major cell surface location for PKC. For instance, PKCα is constitutively present and exhibits binding activity in caveolae, and does not bind to non-caveolae membranes, which constitute over 90% of the plasma membrane (Mineo et al., 1998). Local fluctuations in [Ca^{2+}] may affect the amount of a specific PKC isoforms retained in caveolae, reflecting the ion requirement for PKC binding to caveolae. For instance, caveolae contain PKCα only in the presence of Ca^{2+}, and PKCλ only in the absence of Ca^{2+} from the isolation buffer, while retention of PKCε in caveolae is not dependent on Ca^{2+} (Mineo et al., 1998). Caveolins are scaffold proteins that could help PKCα and ζ localize to the caveolar microdomains where they are subsequently activated (Oka et al., 1997). In rabbit femoral and renal arteries at rest, PKCζ is localized in punctate plasma membrane aggregates alternating with vinculin (supporting its location in caveolae), and in a perinuclear location, and these locations may be conducive to regulating VSM $[Ca^{2+}]_i$ (Ratz and Miner, 2009).

The plasma membrane lipids are also segregated into cholesterol-rich lipid rafts and glycerophospholipid-rich non-raft regions, an arrangement that is critical for preserving the membrane protein architecture and for the translocation of proteins. In VSMC membrane, lipid segregation is supported by annexins that target membrane sites of distinct lipid composition, and each annexin requires different [Ca^{2+}] for its translocation to the plasma membrane, thus allowing a spatially confined graded response to external stimuli and plasmalemmal localization of PKC (Draeger et al., 2005). Several members of the annexin family function as PKC substrates and can promote membrane association of PKC (Dubois et al., 1996; Xu and Rumsby, 2004). PKC isoforms interact with unique members of the annexin family, and PKCβ, ε and α interact with annexin I, II and VI, respectively (Mochly-Rosen et al., 1991). Also, a transient interaction between annexin V and PKCδ occurs in cells after PKCδ stimulation, but before its translocation to the particulate fraction, suggesting that PKCδ requires binding to annexin V for its translocation, and whether other PKCs require annexin binding before translocation is unclear (Kheifets et al., 2006).

Myristoylated alanine-rich C kinase substrate (MARCKS) may play a role in PKC membrane binding. MARCKS is a major PKC substrate that is bound to F-actin and may function as a cross-bridge between cytoskeletal actin and the plasma membrane (Hartwig et al., 1992). Phosphorylation of MARKS by PKC may have an electrostatic effect that affects its protein affinity to the plasma membrane and consequently interferes with its actin cross-linking and causes its displacement from the plasma membrane. On the other hand, dephosphorylation of MARCKS causes its re-association with the plasma membrane via its stably attached myristic acid membrane-targeting moiety (Thelen et al., 1991).

Protein-protein interactions are crucial in signal transduction, and binding sites for arginine-rich polypeptides have been identified in the PKC molecule distal to its catalytic site, allowing targeting of PKC to precise substrates at specific cellular locations. Scaffold

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proteins could participate in the compartmentalization of PKC to the membrane, and include receptor for activated C kinase (RACK), substrates that interacts with C kinase (STICK), receptor for inactive C kinase (RICK), and A-kinase activating proteins (AKAPs) (Ron and Kazanietz, 1999). RACKs and STICKs bind to active PKCs, whereas RICKs and AKAPs interact with inactive PKCs. Binding of a specific activated PKC to its RACK provides access to and phosphorylation of its substrates (Qvit and Mochly-Rosen, 2010). Also, the binding of RACK increases the phosphorylation capacity of PKC several-fold independently from the substrate identity (Ron and Mochly-Rosen, 1994). RACKs may also target PKC to cytoskeletal elements (Ron and Mochly-Rosen, 1994). The interaction of PKC and RACK is isoform specific and is largely mediated by the C2 region of cPKCs (Ron D, 1995), and peptide fragments of this region have been developed as modulators of PKC activity (Mochly-Rosen and Kauvar, 2000). These short peptides induce activation and translocation of the corresponding PKC isoform by mimicking the action of the RACK on the isoform and, therefore, are termed 'pseudo RACKs' (ΨRACK) (Dorn et al., 1999; Churchill et al., 2009). Disruption of the interaction between the ψεRACK and the RACK-binding site is a critical rate-limiting step in translocation of PKCε (Schechtman et al., 2004). Other scaffold proteins including 14-3-3, heat shock protein (HSP), importins, and even actin, have been suggested to tether PKC isozymes to organelles and membranes (Toker et al., 1990; Prekeris et al., 1996; Welch et al., 2010; Adwan et al., 2011; Lum et al., 2013). Additional unique protein-protein interactions between specific PKC isoforms and their substrates might provide further anchoring and specificity at the subcellular sites. For instance, for PKCε, a myofilament-binding site in the C2 region (Huang and Walker, 2004), an intra-sarcoplasmic reticulum calsequestrin-binding site (Rodriguez et al., 1999), a neurocytoskeletal elements-binding site (Zeidman et al., 1999), a unique actin-binding site within the C1 region of εPKC and a Golgi-binding site (Prekeris et al., 1996; Csukai et al., 1997) have been identified (Churchill et al., 2009). PKCε exhibits a unique association with Golgi membranes via its zinc finger domain and specifically modulates Golgi function, and the zinc finger domain might act as a specific localization signal (Lehel et al., 1995a). PKCε may also translocate from Golgi to the plasma membrane by two distinct mechanisms; as a rapid, vesicle-independent process was observed with holo PKCε (which requires the presence of the pseudosubstrate and/or hinge regions), and a slow, vesicle-dependent pathway was observed with the zinc finger fragment (Lehel et al., 1996).

Different parts of the PKC molecule play varying roles in mediating PKC activation and translocation. The pseudosubstrate and hinge regions facilitate plasma membrane and cytoskeletal association (Lehel et al., 1995b). Also, the V5 variable region contributes to the regulation of PKCα activity through multiple mechanisms involving stabilizing the kinase through direct interactions with its N-terminal, interacting with the pseudosubstrate in the N-terminal regulatory domain, and mediating subcellular localization through interaction with RACK (Yang and Igumenova, 2013).

Although the interaction of cPKCs at the plasma membrane has been well-studied, less is known about the activity of nPKCs and aPKCs at the plasma membrane and other cell membranes including those of the nucleus and other cell organelles such as the mitochondria, endoplasmic reticulum (ER) and Golgi (Fig. 3). For instance, c-src-dependent phosphorylation of tyrosine Y256 in PKCι, through enhanced interaction with the nuclear

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transporter protein importin-β, results in its translocation to the nucleus (White et al., 2002). Also, the ER membrane may represent the main target for PKCδ recruitment rather than Golgi or mitochondrial membranes. Other nPKCs such as PKCε also display the same translocation pattern following ATP binding. Considering the importance of the ER in protein synthesis and modification, the functions of nPKCs at the ER membrane need further investigations (Hui X, 2014).

While translocation to cell membranes was traditionally considered the hallmark of PKC activation, this allosteric model for PKC activation by lipid cofactors and the concept that membrane translocation is essential to PKC activation have been challenged. For instance, the model assumes that the cellular actions of PKC are membrane-limited, as it focuses on the role of lipid cofactor in mediating translocation and delivery of the enzyme to the membranes. However, PKC may be localized in the cell periphery in both resting and stimulated tissues. Immunohistochemical studies have shown that the distribution of PKCα in the longitudinal and circular layers of the swine stomach tonic fundus and phasic antrum under resting conditions does not differ, being predominantly localized near the smooth muscle plasma membrane, and stimulation of either tissue with PDBu or carbachol does not alter this peripheral PKCα distribution (Zhang et al., 2013). Also, PKC is found in other cell compartments like the mitochondria and soluble fraction of cells subjected to oxidative stress, a known activator of PKC (Konishi et al., 2001; Steinberg, 2015). PKCs in the soluble fraction of VSM could also phosphorylate sarcomeric proteins in the contractile apparatus, located distant from the membrane lipid bilayer (Steinberg, 2015). PKC translocation may also be dependent on cytoskeletal elements and active transport along the cytoskeleton, suggesting that other forms of protein-protein interactions may be involved in the translocation process (Schmalz et al., 1996; Dykes et al., 2003; Kheifets and Mochly-Rosen, 2007). Another misconception of the canonical model of PKC activation is related to the concept that PKC's catalytic activity is an inherent property of the enzyme that is not altered by the activation process; a model that does not adequately explain the diverse and, in some cases, opposing actions of certain PKCs (Steinberg, 2015).

4. PKC PHOSPHORYLATION

In the inactive state of PKC, the pseudosubstrate binds the catalytic site in the C4 region, and both the regulatory and catalytic domains are folded together (House and Kemp, 1987). In the activated state, the PKC molecule is unfolded, the pseudosubstrate is dissociated from the C4 region, and PKC is ready to target its specific substrate. Before it becomes catalytically competent and able to respond to its allosteric activators, nascent PKCs undergo conformational changes at three conserved serine/threonine residue phosphorylation sites in the C-terminal domain (Newton, 1995) (see Fig. 2). Phosphorylation could change protein conformation or electric charge and consequently affect its lipid affinity and binding to the plasma membrane. Phosphorylation of PKC itself via autophosphorylation or by a putative PKC kinase may determine its localization. The cPKCs, nPKCs and probably aPKCs are dependent to varying degrees on phosphorylation of the sites at the activation loop, turn motifs, and hydrophobic motif (Parekh et al., 2000; Newton, 2003). These phosphorylations are thought to keep PKC in a catalytically competent and protease resistant conformation. Full activation of PKC by allosteric activators induces an open conformation that makes the

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enzyme susceptible to both phosphatases and proteases, leading to either repeated autophosphorylation/dephosphorylation cycles, or proteolytic degradation of the PKC molecule and the synthesis of *de novo* enzyme (Parekh et al., 2000; Newton, 2010).

The first and rate-limiting phosphorylation of the activation loop at the conserved threonine, is catalyzed by phosphoinositide-dependent kinase (PDK), and is critical for activation of PKC (Le Good et al., 1998; Newton, 2001). In the absence of PDK-1, PKC is prone to rapid degradation before turning into catalytically competent enzyme (Balendran et al., 2000). Mutation of phosphorylatable Thr-residues in the activation loop abolishes PKC activity, supporting its essential role in PKC activation (Cazaubon et al., 1994; Liu et al., 2002). As a result of phosphorylation of the activation loop, a negative charge is introduced that properly aligns residues to form a competent catalytic domain and facilitate the subsequent autophosphorylation of 2 sites in the C-terminus, one at the ‘turn motif’, so named because it corresponds to a phosphorylation site in PKA localized at the apex of a turn, and the other at the more C-terminal hydrophobic motif (Behn-Krappa and Newton, 1999). The hydrophobic motif is an important and direct mediator of PKC stability, functioning as a docking-site for PDK-1 through its repeated negatively charged aspartate sequence called PDK-1 interacting fragment (Balendran et al., 2000; Newton, 2003); an interaction that allows PDK-1 to access the activation loop (Hage-Sleiman et al., 2015).

There are differences in the phosphorylation process in different PKCs. In cPKCs, both the turn motif and the hydrophobic motif are autophosphorylated, whereas in nPKCs autophosphorylation occurs only in the turn motif, and phosphorylation in the hydrophobic motif is carried out by other kinases (Hage-Sleiman et al., 2015). For PKCδ, autophosphorylation of its turn motif contributes to its relative stability and solubility. In VSM, autophosphorylation of PKCα and ε may be regulated by α-adrenergic receptor agonists, and the actin-binding protein calponin (CaP) may be involved as α-adrenergic agonists induce translocation of CaP from the contractile filaments to the cortex of VSMCs (Kim et al., 2013). Also, aPKCs are phosphorylated at the activation loop and turn motif, but naturally contain glutamate ‘phosphomimetic’ residues in their hydrophobic motif (Parekh et al., 2000; Newton, 2003; Cameron et al., 2007), while the hydrophobic motif of nPKCs contains an aspartate residue (Cameron et al., 2007).

PKC phosphorylation may occur only during maturation of the newly synthesized enzyme, as has been shown with PKCα, or is dynamically regulated, as has been shown with nPKCs (Cenni et al., 2002; Rybin et al., 2003; Rybin et al., 2004). For example, phosphorylation of multiple sites may be required for activation of mature PKCs as has been shown during H_2O_2-induced tyrosine phosphorylation of PKCδ (Konishi et al., 1997). Also, in cardiomyocytes, PKCδ and ε appear to undergo phosphorylation of the activation loop and the hydrophobic motif even in the absence of allosteric regulators (Rybin et al., 2003), supporting that the regulatory pathways of PKC are isoform- and cell-specific.

There has been some discussion whether phosphorylation of the hydrophobic motif of cPKCs and nPKCs occur via autophosphorylation or through trans-phosphorylation by upstream kinases (Ziegler et al., 1999; Cameron et al., 2007). PDK1 and mTOR are potential upstream kinases that may be key to these phosphorylations (Dutil et al., 1998; Le Good et

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al., 1998; Jacinto and Lorberg, 2008). For instance, phosphorylation of the turn motif by the mTORC2 complex may trigger autophosphorylation of the hydrophobic motif (Sarbassov et al., 2004; Ikenoue et al., 2008).

The scaffold protein 14-3-3 has been identified as a partner of phosphorylated PKCε in mammalian cells. Phosphorylation of PKCε on Ser346 and Ser368 is required for binding to 14-3-3, and in turn locks the enzyme in an open, active and lipid-independent conformation (Saurin et al., 2008; Linch et al., 2014). On the other hand, direct interaction between PKCθ and 14-3-3 tau has been observed in T cells, and 14-3-3 overexpression inhibits PKCθ translocation and function (Meller et al., 1996).

Other phosphorylation patterns may be specific to certain PKC isoforms. For instance, tyrosine phosphorylation has been implicated as a mechanism to regulate PKCδ catalytic activity, and the tyrosine-phosphorylated enzyme is constitutively active and no longer requires DAG as a cofactor (Konishi et al., 2001). PKCδ has tyrosine phosphorylation sites, and a pathway involving tyrosine phosphorylation may underlie redox control of PKCδ activity. A Src family kinase (Lck)-driven phosphorylation at Tyr311 in rodent PKCδ (Tyr313 in human PKCδ) is believed to mediate H_2O_2-dependent increase in PKCδ activity (Konishi et al., 2001; Steinberg, 2015), and has been implicated in different PKCδ-dependent cellular responses (Lu et al., 2007; Nakashima et al., 2008; Steinberg, 2015).

PKC phosphorylation is often used as a marker of its activation and in testing whether certain effects are mediated by PKC. For instance, a PKCα autophosphorylation site has been used as a marker of its activity (Ng et al., 1999; Durgan et al., 2007). However, the use of PKC phosphorylation as a marker of PKC activation may not be definitive. For example, while phospho-S299 could be a useful marker of activated PKCδ, PKCδ is phosphorylated at other sites and undergoes *in vitro* autophosphorylation at three sites within its V3 region (S299, S302, S304), each of which is evolutionarily conserved and unique to PKCδ. S299-phosphorylated PKCδ is localized at both the plasma and nuclear membranes, making it the best marker of the activated enzyme (Durgan et al., 2007). While S643 is also an important PKCδ autophosphorylation site (Li et al., 1997), it is not ideally used as a marker of activation because it is relatively resistant to dephosphorylation and remains phosphorylated even when PKCδ releases its activator DAG and adopts a ‘closed’ conformation (Parekh et al., 2000; Durgan et al., 2007).

The kinase activity of PKCs is terminated by dephosphorylation, and this usually occurs when PKC is in an “open” conformation, unbound by the pseudosubstrate or constitutively active (Dutil et al., 1994; Lee et al., 1996; Gao et al., 2008). For cPKCs and nPKCs, dephosphorylation is carried out by the PP2C member pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) at the hydrophobic motif, which starts the process that drives PKC to be totally dephosphorylated and degraded by PP1/PP2A protein phosphatases at the turn motif (Sontag et al., 1997; Ahn et al., 2007; Gao et al., 2008; Newton, 2010). In some instances, phosphatases may have an indirect effect on PKC, for example dephosphorylation of the PKCθ downstream molecules CARMA1 by PP2A leads to PKCθ deactivation (Eitelhuber et al., 2011). Dephosphorylation predisposes “naked” protein kinases to ubiquitination and degradation (Katzmann et al., 2002). Partial inhibition

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of phosphorylation is caused by binding with HSP70, thus promoting rephosphorylation of PKCs and their subsequent reactivation (Gao and Newton, 2006; Poli et al., 2014).

PKC-priming phosphorylation may also be influenced by the inferred allosteric behavior caused by MgATP binding. Nucleotide pocket occupation confers on PKC a conformation that is both conducive to the action of upstream kinases and protective from the action of antagonistic phosphatases. Studies of kinase-inactive mutant forms of PKC isotypes that typically comprise mutation of the highly conserved lysine residue responsible for coordination of the -β phosphates of ATP, have shown that when the kinase domain is compromised through mutation of the highly conserved lysine it fails to be primed, but can be fully primed upon binding an ATP-competitive high-affinity inhibitor. In one study, investigators expressed an inactive green fluorescent protein (GFP) tagged PKCε K437M mutant (where K437 refers to the conserved lysine that contacts the ATP α-β phosphates) in HEK cells using a tetracycline-inducible system. This led to the accumulation in the cells of inactive PKCε lacking appreciable phosphorylation of the priming sites, while wild-type PKCε expressed under the same conditions was constitutively phosphorylated at all priming sites. Importantly, treatment with the PKC inhibitor bisindolylmaleimide I induced rapid phosphorylation of the priming sites of PKCε K437M–expressing cells, but did not increase phosphorylation of wild-type PKCε, supporting that the conformation induced by occupation of the nucleotide pocket of PKCε K437M with an inhibitor might be sufficient to promote priming. This may be relevant to the natural occupation of the nucleotide pocket with MgATP, as the PKCε M468A gatekeeper mutant confers sensitivity to the PKD inhibitor 1-naphthyl-PP1 (NaPP1) and becomes fully primed at steady-state. However, activation with phorbol esters, which relieves the regulatory domain inhibition, permits catalytic action and turnover of MgATP/ADP, leading to rapid dephosphorylation. This is likely due to reduced steady-state occupancy of the nucleotide-binding pocket, as the mutant PKCε dephosphorylation can be blocked by NaPP1. Also, following complete phorbol ester-induced dephosphorylation of the PKCε M468A mutant, phosphorylation can be re-established by treatment with NaPP1 (Cameron et al., 2009; Linch et al., 2014).

5. PKC ACTIVATORS

PKCs are activated by a variety of hormones such as adrenaline and angiotensin II (AngII), growth factors including epidermal growth factor and insulin, and neurotransmitters like dopamine and endorphin (Mochly-Rosen et al., 2012). These stimulants generally interact with their plasma membrane receptors leading to activation of phospholipase C and hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and DAG. IP_3 stimulates Ca^{2+} release from the intracellular stores in the endoplasmic reticulum, while DAG activates PKC. Activation of PKC can also occur in the absence of receptor activation, as high levels of cytosolic Ca^{2+} can directly activate phospholipase C and lead to activation of PKC (Mochly-Rosen et al., 2012). PKC isoforms respond differently to Ca^{2+}, PS, DAG, and other phospholipid degradation products. cPKCs bind Ca^{2+} in a phospholipid-dependent manner, and Ca^{2+} may form a “bridge” holding the protein and phospholipid complex together at the membrane (Bazzi and Nelsestuen, 1990). PS is indispensable for activation of PKC. Phosphatidylinositol and phosphatidic acid activate PKC at high Ca^{2+} concentrations. DAG activates PKC by reducing its Ca^{2+}

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requirement and enhancing its membrane association (Nishizuka, 1992). PKC activators also include lipids derived from sources other than glycerolipid hydrolysis such as cis-unsaturated free fatty acids and lysophosphatidylcholine, ceramide (a sphingomyelinase product), phosphatidylinositol 3,4,5-trisphosphate, and cholesterol sulfate (Nishizuka, 1995).

Phorbol esters such as phorbol 12,13-dibutyrate (PDBu), phorbol 12-myristate 13-acetate (PMA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) can substitute for DAG in PKC activation. Phorbol esters stabilize PKC–membrane association by reducing its apparent K_m for Ca^{2+} (Kanashiro and Khalil, 1998). PMA binds to PKC 1000-fold more strongly than DAG (Dries and Newton, 2008; Sanchez-Bautista et al., 2009). Interestingly, the binding of PMA to the C1B domain of PKCs alone may not induce a significant conformational change in the protein or release the pseudosubstrate domain from the catalytic core, but may generate a hydrophobic cap covering polar groups and thus helping PKCs to insert into membrane lipid bilayer (Zhang et al., 1995).

DAG analogs and phorbol esters are not specific for a particular PKC isoform, and may have other effects unrelated to PKC. For example, PMA, a widely used PKC activator, recruits members of both cPKCs and nPKCs to the plasma membrane (Hui et al., 2014). Also, 1,2-dioctanoyl-sn-glycerol (DiC8), a DAG analog used commonly to activate PKC, blocks K_v, BK_{Ca} and K_{ATP} channels of mesenteric artery VSM in a PKC-independent manner. 1-oleoyl-2-acetyl-sn-glycerol (OAG) is a related compound that activates PKC but without blocking K^+ channels, and is therefore a preferred pharmacological tool over DiC8 (Rainbow et al., 2011).

Post-translational modifications could activate certain PKC isoforms. For example, proteolysis between the regulatory and the catalytic domains activates PKCδ (Persaud et al., 2005). Other post-translational modifications including oxidation, acetylation and nitration and phosphorylation could also activate PKC (Steinberg, 2008). Oxidants such as H_2O_2 can directly activate PKC, and both the regulatory and catalytic domains of PKC are susceptible to oxidative modification (Gopalakrishna and Anderson, 1989). PKC contains unique structural features that are especially susceptible to oxidative modification, like the zinc-binding cysteine-rich motifs of the N-terminal regulatory domain (Gopalakrishna and Jaken, 2000). Also, hydroquinone, catechol, and whole cigarette smoke condensate have been shown to activate PKC in Lewis lung carcinoma cells (Gopalakrishna et al., 1994).

6. PKC SUBSTRATES

When PKC is not catalytically active, the basic autoinhibitory pseudosubstrate is protected from proteolysis by an acidic patch in the substrate-binding site. When PKC is activated, it phosphorylates arginine-rich protein substrates, which neutralize the acidic patch and displace the pseudosubstrate from its binding site in the kinase core (House and Kemp, 1987; Newton, 1995). The amino acid sequence near the substrate phosphorylation site may assist in PKC substrate recognition. Several PKC substrates have been identified (Table 2), and PKC isotypes may show some substrate specificity. For instance, PKCα, β, and γ are potent histone IIIS kinases, while PKCδ, ε, and η have a poor capacity to phosphorylate histone (Kanashiro and Khalil, 1998). However, PKC isoforms may show overlapping

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specificities for substrates derived from modification of their pseudosubstrate regions. For example, the PKC targeting protein AKAP79 binds the catalytic core of all PKCs through a pseudosubstrate-like mechanism (Faux et al., 1999; Bogard and Tavalin, 2015)

PKC substrates include the anchoring proteins STICKs such as MARCKs, MacMARCKs, α-, β-, and γ-adducin, clone 72 (SseCKs), GTP-binding proteins and cytoskeletal proteins (Ron and Mochly-Rosen, 1994; Ron and Kazanietz, 1999; Hage-Sleiman et al., 2015). PKC causes phosphorylation of the inhibitory GTP-binding protein Gi, facilitating the dissociation of its αi subunit from adenylyl cyclase and thereby relieves it from inhibition (Kanashiro and Khalil, 1998). PKC could also phosphorylate and activate cell migration-related molecules such as focal adhesion kinase, paxillin, and vinculin (Lewis et al., 1996; Li et al., 2003; Kappert et al., 2010; Ding et al., 2011b). PKC phosphorylation of vinculin, a cytoskeletal protein localized at adhesion plaques, could control cell shape and adhesion (Perez-Moreno et al., 1998). PKC could also phosphorylate substrates involved in protein trafficking. Efficient recycling of β1-integrins to the plasma membrane requires PKCε-regulated phosphorylation of vimentin, an intermediate filament protein upregulated upon epithelial cell transformation. Inhibition of PKC and vimentin phosphorylation causes integrins to become trapped in vesicles and attenuates directional cell motility. *In vitro* reconstitution assays showed that PKCε dissociates from integrin containing endocytic vesicles in a selectively phosphorylated vimentin-containing complex. Mutations of PKC-regulated sites on vimentin lead to the accumulation of intracellular PKCε/integrin positive vesicles, while introduction of wild-type vimentin promotes cell motility in a PKCε-dependent manner, supporting that PKC-mediated phosphorylation of vimentin is a key process in integrin trafficking and cell motility (Ivaska et al., 2005). PKC also plays a role in phosphorylation and nucleocytoplasmic shuttling of S6KβII, one of the forms of the ribosomal protein S6 kinase (S6K) involved in the regulation of protein synthesis and the G1/S transition in the cell cycle, and this PKC-mediated phosphorylation is induced by mitogens such as PMA, EGF, IGF-1, and PDGF (Valovka et al., 2003).

The list of PKC substrates is growing and many of these substrates could be playing a role in VSM contraction and growth (Table 2).

7. PKC INHIBITORS

PKC inhibitors include compounds that could interact with the PKC molecule, interfere with PKC binding to its substrates, decrease PKC synthesis, or counteract the effects of PKC. Several PKC inhibitors interact directly with PKC at different sites of the PKC molecule (Table 3). The first generation PKC inhibitors such as H7 and staurosporine are nonspecific pan-PKC inhibitors that block all PKC isoforms and are toxic for clinical use (Clarke and Dodson, 2007). H7 and staurosporine are ATP-competitive small molecule inhibitors that bind to and compete with ATP at the ATP site of the catalytic domain, and therefore display severe side effects *in vivo* (Mochly-Rosen et al., 2012). The poor selectivity of ATP-binding drugs is also due to their interaction with ATP-binding kinases other than PKC, since the hydrophobic pocket is conserved throughout the kinome (Roffey et al., 2009). Some PKC inhibitors targeting the ATP-binding site such as indolcarbazole and bisindoylmaleimide have shown selectivity to specific PKC isoforms. Ruboxistaurin is a class of

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bisindoylmaleimide and a relatively selective PKCβ inhibitor (Koya et al., 1997; Geraldes and King, 2010). PKC inhibitors competing at the DAG/phorbol ester or the PS binding site may be more specific. Calphostin C bind to the C1 domain, mimicking DAG-binding (Mochly-Rosen et al., 2012). Interestingly, extended exposure to phorbol esters can specifically downregulate PKCα, β and γ (Kanashiro et al., 2000a), but the tumor-promoting properties of phorbol esters limit their clinical use.

Peptides that interfere with the intramolecular interactions within PKC have been developed (Churchill et al., 2009). For instance, myr-ΨPKC is a myristoylated peptide based on the substrate motif of PKCα and β that inhibits TPA-Induced PKC activation and phosphorylation of MARCKS (Eichholtz et al., 1993). Other peptides disrupt protein/protein interactions between the PKC regulatory domain and RACK (Mochly-Rosen et al., 2012). The interaction of PKC and RACK is isoform selective and largely involves the C2 region of cPKC, and peptide fragments of this region may function as selective cPKCs inhibitors (Ron et al., 1995). Also, a peptide derived from the PKC binding proteins annexin I and RACKI inhibits translocation of PKCβ (Ron and Mochly-Rosen, 1994).

Peptides derived from the pseudosubstrate region show autoinhibitory effect on PKC activity and are attractive PKC inhibitors (House and Kemp, 1987; Eichholtz et al., 1993; Bogard and Tavalin, 2015). The autoinhibitory role of the PKC pseudosubstrate has been suggested as deletion of the pseudosubstrate site abrogates the inhibitory effect of the regulatory domain of PKCα on the full-length enzyme (Parissenti AM, 1998). Synthetic oligopeptides based on pseudosubstrate sequence are specific PKC inhibitors because they exploit its substrate specificity and do not interfere with ATP binding. The synthetic peptide (19–36) inhibits PKC autophosphorylation and protein substrate phosphorylation. Replacement of Arg-27 with alanine in the peptide [Ala-27] PKC (19–31) increases the IC_{50} for inhibition of substrate phosphorylation. A structure-function study of the PKC pseudosubstrate sequence R19FARK-GALRQKNV31 examined the role of specific residues using an alanine substitution scan. Arg-22 was the most important determinant in the inhibitor sequence, since substitution of this residue by alanine gave a 600-fold increase in the IC_{50}. Substitutions of other basic residues with Ala-19, Ala-23 and Ala-27 also increased the IC_{50} 5-, 11- and 24-fold, respectively. The importance of basic residues in determining the potency of the pseudosubstrate peptide reflects the requirement of these residues in peptide substrate phosphorylation. Gly-24, Leu-26 and Gln-28 residues were also important for pseudosubstrate inhibitor potency. The large increase in the IC_{50} for the [A22]PKC(19–31) peptide makes it a valuable control in studies utilizing the pseudosubstrate peptide to examine functional roles of PKC (House and Kemp, 1990). Another reason pseudosubtrate inhibitors were thought to be more specific inhibitors for PKC isoforms is that the pseudosubstrate region provides a large interface for multiple points of contact (Churchill et al., 2009; Bogard and Tavalin, 2015). However, this is not always the case as a cell-penetrating myristoylated peptide derived from the pseudosubstrate domain of PKCζ, and termed PKCζ pseudosubstrate inhibitor peptide (ZIP) shows affinity for all PKC isoforms causing disruption of PKC targeting and translocation, suggesting that pseudosubstrates of PKC isoforms may possess several invariant well-conserved residues (Bogard and Tavalin, 2015). Also, mutation of the alanine in the pseudosubstrate with serine or glutamate, mimics

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the charge of a phosphorylated residue and in effect activates PKC (Pears et al., 1990; Parissenti et al., 1998; Kheifets and Mochly-Rosen, 2007).

Compounds that counteract the effects of PKC include activators of β-adrenoceptors and antioxidants. For example, in portal vein, stimulation of β-adrenoceptors opposes the effects of PKC and causes vasodilatation and reduces the activity of store-operated channels via a cAMP-dependent protein kinase (PKA) pathway (Liu et al., 2005; Albert and Large, 2006). Also, antioxidants may inactivate PKC. The PKC catalytic domain contains several reactive cysteines that can be targeted by antioxidants such as selenocompounds, vitamin E, and polyphenolic agents such as curcumin (Boscoboinik et al., 1991; Liu et al., 1993; Gopalakrishna and Jaken, 2000). In VSM, α-tocopherol inhibits the expression, activity, and phosphorylation of PKCα and decrease VSM proliferation, and PKC activity in VSM gradually declines as the α-tocopherol level rises. These effects are not mimicked by β-tocopherol or probucol (Engin, 2009), and, in effect, β-tocopherol may oppose the inhibitory effects of α-tocopherol (Clement et al., 1997). Interestingly, hyperglycemia-induced retinal vascular dysfunction in different animal models can be prevented by α-tocopherol via inhibition of the DAG-PKC pathway (Engin, 2009). Also, high doses of vitamin E may decrease hyperglycemia-induced DAG and PKC activity and reverse some of the changes in the retinal and renal vessels in diabetes (Bursell and King, 1999). On the other hand, glutathione may inhibit PKC by a nonredox mechanism (Ward et al., 1998).

Post-translational modifications of PKC may alter its function. S-nitrosylation, a ubiquitous protein modification in redox-based signaling that forms S-nitrosothiol from nitric oxide (NO) on cysteine residues, decreases PKC activity and signaling and impairs contraction in mouse aorta, and may represent a key mechanism in conditions associated with decreased vascular reactivity (Choi et al., 2011).

Transgenic animals, knockout mice and antisense techniques have been useful in studying the effects of PKC down-regulation *in vivo* (Table 4). Isoform-specific PKC knockout mice have demonstrated a critical role of PKC in several tissues including endocrine and vascular cells, and further characterization of the PKC knockout vascular phenotype should shed more light on the role of PKC in the vascular system. Also, antisense and siRNA for specific PKC isoforms are now available and can be used to study the role of PKC in various cell functions. ISSI-3521 is a phosphorothioate antisense oligonucleotide that has been targeted to the 3′-untranslated region of PKCα mRNA, and has shown a highly specific reduction of PKCα protein expression in cancer cell lines and human tumor xenograft models (Song et al., 2003; Roffey et al., 2009).

Thus some challenges remain with the development of drugs that target specific PKC isoforms. These challenges are largely posed by the ~70% homologous structure of the catalytic domain within the PKC family. Pharmacological tools that target the C2 region could be more selective, as the C2 region is the less conserved among different PKCs (Mochly-Rosen et al., 2012). The V5 region may also be a good target for isoform-specific modulators of PKC activity. PKC isoforms interact with their substrates at sequences unique for the individual isoforms and this interaction can be selectively disrupted by peptide inhibitors that share the same substrate sequence. Also, protein-protein interactions can

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regulate the subcellular localization of specific PKC isoforms. Further research of PKC substrate interaction sites and PKC protein-protein interactions would shed more light on the various PKC-mediated effects in different systems and provide more specific targets for future therapy of PKC-related disorders (Mochly-Rosen et al., 2012).

8. Vascular Effects of PKC

PKC isoforms have diverse effects in different vascular cell types, with prominent effects on VSM. The role of each PKC isoform in certain vascular responses has been supported by measuring PKC gene expression, protein levels and PKC activity, and by determining the effects of pharmacological isoform-specific PKC inhibitors as well as knockout mice and transgenic rats (Mehta, 2014).

PKC and VSM Contraction

It is widely accepted that Ca^{2+}-dependent myosin light chain (MLC) phosphorylation is a major determinant of VSM contraction (Rembold and Murphy, 1988; Kamm and Stull, 1989) (Fig. 4). Agonist-induced activation of membrane receptors causes an increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) due to initial Ca^{2+} release from the sarcoplasmic reticulum and maintained Ca^{2+} entry from the extracellular space. Ca^{2+} binds calmodulin (CAM) to form a Ca^{2+}-CAM complex, which activates MLC kinase (MLCK), causes phosphorylation of the 20-kDa MLC, and increases the activity of actin-activated Mg^{2+}-ATPase, leading to actin-myosin interaction and VSM contraction (Rembold and Murphy, 1988; Kamm and Stull, 1989). VSM relaxation is initiated by a decrease in $[Ca^{2+}]_i$ due to Ca^{2+} uptake by the sarcoplasmic reticulum and Ca^{2+} extrusion by the plasmalemmal Ca^{2+} pump and Na^+-Ca^{2+} exchanger. The decrease in $[Ca^{2+}]_i$ causes dissociation of the Ca^{2+}-CAM complex and the phosphorylated MLC is dephosphorylated by MLC phosphatase.

PKC can affect VSM contraction by several mechanisms including regulation of ion channels and pumps and in turn $[Ca^{2+}]_i$, Ca^{2+} sensitization of the contractile proteins, or activation of Ca^{2+} independent contraction pathways. PKC translocation to the cell surface could also trigger a cascade of protein kinases that ultimately interact with the contractile myofilaments and cause VSM contraction. In some instances, PKC may inhibit VSM contraction.

PKC, ion Channels, and $[Ca^{2+}]_i$

PKC can change $[Ca^{2+}]_i$ by modulating the activity of plasmalemmal K^+ and Ca^{2+} channels. K^+ channels play a role in the regulation of the resting membrane potential, and inactivation of K^+ channels in VSMCs causes membrane depolarization, elevation of $[Ca^{2+}]_i$ and VSM contraction (Nelson and Quayle, 1995). Membrane depolarization activates Ca^{2+} entry via L-type voltage-gated Ca^{2+} channels (L-VGCC) and may also cause Ca^{2+} release from IP_3- and ryanodine-sensitive intracellular Ca^{2+} stores leading to increase in $[Ca^{2+}]_i$ (Nauli et al., 2001; Kizub et al., 2014). Large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) are the predominant K^+ channels in VSMCs (Nelson and Quayle, 1995; Ghatta et al., 2006). PKC activators such as PDBu inhibit BK_{Ca} leading to increases in vascular tone in both

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physiological and pathophysiological conditions (Taguchi et al., 2000; Barman et al., 2004; Kizub et al., 2010; Novokhatska et al., 2013), and in various vascular beds including pulmonary (Barman et al., 2004), coronary (Minami et al., 1993), cerebral (Lange et al., 1997), and uterine vessels (Hu et al., 2011). PKC activators inhibit BK_{Ca} by phosphorylation of the channel protein and decreasing its sensitivity to activation by cGMP-dependent protein kinase (Crozatier, 2006; Ledoux et al., 2006).

Voltage-gated K^+ channels (K_v) also play a role in the regulation of VSM function, and can be modulated by vasoconstrictors such as arginine vasopressin, ET-1 and AngII via a mechanism involving PKC. In rat mesenteric artery VSMCs, vasopressin regulates Kv7.4 and Kv7.5 subunits of Kv7 channels via activation of PKC. PKCα-dependent phosphorylation of the K^+ channel proteins on serine residues is sufficient to reduce Kv7 channel activity, and the extent of PKC-mediated Kv7.4 and Kv7.5 phosphorylation and K^+ current suppression depends on the subunit composition of the channel proteins (Brueggemann et al., 2014). Also, thromboxane A2 may induce pulmonary vasoconstriction by a mechanism involving PKCζ and inhibition of Kv (Cogolludo et al., 2003). PKC isoforms may contribute differently to the vasoconstrictor-induced effects on different K^+ channels. In rabbit coronary arterial VSMCs, ET-1 and AngII inhibit Kv currents by activating PKCε, and inhibit K_{IR} channel activity by activating PKCα (Park et al., 2005; Park et al., 2006).

PKC may also regulate K_{ATP} channels, and vasoconstrictor agonists may inhibit K_{ATP} through PKC signaling (Nelson and Quayle, 1995; Quayle et al., 1997). Phorbol esters inhibit K_{ATP} currents in mesenteric arteries (Bonev and Nelson, 1996). Although the mechanism via which PKC regulates K_{ATP} is not well defined, in human embryonic kidney cells (HEK293) PKC-mediated AngII- and PDBu- induced inhibition of K_{ATP} channel may involve channel complexes composed of four Kir6.1 and their associated SUR2B subunits (Thorneloe et al., 2002). Also, trafficking studies have shown that PKC may initiate internalization of the channel complex leading to decreased K_{ATP} channel activity (Manna et al., 2010). PKC-mediated phosphorylation of K_{ATP} may also alter the channel properties, kinetics and/or number at the cell membrane (Levitan, 1994; Light, 1996).

PKC, Ion Pumps and Co-transporters, and $[Ca^{2+}]_i$

Plasmalemmal Ca^{2+}-ATPase (PMCA) and sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) are important Ca^{2+} homeostasis mechanisms in VSM. PKC may activate PMCA or SERCA, an action that promotes Ca^{2+} extrusion and re-uptake and lead to a decrease in VSM $[Ca^{2+}]_i$. In isolated cardiac sarcoplasmic reticulum preparations, PKC activates the Ca^{2+}-transport ATPase (Limas, 1980). Also, the α1 subunit of Na^+/K^+-ATPase may serve as a PKC substrate, and PKC-mediated inhibition of Na^+/K^+ pump causes changes in the membrane potential and the intracellular concentrations of Na^+ and K^+ (Bertorello et al., 1991). PKC activation by phorbol esters and permeable DAG analogs may also phosphorylate and activate the Na^+/H^+ antiport exchanger and thereby increase the cytoplasmic pH leading to alkalinization, which generally increases vascular contraction (Rosoff et al., 1984; Aviv, 1994; Austin and Wray, 2000; Wray and Smith, 2004).

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KC and Ca^{2+}-Sensitization of Contractile Proteins

Activation of PKC could increase the myofilament force sensitivity to $[Ca^{2+}]_i$, thereby maintaining VSM contraction with smaller increases in $[Ca^{2+}]_i$. The nPKC isoforms play an important role in mediating VSM contraction through a Ca^{2+} sensitizing pathway, and inhibition of nPKCs attenuates norepinephrine-induced VSM contraction (Wang et al., 2015). PKC-induced Ca^{2+} sensitization could involve phosphorylation of regulatory proteins in the VSM contractile myofilaments and the cytoskeleton. PKC phosphorylates CPI-17, which in turn inhibits MLC phosphatase, increases MLC phosphorylation, and thereby enhances VSM contraction (Woodsome et al., 2001). PKC could also inhibit MLC-phosphatase via the phosphorylation of the myosin targeting subunit of myosin phosphatase (MYPT1) (El-Yazbi et al., 2015). Activation of PKCα could cause phosphorylation of CaP, a VSM differentiation marker and an actin-associated regulatory protein, and thereby reverses its inhibition of actin-activated myosin ATPase, allowing more actin to bind myosin, and enhancing VSM contraction. Interestingly, CaP may activate PKC *in vitro* in the absence of lipid cofactors, and knockdown of CaP inhibits PKC-dependent contraction in ferret arterial VSM (Je et al., 2001; Kim et al., 2008).

PKC may contribute to VSM force production in a MLC phosphorylation-independent manner. In rat middle cerebral artery, PKC activation by PDBu is associated with sustained force generation and vasoconstriction that is much larger than that expected with the same level of MLC phosphorylation achieved by 5-HT (El-Yazbi et al., 2015).

PKC may also be involved in mechanical stretch-induced vascular myogenic response. In rat cerebral artery VSMCs, PKC activators increase stretch-activated channel activity and induce depolarization, and these effects are blocked by PKC inhibitors (Slish et al., 2002). It is possible that increases in tension on vascular myocytes lead to stimulation of phospholipase C (PLC), hydrolysis of phosphoinositides and production of DAG, which activates PKC and stimulate the myogenic response (Albert and Large, 2006). PKC may primarily affect the maintained phase of stretch-induced contraction by changing the Ca^{2+} sensitivity of the contractile elements (Nakayama and Tanaka, 1993). Some studies suggest that PKCθ and μ may participate in stretch-induced VSM mechanotransduction, as cyclic-stretch of VSM specifically activates these PKC (Yang et al., 2014). However, other studies have shown activation of PKCδ by cyclic stretch in VSM (Li et al., 2003) and PKCε activation by mechanical stretch in cardiomyocytes (Klein et al., 2005; Bullard et al., 2007).

The nature and extent of the PKC-activated pathway could vary depending on the vasoconstrictor tested, the vascular bed examined and in arteries versus veins. Vasoconstrictors such as AngII, ET-1, serotonin, norepinephrine, and neuropeptide Y activate PKC-dependent pathways, causing VSMC membrane depolarization and contraction (Quayle et al., 1997; Cole et al., 2000; Zhu et al., 2013). Of note, AngII activates multiple PKC isoforms in VSM (Griendling et al., 1997) and PKC may increase VSM contraction via other pathways involving downregulation of atrial natriuretic peptide (ANP) receptor and the binding of ANP to VSM, and thereby preventing ANP-induced inhibition of contraction (Jiao and Yang, 2015). Although the role of venous function in blood pressure (BP) control has been underappreciated, its contribution is significant in the deoxycorticosterone salt rat model of HTN where ET-1 was found to elevate venomotor tone

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and contribute to HTN (Tykocki et al., 2014). The PKC inhibitor chelerythrine attenuates ET-1-induced contraction in both the aorta and vena cava, suggesting that ET-1 acts via PKC to mediate VSM contraction of both arteries and veins. However, in the aorta, ET-1-induced contraction is largely dependent on PLC activation and IP_3-mediated Ca^{2+} release, while in the vena cava ET-1 induced contraction is unaffected by the IP_3 receptor antagonist 2-APB. Also, only the vena cava contracts in response to the DAG analog OAG, highlighting the differences in the venous and arterial pathways of contraction (Tykocki et al., 2014). It should be noted that endothelium-derived NO regulates VSM tone by activating guanylate cyclase, increasing cGMP and producing vasodilation, and PKC could inhibit NO-mediated vasodilation by inhibiting guanylate cyclase, leading to decreases in intracellular cGMP and increased vasoconstriction (Johnson and Barman, 2004).

PKC and Cytoskeletal Proteins

Studies in cerebral resistance arteries have shown that PKC could mediate myogenic constriction through dynamic reorganization of the cytoskeleton and increased actin polymerization (Moreno-Dominguez et al., 2014). Also, both in the presence and absence of Ca^{2+}, PKC may promote cerebral vasoconstriction by increasing the phosphorylation of paxillin and HSP27, reducing G-actin content, and promoting actin cytoskeleton reorganization (El-Yazbi et al., 2015). The relative contribution of PKC to cytoskeletal modification versus other mechanisms of VSM contraction appears to be more significant in the cerebral circulation. In rat middle cerebral arteries, PDBu-induced PKC constriction is more sensitive to disruption of actin cytoskeleton compared to inhibition of cross-bridge cycling, providing evidence for the pivotal contribution of PKC-mediated cytoskeletal actin polymerization to force generation in cerebral resistance arteries (El-Yazbi et al., 2015).

PKC may also modulate certain genes that code for structural proteins such as fibronectin and type IV collagen, by changing the binding of nuclear transcription factors to the promoter regions on responsive genes (Clarke and Dodson, 2007). PKC also affects the gene expression of the regulator of G-protein signaling 2 (RGS2), which may affect vascular tone. In cultured VSMCs, adrenotensin increases RGS2 expression, while the PKC inhibitor chelerythrine reduces RGS2 expression, suggesting that adrenotensin increases gene expression via a PKC-dependent pathway (Mao et al., 2013).

PKC-Dependent Signaling Cascades

The interaction of PKC with its substrate may trigger a cascade of protein kinases that ultimately stimulate VSM contraction (Fig. 4). PKC may affect Akt signaling PKC (Radhakrishnan et al., 2008; Ding et al., 2011b). Also, mitogen-activated protein kinases (MAPK) such as extracellular signal-regulated kinase (ERK), p38 and JNK, are common downstream effectors of PKC (Yamaguchi et al., 2004; Ginnan and Singer, 2005). PKC, MAPK, and c-Raf-1 have been implicated in VSM growth. MAPK is a Ser/Thr kinase that is activated by its dual phosphorylation at Thr and Tyr residues. In quiescent undifferentiated cultured VSMCs, MAPK is mainly cytosolic, but translocates to the nucleus during activation by mitogens (Mii et al., 1996). Tyrosine kinase and MAPK activities have also been identified in differentiated contractile VSM. MAPK transiently translocates to the surface membrane during early activation of VSM, but undergoes redistribution to the

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cytoskeleton during maintained VSM activation (Khalil et al., 1995). It has been suggested that during VSM activation, DAG promotes translocation of cytosolic PKCε to the surface membrane, where it is fully activated. Activated PKCε stimulates the translocation of cytosolic MAPK kinase (MEK) and MAPK to the plasmalemma, where they form a surface membrane kinase complex. PKC causes phosphorylation and activation of MEK, which in turn phosphorylates MAPK at both Thr and Tyr residues. Tyrosine phosphorylation targets MAPK to the cytoskeleton, where it phosphorylates the actin-binding protein caldesmon (CaD) and reverses its inhibition of MgATPase activity and thus increases actin-myosin interaction and VSM contraction (Khalil et al., 1995; Kim et al., 2008). This is supported by the observations that in aortic VSM, phenylephrine activates a pathway involving CaP-dependent PKC autophosphorylation and activation followed by a much delayed ERK activation, CaD phosphorylation and VSM contraction (Kim et al., 2013). These PKC-dependent pathways occurs in parallel with the previously described transient spike in $[Ca^{2+}]_i$ and MLC phosphorylation in VSM (Kim et al., 2013). Interestingly, biochemical studies have shown that in either the presence or absence of extracellular Ca^{2+}, PKC activation by PDBu does not directly change the phospho-content of the thin filament proteins CaP or CaD (El-Yazbi et al., 2015), supporting that other kinases downstream of PKC may be needed to cause phosphorylation of CaP or CaD *in vivo*, and further demonstrating the complexity of signaling at the whole cell level (Kim et al., 2013).

PKC and Vasodilation

PKC may directly affect the permeability of VSM Ca^{2+} channels. In VSMC, agonists of G-protein-coupled receptors could activate receptor-operated Ca^{2+} channels (ROCs) including store-operated Ca^{2+} channels (SOCs) and transient receptor potential channels (TRPCs), and PKC may modulate these channels. Studies have suggested that low levels of DAG could activate TRPC6 via a PKC-independent mechanism, while high levels of DAG inhibit TRPC6 SOCs activity. In mesenteric and ear artery VSMC, DAG exerts an inhibitory action on TRPCs through a PKC-dependent pathway, and such mechanism may limit ROC activity at high agonist concentrations (Large et al., 2009). PKC inhibits the TRPC6 SOCs activity in a Ca^{2+}-dependent manner (Shi et al., 2004; Albert and Large, 2006).

The 20-kDa MLC and MLCK also serve as substrates for PKC, and their phosphorylation could counteract the Ca^{2+}-induced actin-myosin interaction and force development (Inagaki et al., 1987). In human VSMCs, PKC activation stimulates secretion of C-type natriuretic peptide (CNP) (Mendonca et al., 2006), which could function as an endogenous vasodilator (Ahluwalia et al., 2004). PKCα and δ mediate most of the increase in CNP mRNA induced by the PKC activator PMA, and PDGF increases CNP in SMCs via a PKCδ-dependent pathway (Mendonca et al., 2012).

9. PHYSIOLOGICAL CHANGES IN PKC

PKC levels may vary with certain physiological changes such as age, exercise, gender, sex hormone status and pregnancy.

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Age-related Changes in PKC

Studies have shown age-dependent decrease in PKC activity and its translocation has in postmortem human brains (Wang et al., 1994). Also, in platelets, PKC activity in both the cytosolic and membrane fractions and its redistribution in response to stimulation of cell surface receptors are reduced in elderly men. Interestingly, age-related decrease in PKC activity is mitigated in older men who maintain moderately high levels of aerobic fitness as they age (Wang et al., 1995). Also, in rats, PKCε expression decreases gradually with age particularly among male rats (Li et al., 2014). Thus, PKC activity and its translocation may serve as biological markers of aging, and physical exercise may slow the changes in PKC during the aging process (Wang et al., 2000).

Sex Differences in PKC

Sex hormone status has emerged as an important modulator of vascular physiology and cardiovascular risk, and PKC expression/activity may be different in males versus females. Low testosterone levels in men may be associated with a higher risk of cardiovascular disease (Weidemann and Hanke, 2002), and testosterone reduces neointimal plaque development in male rabbit aortas (Hanke et al., 2001). PKCδ plays a role in mediating testosterone-induced apoptosis and inhibition of VSMC proliferation (Bowles et al., 2007). Overexpression of PKCδ in rat aortic VSMCs inhibits growth and proliferation, decreases thymidine incorporation, induces G0/G1 arrest, reduces cyclin D1 and E, and increases p27kip1, and PKCδ knockdown with siRNA diminishes the downregulation of cyclin D1 and E, and the upregulation of p21cip1 (Fukumoto et al., 1997; Bowles et al., 2007). Cleavage of PKCδ by caspase 3 and nuclear accumulation of catalytic PKCδ could be an important component of the apoptotic response induced by testosterone. It is believed that testosterone-induced increase in full-length PKCδ could cause an increase in caspase 3-mediated production of the 40-kDa catalytic fragment of PKCδ and lead to VSMC apoptosis (Bowles et al., 2007). PKCδ null mice exhibit decreased VSMC apoptosis and exacerbated vein graft arteriosclerosis (Leitges et al., 2001). The testosterone-induced PKCδ-dependent G1/S cell cycle arrest and stimulation of apoptosis may explain some of its beneficial effects on coronary vasculoproliferative disease, restenosis and atherosclerosis (Bowles et al., 2007). While local conversion of testosterone to estrogen via aromatase could mediate some of the beneficial effects of testosterone (Yamada et al., 1990), it may not be involved in testosterone-induced PKCδ-mediated inhibition of coronary VSMC proliferation (Bowles et al., 2007).

PKC may also mediate some of the vascular effects of female sex hormones. For instance, females tolerate shock and sepsis better than males likely through a protective GPR30-estrogen receptor mediated vascular response involving PKC (Angele et al., 2006; Li et al., 2014). Also, in mesenteric arteries from normal and shocked rats, estrogen increases the expression/activity of PKCε, and PKCε psuedosubstrate inhibitory peptide antagonizes the effect of estrogen on vascular reactivity in shocked rats (Li et al., 2014).

Sex differences in the expression/activity of PKC isoforms have also been observed in aortic VSM of male and female Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR). VSM contraction and the expression/activity of PKCα, δ and ζ are less in intact female

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compared with male WKY, and the sex-related differences are greater in VSM from SHR compared with WKY rats (Kanashiro and Khalil, 2001). PDBu-induced contraction and PKC activity are greater in ovariectomized (OVX) females than in intact female rats, and treatment of OVX females with 17β-estradiol subcutaneous implants reduces PDBu contraction and PKC activity to a greater extent in SHR than WKY rats. These observations have suggested sex-related reduction in VSM contraction and the expression/activity of PKCα, δ and ζ in females compared with males, and that these differences are possibly mediated by estrogen and are enhanced in hypertension (Kanashiro and Khalil, 2001).

Pregnancy-related Changes in PKC

Normal pregnancy is associated with physiological changes in uterine blood flow caused by changes in uterine arterial Ca^{2+}-dependent phasic contraction and maintained DAG/PKC - mediated tonic contraction (Ford, 1995). PKC inhibitors decrease thromboxane A2-induced contraction in uterine and mesenteric arteries of non-pregnant rats and in mesenteric of pregnant rats, supporting a role of PKC in mediating VSM contraction during pregnancy (Goulopoulou et al., 2012). PKC activity changes during the course of pregnancy, and PKC activity and vascular contraction are reduced in uterine artery of late pregnant ewes and gilts and aorta of late pregnant rats (Magness et al., 1991; Farley and Ford, 1992; Kanashiro et al., 2000b). Also, the expression and subcellular redistribution of PKCα, δ and ζ are reduced in aortic VSM of late pregnant rats (Kanashiro et al., 1999; Kanashiro et al., 2000b). A decrease in PKC signaling is chiefly responsible for the decreased contractions in pregnant uterine arteries in order to maintain low basal uterine vascular tone and to accommodate the increased uterine blood flow during pregnancy (Xiao et al., 2006). The pregnancy-associated decrease in uterine vascular tone and increase in uterine blood flow may be caused by increased steroid hormones and their receptors. The sex steroids estrogen and progesterone have been shown to attenuate PKC-mediated signaling in uterine arterial VSMCs and uterine artery contraction and myogenic tone, partly through upregulation of K^+ channel expression/ activity (Xiao et al., 2006; Zhu et al., 2013).

10. PKC IN VASCULAR INJURY AND DISEASE

In addition to its effects on vascular contraction/relaxation mechanisms, PKC has been implicated in multiple pathological processes involving VSM growth/proliferation, angiogenesis/vasculogenesis, apoptosis, vascular inflammation, restenosis, oxidative stress and ischemia-reperfusion injury. Pathological changes in PKC expression/activity could cause vascular hyper-reactivity and vascular remodeling leading to vascular disorders such as systemic and pulmonary HTN, preeclampsia, diabetic vasculopathy, atherosclerosis, and coronary artery disease (Fig. 5).

PKC, VSM Growth, and Angiogenesis/Vasculogenesis

Studies have shown PKC translocation and localization to the nucleus in different cell types including VSM, suggesting interaction with nuclear factors and genes and a role in the regulation of VSM growth and proliferation (Salamanca and Khalil, 2005). PKC isoforms exert different effects leading to either stimulation or suppression of cell growth (Clarke and Dodson, 2007). PKC regulates vascular endothelial growth factor (VEGF) at the gene

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transcription level (Monti et al., 2013; Carracedo et al., 2014). Also, PKCε is a powerful oncogene promoting cell growth and proliferation (Nishizuka, 1995) and has been used as a tumor biomarker (Gorin and Pan, 2009; Duquesnes et al., 2011). PKCβII is also an upstream regulator of Early growth response-1 (Egr-1), a master switch that orchestrates the expression of diverse gene families that elicit a pathological response to hypoxia, ischemia/ reperfusion, and vascular stress (Yan et al., 2006). In contrast, PKCδ is pro-apoptotic, anti-oncogene and tumor suppressor (Reddig et al., 1999; Duquesnes et al., 2011), that suppresses the expression of positive regulatory factors required for cell cycle progression (Fukumoto et al., 1997; Bowles et al., 2007).

PKC may be involved in the angiogenesis and vasculogenesis associated with cancer and metastasis (Kim et al., 2011a; Mochly-Rosen et al., 2012). VEGF activates PKCε in endothelial cells, and the selective PKCε agonist ψεRACK promotes fibroblast growth factor-2 (FGF-2) release and export to cell membrane, and induces pro-angiogenic responses in endothelial cells and the formation of capillary-like structures and endothelial cell growth, proliferation and sprouting (Monti et al., 2013), PKCε-dependent formation of blood vessels may involve downstream signaling cascades including Akt and eNOS (Rask-Madsen and King, 2008). Double null mutation of PKCδ and ε causes embryonic lethality with defective blood vessel formation, impaired endothelial cell organization, dilated vessels, reduced endothelial-specific adherent junctions, decreased contact of endothelial cells with mural cells, deficient angiogenesis related transcripts, and almost undetectable α-smooth muscle actin, a classical marker for VSMC (Carracedo et al., 2014). On the other hand, PKCδ-deficient mice show increased number of SMCs and macrophages, accelerated neointimal lesions and intimal hyperplasia and delayed reendothelialization in mouse wire-injured femoral artery. PKCδ knockdown using small hairpin RNA (shRNA) in cultured endothelial cells is also associated with reduced cell migration and accumulation of the antiangiogenesis protein vasohibin-1, and downregulation of vasohibin-1 restores the migration rate in PKCδ-deficient cells (Bai et al., 2010).

PKC and VSM Apoptosis

Apoptosis has been observed in cardiovascular diseases such as myocardial infarction, aneurysm and ischemia/reperfusion injury. Whether apoptosis is beneficial or detrimental in vascular disease has been debated, but the finding of marked endothelial cell apoptosis in patients with peripheral vascular disease suggest that it may induce cell and tissue damage in certain conditions (Gardner et al., 2014).

PKCδ plays a role in apoptosis, and overexpression of the catalytic fragment of PKCδ alone is sufficient to induce apoptosis (Zhao et al., 2012). PKCδ is activated by a variety of pro-apoptotic stimuli including DNA damaging agents, ultraviolet (UV) radiation, the phorbol ester PMA and reactive oxygen species (ROS). VSMCs from PKCδ null mice are resistant to apoptosis induced by UV, TNFα, or H_2O_2, and show defective caspase-3 activation in response to oxidative stress (Zhao et al., 2012). PKCδ is an early regulator of apoptosis, and may function upstream of the mitochondria as an integrator for various death signals in multiple cell types and under various stimuli. Cytosolic PKCδ may function at the initial stages of apoptosis. Tyrosine phosphorylation of PKCδ also occurs at the beginning of

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apoptosis and may be responsible for its translocation to the cell membrane, mitochondria, ER, and lysosomes (Zhao et al., 2012). A positive loop between mitochondrial-mediated caspase activation and PKCδ cleavage and activation supports the pro-apoptotic role of PKCδ in the cytoplasm. The nucleus may also act as a major target for PKCδ to amplify the apoptosis signal. Translocation of PKCδ to the nucleus may be essential for inducing apoptosis, and the proteolytically cleaved constitutively active catalytic fragment of PKCδ accumulates in the nucleus (DeVries et al., 2002; Zhao et al., 2012). PKCδ knockout mice are resistant to apoptosis in models of abdominal aortic aneurysm, and adenovirus-mediated delivery of PKCδ locally to the arterial wall is sufficient to restore aneurysm development in PKCδ knockout mice (Morgan et al., 2012).

In contrast with the pro-apoptotic properties of PKCδ, PKCδ may have anti-apoptotic effects, as demonstrated in the response to the cytokine TNFα (Lu et al., 2009; Ren et al., 2014). Silencing PKCδ expression by siRNA inhibits TNFα-mediated ERK1/2 activation (Kilpatrick et al., 2006; Ren et al., 2014). PKCδ also interacts with the mitochondrial protein Smac, and exposure to apoptotic stimuli such as paclitaxel, disrupts the PKCδ-Smac interaction resulting in the release of Smac into the cytosol, activation of caspases in the cytochrome c/Apaf-1/caspase-9 pathway and promotion of apoptosis. On the other hand, activation of PKCδ rescues the PKCδ-Smac interaction and suppresses paclitaxel-induced cell death (Masoumi et al., 2012; Ren et al., 2014). The factors that determine whether PKCδ exerts a pro- or anti-apoptotic role in a given cell remain to be examined.

PKC and Vascular Inflammation

Vascular inflammation is observed in cardiovascular diseases such as atherosclerosis and myocardial infarction (Ross, 1999). Pro-inflammatory chemokines released by VSMCs play a role in vascular inflammation and recruit inflammatory cells to the vascular wall (Brasier, 2010; Ren et al., 2014). PKCδ is upregulated in VSMCs of injured arteries such as in aneurysmal aortic tissues and in restenotic lesions, and could be involved in vascular inflammation (Morgan et al., 2012; Si et al., 2012; Ren et al., 2014). PKCδ knockout mice show diminished expression of VSM pro-inflammatory factors and inflammatory cell infiltration (Morgan et al., 2012). PKCδ may promote chemokine expression at the transcription level by activating NF-κB through an IκB-independent cytosolic interaction, which subsequently leads to enhanced p65 phosphorylation and DNA binding affinity (Ren J, 2014). Delivery of PKCδ to the aortic wall of PKCδ$^{-/-}$ mice restores aneurysm, whereas overexpression of a dominant negative PKCδ mutant in the aorta of wild-type mice attenuates aneurysm. Monocyte chemoattractant protein-1 (MCP-1) is one of several inflammatory chemokines in VSMCs induced by PKCδ-regulated genes, and could be involved in the PKCδ role in aneurysm formation (Ren et al., 2014). This is supported by reports that PKCδ gene deficiency reduces the production of MCP-1 and other cytokines by aortic VSMCs, and the ectopic administration of MCP-1 to the aortic wall of PKCδ knockout mice restores aneurysm development (Morgan et al., 2012).

PKCε is also likely involved in inflammation, as PKCε inhibition both by knockout in mice and peptide modulators suppress the acute and chronic inflammatory pain response (Hucho et al., 2005; Koyanagi et al., 2007). Also, selective inhibition of PKCε with εV1-2 prolongs

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graft survival and improves functional recovery of the heart in cardiac transplantation models. PKCε inhibition attenuates the inflammatory response, decreases infiltration of macrophages and T cells and the attachment of mononuclear inflammatory cells to the arterial wall, and reduces luminal narrowing and parenchymal fibrosis, thereby preserving cardiac tissue architecture after transplantation (Koyanagi et al., 2007).

PKC may play a role in the inflammation caused by prolonged Mg^{2+} deficiency (Altura et al., 2012). PKC is activated in rat VSMC exposed to short-term Mg^{2+} deficiency. In Mg^{2+} deficient animals there may be cross-talk between PKC and the ceramide, sphingosine, NF-κB and cytokine pathways in vascular cells. PKCζ, in particular, plays a role in *de novo* formation of ceramide, through a sphingolipid salvage pathway. Mg^{2+} supplements in drinking water prevents the upregulation of PKC isoforms in VSMCs when exposed to low $[Mg^{2+}]_o$ providing an effective solution to prevent inflammation induced by Mg^{2+} deficiency (Altura et al., 2014).

PKC and Vascular Restenosis

Long-term success of vascular bypass and angioplasty procedures is limited by restenosis particularly in obese and diabetic patients and PKC may contribute to vascular restenosis through the initial thrombosis and inflammation and the subsequent VSMC migration and proliferation (Ding et al., 2011a).

Thrombosis is involved in the early stages of vascular restenosis. PKCα, β, δ and θ are expressed in platelets, and cPKCs may promote while nPKCs inhibit platelet aggregation and thrombus formation (Gilio et al., 2010). This is illustrated by reports that knocking out PKCδ or PKCθ potentiates murine platelet aggregation, and that the PKCδ inhibitor rottlerin potentiates human platelet aggregation (Pula et al., 2006; Ding et al., 2011a).

PKCα, β and ζ also potentiate the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), a key step in leucocyte recruitment, that leads to VSMC migration and proliferation and vascular stenosis (Javaid et al., 2003; Kouroedov et al., 2004; Abdala-Valencia and Cook-Mills, 2006). PKCα, β and δ further affect VSMC migration by promoting actin polymerization and enhancing cell adhesion (Okazaki et al., 2000; Campbell and Trimble, 2005; Liu et al., 2007). PKCε promotes VSMC migration by upregulating matrix metalloproteinases (MMPs), particularly MMP-2 and -9 (Rodriguez-Pla et al., 2005; Thomas and Newby, 2010; Ding et al., 2011a).

PKC also contributes to VSMC proliferation, the final step of vascular restenosis. PKCβ mediate synergistic proliferative effect of PDGF and high glucose on human coronary VSMCs (Ling et al., 2002), and the selective PKCβ inhibitor LY-379196 attenuates DNA synthesis and cell growth (Ding et al., 2011b). PKCε seems to be involved in the development of neointimal hyperplasia. In rat models of aortic balloon injury the PKCε activator ψεRACK promotes neointimal development, while the PKCε inhibitor εV1-2 reduces luminal narrowing, neointimal proliferation and VSMC ERK phosphorylation *in vivo*, and PDGF-induced VSMC proliferation/migration *in vitro* (Deuse et al., 2010).

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PKC and Oxidative Stress

Oxidative agents such as H_2O_2 and superoxide activate PKC independent of classical PKC cofactors such as DAG. H_2O_2-induced activation of PKC may cause stimulation of arterial VSM L-type Ca^{2+} channels, and these effects are abolished by PKC inhibition. Also, hypoxia and vasoconstrictors such as AngII increase mitochondrial ROS production via PKC-mediated activation of NADPH oxidase (Nox) in pulmonary artery VSMCs (Doughan et al., 2008; Rathore et al., 2008; Perez-Vizcaino et al., 2010).

PKC and ROS appear to be tightly coupled in causing vascular dysfunction, as ROS can activate PKC and vice versa (Novokhatska et al., 2013). Also, a positive feedback mechanism may amplify the production of ROS and PKC. For instance, excess ROS production may occur through PKC activation, and subsequent phosphorylation of p47 phox subunit and activation of NADPH oxidase. ROS then creates a positive feedback loop through activation of c-Src, which then amplifies NADPH oxidase activity to produce more ROS (Lyle and Griendling, 2006; Novokhatska et al., 2013).

ROS activates different PKC isoforms. In isolated pulmonary artery, H_2O_2-induced Ca^{2+} sensitization and constriction is associated with PKCα activation and abolished by PKC inhibitors (Pourmahram et al., 2008; Perez-Vizcaino et al., 2010). Also, exogenous H_2O_2, mimics hypoxia and increases PKCε activity (Rathore et al., 2006; Perez-Vizcaino et al., 2010). In pulmonary artery VSMCs, mitochondrial-derived ROS may activate PKCε, which subsequently activates Nox-dependent ROS generation, further illustrating the positive feedback mechanism involved in hypoxia-induced increase in ROS (Rathore et al., 2008; Perez-Vizcaino et al., 2010).

PKC isoforms affect ROS production via different pathways. For instance, PKCε siRNA knockdown blocks ROS production by sphingosylphosphorylcholine (Shaifta et al., 2015). On the other hand, PKCζ appears to increase ROS through an Insulin Growth factor IGF-I–stimulated pathway, as high glucose induces NADPH oxidase 4 (Nox4) upregulation in a PKCζ/NF-κB–dependent manner in VSMCs and diabetic mice (Xi et al., 2012).

Oxidative stress may have other PKC-mediated vascular effects. In pulmonary artery, H_2O_2 may inhibit K_v channel by activating PKCα and ε. Also, in mesenteric artery, PKCα and ε may mediate the K_v channel inhibitory effect of ET-1 and AngII, respectively (Rainbow et al., 2009; Perez-Vizcaino et al., 2010). PKCζ also plays a role in the inhibition of K_v channels by U46619 and hypoxia in rat pulmonary artery VSMCs (Cogolludo et al., 2003; Cogolludo et al., 2009). Although PKC is involved in hypoxia-induced ROS generation, PKCζ does not mediate K_v channel inhibition through ROS (Perez-Vizcaino et al., 2010).

PKC and Ischemia/Reperfusion Injury

PKC activity has been observed in ischemic injury in multiple tissues including the heart (Speechly-Dick et al., 1994), liver (Piccoletti et al., 1992) and kidney (Padanilam, 2001). The regulation of cellular viability during an ischemic event may be influenced by the ratio of PKCδ and ε, as they display detrimental and protective effects, respectively (Churchill and Mochly-Rosen, 2007; Duquesnes et al., 2011).

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Prolonged ischemia and reperfusion activates PKCδ more than PKCε, leading to translocation of PKCδ into the mitochondria and phosphorylation of pyruvate dehydrogenase kinase, which in turn phosphorylates pyruvate dehydrogenase, leading to a reduction in the tricarboxylic acid (TCA) cycle and ATP regeneration (Inagaki et al., 2003; Churchill et al., 2005; Mochly-Rosen et al., 2012). Mitochondrial dysfunction causes increases in ROS production and lipid peroxidation leading to accumulation of ROS and toxic aldehydes, such as 4-hydroxynonenal (4HNE), that interact and inactivate macromolecules including proteins, DNA and lipids. Mitochondrial dysfunction and increase in ROS leads to apoptosis, necrosis and severe cardiac dysfunction (Armstrong and Whiteman, 2007; Mochly-Rosen et al., 2012).

Short bouts of ischemia and reperfusion prior to the prolonged ischemic event (ischemic preconditioning) provides cardioprotection by preferentially activating PKCε (Inagaki et al., 2006), which translocates into the mitochondria and prevents mitochondrial dysfunction induced by prolonged ischemia and reperfusion (Budas et al., 2010; Mochly-Rosen et al., 2012). PKCε-mediated protection occurs, in part, by PKCε-induced phosphorylation and activation of aldehyde dehydrogenase 2 (ALDH2) (Chen et al., 2008), which metabolizes aldehydes such as 4HNE, thus reducing the aldehyde load and the mitochondrial and cellular damage (Mochly-Rosen et al., 2012). In addition, mitochondrial function is preserved in preconditioned hearts through the inhibition of the mitochondrial permeation pore and K_{ATP} channel opening (Costa et al., 2006; Duquesnes et al., 2011). The reduced 4HNE levels also prevent direct inactivation of peroxisome and thus enable fast removal of aggregated proteins. Furthermore, ischemic preconditioning prevents I/R injury at reperfusion by protecting ATP-dependent 26S proteasomal function. The active proteasome also selectively degrades activated PKCδ, thus decreasing the accumulation of the pro-apoptotic PKCδ at cardiac mitochondria and increasing the balance in favor of the cardiac protective and pro-survival PKCε. (Budas et al., 2007; Churchill et al., 2010; Mochly-Rosen et al., 2012). PKC-induced closure of connexons may also participate in ischemic preconditioning by an unclear mechanism (Naitoh et al., 2009; Duquesnes et al., 2011).

Ischemic stroke represents a major cause of death and disability among elderly, and the presence or absence of reperfusion is an important variable affecting outcome (Aronowski and Labiche, 2003). PKCβI and βII are increased in infarcted tissue of an ischemic stroke, whereas PKCγ increases 2 to 24 fold in the ischemic penumbra, but not in the infarcted tissues (Krupinski et al., 1998; Young et al., 2005). PKCγ may play a contrasting role in regulating the vulnerability of tissue to I/R-induced damage, as it functions first as a deleterious factor during evolution of intra-ischemic neuronal damage, then as a neuroprotective factor during post-ischemic reperfusion (Aronowski and Labiche, 2003). PKCγ may carry out its neuroprotective role in reversible focal ischemia by protein phosphorylation, as impaired protein phosphorylation in PKCγ knockout mice influences the overall infarct volume. Studies have shown larger infarct volumes in PKCγ knockout compared with wild-type, and inhibitors of the protein phosphatase calcineurin reduced infarct volume in the PKCγ knockout mice (Aronowski et al., 2000; Young et al., 2005).

In the cerebral circulation, PKCδ is believed to have a deleterious role in cerebral reperfusion. A model of transient middle cerebral artery occlusion demonstrated that PKCδ-

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null mice showed a 70% reduction in stroke size compared with wild-type mice (Chou et al., 2004; Young et al., 2005). PKCδ may mediate its detrimental effects in cerebral reperfusion by affecting neutrophil migration into ischemic tissue. PKCδ null mice show impaired neutrophil function and decreased neutrophil migration into ischemic tissues, and transplantation of bone marrow from the PKCδ-null mice into the wildtype mice reduces infarct size while bone marrow transplantation from wildtype donors increased infarction size and worsened neurological scores in PKCδ-null mice (Chou et al., 2004; Young et al., 2005). Inhibition of PKCδ improves microvascular pathology and function in transient focal ischemia in normotensive animals and chronic hypertension, and reduces ischemic damage following an ischemic event. Thus PKCδ could be an important therapeutic target for the preservation of microcerebrovascular function following stroke, and its inhibition may reduce stroke risk and damage in hypertensive patients (Bright et al., 2007). PKCζ also appears to be a downstream component of NMDA-induced excito-toxic neuronal cell death, as inhibiting PKCζ and its translocation, prevents NMDA-induced cell death. PKCζ mRNA is also induced in the cerebral cortex after focal brain ischemia (Koponen et al., 2003; Young et al., 2005). In contrast, PKCε is activated during cerebral ischemia *in vivo* and may play a role in mediating the early cellular response to ischemic stress, possibly mediating ischemic tolerance. Systemic delivery of PKCε-selective peptide activator ψεRACK confers neuroprotection against a subsequent cerebral ischemic event when delivered immediately prior to stroke. In addition, activation of PKCε by ψεRACK decreases vascular tone and microvascular cerebral blood flow, which may contribute to the conferred protection (Bright et al., 2008).

PKC and Coronary Artery Disease

PKCδ may contribute to coronary artery disease, through increased ROS formation, decreased ATP generation and increased apoptosis and necrosis (Inagaki et al., 2003; Churchill and Mochly-Rosen, 2007; Mochly-Rosen et al., 2012). PKCε, on the other hand, is protective, as it protects mitochondrial functions and proteasomal activity, activates ALDH2 and reduces aldehyde load (Mochly-Rosen and Kauvar, 2000; Budas et al., 2007; Chen et al., 2008; Mochly-Rosen et al., 2012). A combination of a PKCδ inhibitor and a PKCε activator could be useful for organ preservation and in prevention of ischemia-reperfusion injury and graft coronary artery disease in cardiac transplantation (Tanaka et al., 2004). In a case-control study, ischemia-reperfusion injury was the strongest alloantigen-independent factor for the subsequent development of graft coronary artery disease (Gaudin et al., 1994), and PKC modulators could modulate this pathological process.

PKC and Hypertension

Increased PKC activity could play a role in HTN, and mutations of PKC may influence the individual susceptibility to vascular hyper-reactivity. For instance, a consistent association is found between the single nuclear polymorphism (SNP) rs9922316 in PKCβ gene (*PRKCB*) and inter-individual variation in the constriction responses of dorsal hand vein to the selective α2 adrenergic receptor agonist dexmedetomidine (Posti et al., 2013). Also, PKCδ mRNA expression and protein levels are increased in VSM from SHR rats. PKC could increase VSM contraction in HTN by altering BK_{Ca} channel conductance, as the PKC inhibitor chelerythrine restores K^+ channel activity in SHR (Novokhatska et al., 2013).

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Sleep apnea could cause systemic and pulmonary HTN (Campen et al., 2005; Snow et al., 2011). Rat models of sleep apnea produced by exposure to eucapnic intermittent hypoxia display increased circulating ET-1 levels and ET-1-dependent systemic HTN, that is likely mediated by PKCδ-dependent VSM Ca^{2+} sensitization in systemic arteries (Allahdadi et al., 2008; Snow et al., 2011). On the other hand, in the pulmonary circulation, intermittent hypoxia appears to mediate a PKCβ-dependent increase in reactivity to different receptor-mediated vasoconstrictor agonists including ET-1 (Snow et al., 2008).

PKC isoforms have been implicated in hypoxia-associated pulmonary HTN by affecting both Ca^{2+} influx and Ca^{2+} sensitization in pulmonary artery VSM. In normal small pulmonary arteries, PKC inhibitors attenuate ET-1 induced constriction and $[Ca^{2+}]_i$ as well as vasoconstrictor responses associated with store-operated Ca^{2+} entry, suggesting that PKC contributes to both Ca^{2+} sensitization and Ca^{2+} influx (Jernigan and Resta, 2014). Also, in fawnhooded rat model of pulmonary HTN, PKC inhibits BK_{Ca}, resulting in indirect activation of VGCC and pulmonary vasoconstriction (Zhu et al., 2008). PKC-mediated Ca^{2+} sensitization is also demonstrated by an increase in total and phosphorylated active CPI-17 levels in pulmonary arteries from newborn swine exposed to hypoxia (Dakshinamurti et al., 2005).

PKCε may have divergent effects in pulmonary HTN. PKCε null mice show decreased acute hypoxic pulmonary vasoconstriction, increased Kv3.1b channel expression and membrane hyperpolarization (Littler et al., 2003). On the other hand, PKCε null mice show a greater increase in pulmonary arterial pressure compared to wild-type mice following chronic hypoxia exposure. The increase in pressure is reversed by inhaled NO suggesting that PKCε may be an important signaling intermediate in the hypoxic regulation of NO synthase (Littler et al., 2005).

Hypertension in pregnancy and preeclampsia are major complications of pregnancy and placental ischemia/hypoxia could be an initiating event. Chronic hypoxia enhances uterine vascular tone in pregnant sheep and is associated with an increase in PKC activity (Chang et al., 2009). Hypoxia during pregnancy may attenuate the effects of sex steroid hormones/receptors, leading to enhanced PKC activation in pregnant uterine arteries. Increased BK_{Ca} channel activity inhibits PKC-mediated contraction in ovine uterine arteries during pregnancy, and gestational hypoxia may upregulate PKC and inhibit BK_{Ca} (Xiao et al., 2014). Hypoxia may also inhibit K_{IR} channels via PKC-dependent mechanism, and may contribute to the maladaptation of uterine vascular hemodynamics in preeclampsia and the fetal intrauterine growth restriction in response to hypoxia (Zhu et al., 2013). Also, in cultured rat cardiomyocytes, treatment with IgG obtained from preeclamptic women enhances AT_1R-mediated response, which is ameliorated with the PKC inhibitor calphostin C, further supporting a role of PKC in preeclampsia (Wallukat et al., 1999).

PKC and Diabetic Vasculopathy

Diabetes mellitus is a complex syndrome of multiple disorders including vascular dysfunction. PKC could play a role in diabetes-related vascular pathology through multiple mechanisms including cell growth and proliferation, cell permeability, oxidative stress, increased vascular reactivity, inhibition of K^+ channels and Na^+-K^+-ATPase, activation of

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cytosolic phospholipase A2, vascular remodeling and increased ECM, and vascular inflammation and increased pro-inflammatory cytokines (Nishizuka, 1992; Koya and King, 1998; Meier and King, 2000). In diabetes, PKC is activated by advanced glycation end (AGE) products and polyol pathway flux (Thallas-Bonke et al., 2008; Geraldes and King, 2010; Kizub et al., 2014). Also, chronic hyperglycemia stimulates synthesis of DAG and activates DAG-dependent cPKCs and nPKCs in cultured bovine aortic endothelial cells and VSM (Inoguchi et al., 1992). Fatty acids, especially the unesterified forms and their coenzyme A (CoA) esters, work synergistically with DAG to activate PKC (Clarke and Dodson, 2007). PKC is also activated by ROS generated by different oxidases and the mitochondrial electron transport chain, and following AGE:RAGE (AGE receptor) interactions (Liu and Heckman, 1998).

High glucose-induced activation of PKC could cause vascular dysfunction by altering the expressions of growth factors such as VEGF, PDGF, and transforming growth factor-β (Yokota et al., 2003; Lizotte et al., 2013), which in turn affect the expression of ECM proteins (Jakus and Rietbrock, 2004). Also, in diabetes, activated PKC increases endothelial cell permeability and decreases blood flow and the production of and responsiveness to angiogenic factors, and this may contribute to the loss of capillary pericytes, retinal permeability, ischemia, and neovascularization (Aiello et al., 1994; Huang and Yuan, 1997; Williams et al., 1997; Pomero et al., 2003; Lizotte et al., 2013). PKC also activates NADPH oxidases and increases ROS (Inoguchi et al., 2000; Gao and Mann, 2009; Kizub et al., 2014). In hyperglycemia, VSMCs show increased DNA synthesis and contraction to PMA and reduced apoptosis, and these effects are blocked by the PKC inhibitor calphostin C (Hall et al., 2000; Geraldes and King, 2010). In diabetes, PKC may enhance vascular reactivity by inhibition of K^+ channels and promoting Ca^{2+} sensitization in VSM myofilaments (Nelson and Quayle, 1995; Kizub et al., 2014). High glucose via PKC activation and oxidative stress also reduces arterial SMC K_v current resulting in VSM depolarization and vasoconstriction (Liu et al., 2001; Rainbow et al., 2006; Straub et al., 2009). Diabetic patients also often have reduced nocturnal BP dip and increased vascular complications regardless of the average BP, partly due to lack of diurnal PKC inhibition (Nakano et al., 1991; Palmas et al., 2008).

PKC may contribute to diabetic nephropathy by increasing endothelial permeability to albumin and other macromolecules. PKC also induces ECM protein synthesis by mesangial cells and promotes sclerosis (Rovin et al., 1992; Henry et al., 1999; Heilig et al., 2013). In cultured mesangial cells, cGMP suppresses PKC-mediated actions including matrix protein production, and impaired NO-mediated cGMP generation in mesangial cells could amplify the PKC signal and increase matrix protein synthesis in diabetes (Williamson et al., 1993; Craven et al., 1994; Derubertis and Craven, 1994). Podocyte injury or loss is a hallmark of diabetic nephropathy and PKC contributes to the progression of glomerular injury (Teng et al., 2014). PKC also mediates diabetic glomerulosclerosis partially through its interaction with GLUT1, which facilitates the movement of glucose into the cell (Koya et al., 1997; Heilig et al., 2013). PKC stimulates TGF-β-mediated effects including activation of the highly pro-fibrotic cytokine CTGF, and engagement of the TGF-β receptor, triggering more GLUT1 synthesis via MAPK, and ECM protein synthesis via Smads (Twigg et al., 2001; Qi et al., 2005; Heilig et al., 2013). Also, AngII interaction with AT1 receptor stimulates DAG/PKC causing additional GLUT1 synthesis, and AngII and GLUT1 activation of PKC

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could promote glomerulosclerosis through both TGF-β-dependent and -independent pathways (Koya et al., 1997; Henry et al., 1999; Heilig et al., 2013).

PKC isoforms play different roles in diabetic vasculopathy. In VSMCs, high glucose activates PKCα, β, δ, and ε but not PKCζ (Haller et al., 1995; Igarashi et al., 1999; Lizotte et al., 2013). PKCβ and δ appear to be the dominant PKCs involved in diabetes. In rat VSMCs, high glucose increases the membrane fraction expression of PKCβ and PKCδ, p38 MAPK phosphorylation and arachidonic acid release (Igarashi et al., 1999; Geraldes and King, 2010). PKCβ is implicated in insulin resistance. Transgenic mice overexpressing PKCβII exhibit decreased Akt activation in vascular cells after insulin stimulation (Naruse et al., 2006; Geraldes and King, 2010). PKC could prevent insulin actions on the PI_3K pathway at the insulin receptor substrate (IRS) level (Sampson and Cooper, 2006), but could accentuate insulin actions on the ERK1/2 pathway (Bakker et al., 2008). Thus, PKC could mediate selective insulin resistance by enhancing insulin's pro-atherosclerotic mechanisms via ERK1/2 signaling or inhibiting its anti-atherosclerotic mechanisms by inhibiting the PI_3K/Akt pathway (Geraldes and King, 2010). PKCβ activation by hyperglycemia may play a role in mediating the microvascular disease complications of retinopathy, nephropathy, and neuropathy. Hyperglycemia leads to chronic activation of PKCβ, causing aberrant signaling and other pathologies including cytokine activation and inhibition, vascular alterations, cell cycle and transcriptional factor dysregulation, and abnormal angiogenesis (Geraldes and King, 2010; Mochly-Rosen et al., 2012). PKCβ is chiefly responsible in causing diabetic retinopathy by affecting VEGF expression through the mRNA-stabilizing human embryonic lethal abnormal vision protein, HuR, in the retina (Amadio et al., 2010; Gogula et al., 2013). PKCβ may mediate diabetes-induced increase in vascular contraction by inhibiting BK_{Ca} channel, and PKCβ inhibition restores BK_{Ca}-mediated vasodilation in diabetic mice. Also, reduced expression of the $BK_{\beta I}$ channel subunit in arteries of STZ-induced diabetic mice and in human coronary artery VSMCs cultured with high glucose has been related to increased PKCβ expression (Lu et al., 2012; Kizub et al., 2014).

The nPKCs could also contribute to insulin resistance by serine phosphorylation and inhibition of IRS1 (Yu et al., 2002; Ritter et al., 2015). PKCδ plays a role in islet cell function and insulin response, and changes in PKCδ expression/activity among mice strains correlate with insulin resistance and glucose intolerance. Also, mice with global or liver-specific downregulation of the PKCδ gene (*PRKCD*) display increased hepatic insulin signaling and improved glucose tolerance with aging. Conversely, mice with liver-specific overexpression of PKCδ develop hepatic insulin resistance and decreased insulin signaling (Bezy et al., 2011). Diabetes-induced PKCδ activation also decreases responsiveness to PDGF leading to pericyte apoptosis, acellular capillaries, and diabetic retinopathy (Geraldes et al., 2009). PKCδ is also likely involved in poor collateral vessel formation in diabetes, as the ischemic adductor muscles of diabetic *PRKCD* knockout mice show increased blood flow and capillary density compared with diabetic *PRKCD*$^{+/+}$ mice. The poor angiogenesis response in ischemic diabetic muscles could be caused by PKCδ-induced expression of Src homology-2 domain-containing phosphatase-1 (SHP-1), contributing to VEGF and PDGF unresponsiveness (Lizotte et al., 2013). PKCδ may also mediate inhibition of K^+ current in aortic SMCs, and PKCδ gene silencing by siRNAs restores VSMCs K^+ current and endothelium-dependent vasodilatation in aorta of streptozotocin-induced diabetic rats (Kizub

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et al., 2014; Klymenko et al., 2014). Endothelium-independent vasoconstriction mediated by EP1-/EP3-receptors activation is also enhanced in mesenteric arteries of diabetic rats and highly sensitive to PKCδ inhibition (Ishida et al., 2012; Kizub et al., 2014).

Ruboxistaurin (LY333531) is an oral PKCβII inhibitor commonly used in cellular, animal and human studies (Geraldes and King, 2010). Ruboxistaurin has been tested in diabetic retinopathy, nephropathy and neuropathy, and is well-tolerated (Mehta et al., 2009; Kizub et al., 2010; Aiello et al., 2011). Ruboxistaurin decreases vessel permeability and the onset of diabetic macular edema, improves retinal condition in diabetic patients, and prevents reduction of visual acuity (Geraldes and King, 2010; Gogula et al., 2013). While ruboxistaurin preserves visual acuity by decreasing capillary permeability or targeting the neural retina it may not delay the progression of diabetic retinopathy, and inhibiting PKCβ alone may not be sufficient to stop the early metabolic changes that drive the progression of pre-proliferative diabetic retinopathy (Geraldes and King, 2010). Indolylmaleimide and its derivatives are nonselective PKC inhibitors that reduced diabetic complications such as nephropathy, cardiomyopathy and neuropathy in clinical trials (Sobhia et al., 2013; Kizub et al., 2014), but the lack of selectivity on PKC isoforms raises concerns regarding safety. Interestingly, some of the drugs already in clinical use for vascular disease may mediate some of their effects through inhibition of PKC. For instance, metformin and liraglutide (a glucagon like peptide-1 (GLP-1)) could prevent diabetic cardiovascular complications and atherosclerosis (Batchuluun et al., 2013). In cultured human endothelial cells, both metformin and liraglutide prevent high glucose-induced oxidative stress through inhibition of PKC-NADPH oxidase pathway, and these effects are enhanced when the drugs are combined. In cells treated with metformin and liraglutide, hyperglycemia fails to induce PKCβII translocation and phosphorylation of endogenous PKC. Also, both drugs inhibit p47phox translocation and NADPH oxidase activation, and prevent high glucose-induced changes in intracellular DAG level and phosphorylation of AMP-activated protein kinase (AMPK) (Batchuluun et al., 2013).

PKC and Atherosclerosis

Atherosclerosis results from deposition of lipid and chronic inflammation in the arterial wall, and PKC has been linked to many of the pathways involved in atherosclerosis. PKC expression is higher in plaques from atherosclerotic patients compared with control subjects. Also, PKC expression is higher in atherosclerotic rabbit aorta VSMC than in the control group and in unstable versus stable plaques (Sirikci et al., 1996). PKC is also positively correlated with the increase in adipose differentiation-related protein, a protein present in higher amounts in unstable atherosclerotic versus stable plaques. PKC may further potentiate plaque-formation through endothelial dysfunction, foam cell formation, and VSMC proliferation. PKC may also contribute to the increased thickness of the intima and media of the vessel wall in atherosclerosis due to an imbalance between proliferation and apoptosis (Xu et al., 2015).

PKC isoforms play a varying role in the atherosclerotic process. Both PKCβ and δ are potential therapeutic targets as PKCβ potentiates atherosclerotic formation, and PKCδ appears to have an opposite effect (Fan et al., 2014). Depletion of PKCβ gene or treatment

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with LY333531 in apolipoprotein E-deficient mice decreases atherosclerosis by inhibiting the early growth response (Egr)-1 protein, which regulates VCAM expression and matrix metalloproteinase-2 activity (Harja et al., 2009; Geraldes and King, 2010). On the other hand, PKCδ deletion promotes arteriosclerosis, partly due to the lack of PKCδ-mediated VSMC apoptosis (Leitges et al., 2001; Geraldes and King, 2010). Also, PKCδ mediates collagen I secretion in VSMCs, and tight regulation of collagen is critical to the stability of atherosclerotic plaque. PKCδ knockout mice show marked reduction of collagen I in the arterial wall. PKCδ may also regulate the trafficking of collagen by controlling its exit from the trans-Golgi network through a mechanism involving cell division cycle 42 (Cdc42) protein (Lengfeld et al., 2012).

Vascular calcification contributes to atherosclerosis, as it reduces elasticity of blood vessels, and PKC may coordinate between cytoskeletal changes and hyperphosphatemia-induced vascular calcification. Expression and phosphorylation of both PKCα and δ decreases during inorganic phosphate (Pi)-induced VSMC calcification. Knockdown of PKCα and δ accelerates Pi-induced calcification in VSMCs and aorta in culture through upregulation of osteogenic signaling. Inhibition of PKCα and δ may also induce disassembly of microtubule and actin, respectively (Lee et al., 2014).

11. CONCLUSION AND PERSPECTIVE

PKC is a major regulator of vascular function and a potential target in several pathological processes. Although significant information is currently available on PKC, it is important to further our knowledge of the role of PKC in vascular disease and the mechanisms behind its contribution. Research efforts have been limited by the existence of several PKC isoforms, the non-uniform expression and distribution of PKC throughout the vascular tree, and the poor specificity of chemical inhibitors (Schubert et al., 2008). Continued research should further define the specific characteristics of the different PKC isoforms that determine their subcellular localization, phosphorylation pattern and potential substrates. The precise knowledge of the structural aspects of PKC isoforms should allow the development of new tools to evaluate PKC function and potential new therapies. The development of FRET-based reporters of PKC activity (Violin et al., 2003; Braun et al., 2005) and new peptides directed towards other domains than those presently utilized in the V1 region is a step in the right direction (Churchill et al., 2009; Duquesnes et al., 2011). Also, while PKC could play a role in vascular disease, this should not minimize its role in other pathological processes and diseases (Fig. 5). It is important to continue research of the role of PKC isoforms in various diseases, as there are still many uncertainties about their exact mechanism of action. For instance, while pro-apoptotic PKCδ likely acts as a tumor suppressor (Hampson et al., 2005; Zhao et al., 2012), in some cases it enhances tumorigenesis, and PKCδ-deficient mice are protected from urethane-induced lung tumor (Symonds et al., 2011; Zhao et al., 2012).

In order to enhance selectivity, it is important to determine the precise cellular location of various PKC isoforms, both in the resting and active state. The subcellular location of PKC may determine the state of VSM activity, and may be useful in the diagnosis/prognosis of HTN (Salamanca and Khalil, 2005). Since some PKC-mediated pathways involve PKC translocation to the nucleus, such as in apoptosis-induction by PKCδ, new nuclear targets of

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PKCδ such as the recently identified C/EBPa and hnRNPK could limit apoptosis (Gao et al., 2009; Zhao et al., 2012). Similarly, the translocation activator peptide ψδRACK attenuates Ccl2 production, providing a way to specifically block PKCδ-regulated proinflammatory chemokines (Ren et al., 2014). Genetic differences in PKC may also alter its effects, and studies have suggested a new role for PKC in inhibiting store-operated Ca^{2+} entry in the hypertensive pulmonary circulation of Sprague-Dawley, but not Wistar rats. The precise genetic differences responsible for this discrepancy in VSM Ca^{2+} regulation, as well as in other PKC-mediated effects, should be further explored (Snow et al., 2009).

Modulation of PKC activity presents an attractive target for drug development in vascular disease and other related conditions. Despite the promise of PKC modulators, results in clinical trials have been mixed and often negative (Mochly-Rosen et al., 2012). Isoform-specific PKC inhibitors have shown some promise in clinical trials (Table 5). Partial prevention of the progress of malignancies were found in early phases of clinical trials of the PKC inhibitors UCN-01 and CGP41251 (Shen, 2003). PKC inhibitors could also be useful in treatment of Ca^{2+} antagonist-resistant forms of HTN where the Ca^{2+} independent PKC isoforms could be targeted (Salamanca and Khalil, 2005). Inhibitors of PKCβ and δ may reduce fat accumulation, improve glucose tolerance, decrease hepatosteatosis, and suppress foam cell formation in obesity and hyperlipidemia-induced atherosclerosis (Bezy et al., 2011; Huang et al., 2012; Fan et al., 2014). Also, a PKCβ inhibitor or PKCε activator may reduce damage secondary to endothelial dysfunction and VSMC proliferation in patients with atherosclerosis caused by long-term smoking, HTN or diabetes (Harja et al., 2009; Huang et al., 2010; Monti et al., 2010; Fan et al., 2014). Activators of PKCε may also be useful in coronary artery disease, and the PKCε activator acadesine reduced the 2 year mortality in patients with postoperative acute myocardial infarction after coronary bypass grafting (Mochly-Rosen et al., 2012).

Since PKC modulates many physiological functions, unwanted effects may occur when non-selective PKC inhibitors are administered systemically. Sustained delivery of peptide inhibitors of PKC for two months is safe in animals (Tanaka et al., 2004; Inagaki et al., 2008; Ding et al., 2011b). Nevertheless, local delivery of PKC inhibitors may be a better approach. For example, to prevent restenosis, PKC inhibitors could be coated onto stents or balloons to be directly released into the injured area at effective concentrations. PKCβII and δ inhibitors coated stents or balloons showed efficacy and safety in animal trials (Ding et al., 2011b).

PKC siRNA may hold the promise to target specific PKC isoforms in vascular disease. PKCδ gene silencing with the short hairpin RNAs (shRNAs)-plasmid delivery system administered intravenously restores the vasodilator potential and normalize vascular function and high BP in SHR (Novokhatska et al., 2013). Also, PKCδ siRNA attenuates the proinflammatory effect of human CRP in diabetic rats (Jialal et al., 2013). Further research should help design more specific and effective remedies of PKC-mediated vascular disease and other PKC-related conditions.

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ABREVIATIONS

AGE	Advanced Glycation End products
ALDH2	aldehyde dehydrogenase 2
AngII	angiotensin II
BK_{Ca}	large conductance Ca^{2+}-activated K^+ channel
BP	blood pressure
Ca^{2+}	calcium
$[Ca^{2+}]_i$	intracellular free Ca^{2+} concentration
CaD	caldesmon
CAM	calmodulin
CaP	calponin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
CPI-17	PKC-potentiated phosphatase inhibitor protein-17
DAG	diacyglycerol
ECM	extracellular matrix
ER	endoplasmique reticulum
ERK	extracellular signal-regulated kinase
ET-1	endothelin-1
HSP	heat shock protein
HR1	homology region 1
ICAM-1	intercellular adhesion molecule-1
IP_3	inositol 1,4,5-trisphosphate
IRS1	insulin receptor substrate 1
K_v	voltage-gated K^+ channel

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MARCKS	myristoylated alanine-rich C kinase substrate
MCP-1	monocyte chemoattractant protein-1
MLC	myosin light chain
PDBu	phorbol 12,13-dibutyrate
PDGF	platelet-derived growth factor
PDK	phosphoinositide-dependent kinase
PKC	protein kinase C
PKG	cGMP-dependent protein kinase
PMA	phorbol 12-myristate 13-acetate
PLC	phospholipase C
PS	phosphatidylserine
RAGE	AGE receptor
ROS	reactive oxygen species
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VGCC	voltage-gated Ca^{2+} channel
VSM	vascular smooth muscle

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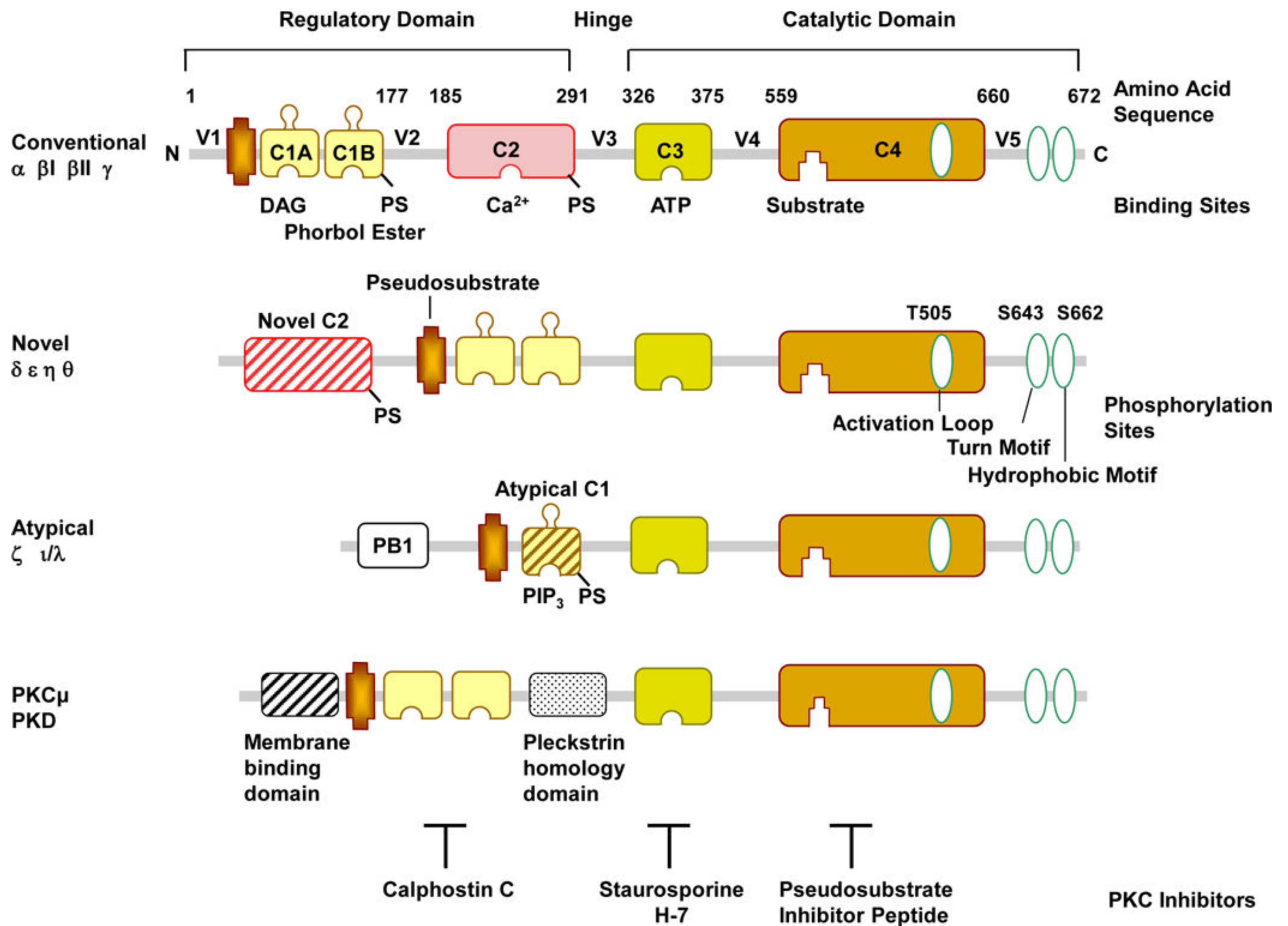

Fig. 1.

PKC Structure. PKC contains a N-terminal 'regulatory domain' and a C-terminal 'catalytic domain', between which lies the V3 hinge region. The regulatory domain contains two conserved C1 and C2 regions, and the pseudosubstrate region. The catalytic or kinase activity domain contains a C3 ATP/Mg-binding site and a C4 binding site for the phospho-acceptor sequence in the substrate proteins. The catalytic domain also contains phosphorylation sites in the activation loop, turn-motif and hydrophobic-motif. The figure illustrates the amino acids for PKCδ phosphorylation sites, which may vary among different PKCs. Based on the domain composition of the N-terminals, the PKC family is classified into conventional cPKCs α, βI, βII, and γ; novel nPKCs δ, ε, η and θ; and atypical aPKCs ι/λ and ζ isoforms. cPKCs consist of 4 conserved (C1–C4) and 5 variable regions (V1–V5) and are activated by Ca^{2+}, DAG, and PS. The C1 region contains the recognition site for phosphatidylserine (PS), DAG, and phorbol esters, while the C2 region contains the binding site for Ca^{2+}. PS can also be bound by the C2 region. Both cPKCs and nPKCs have twin C1 regions (C1A and C1B) and a C2 region. The order of C1 and C2 regions is switched in nPKCs compared with cPKCs. The nPKCs have a variant form of C2 region that is insensitive to Ca^{2+} activation, but still binds lipids. The aPKCs do not have a C2 region and hence not activated by Ca^{2+} or DAG, and have a variant form of C1 that is not duplicated,

but retains lipid-binding activity and are activated by DAG and PS. The aPKCs also encode the protein–protein-interacting region Phox and Bem 1 (PB1) that binds with ZIP/p62, Par6, or MEK5 through a PB1-PB1 domain interaction and hence control the localization of aPKCs. Other related kinases include PKCμ (PKD). PKC inhibitors could compete with DAG at the C1 region (calphostin) or ATP at the ATP-binding site (staurosporine) or the true PKC substrate (pseudosubstrate inhibitor peptide).

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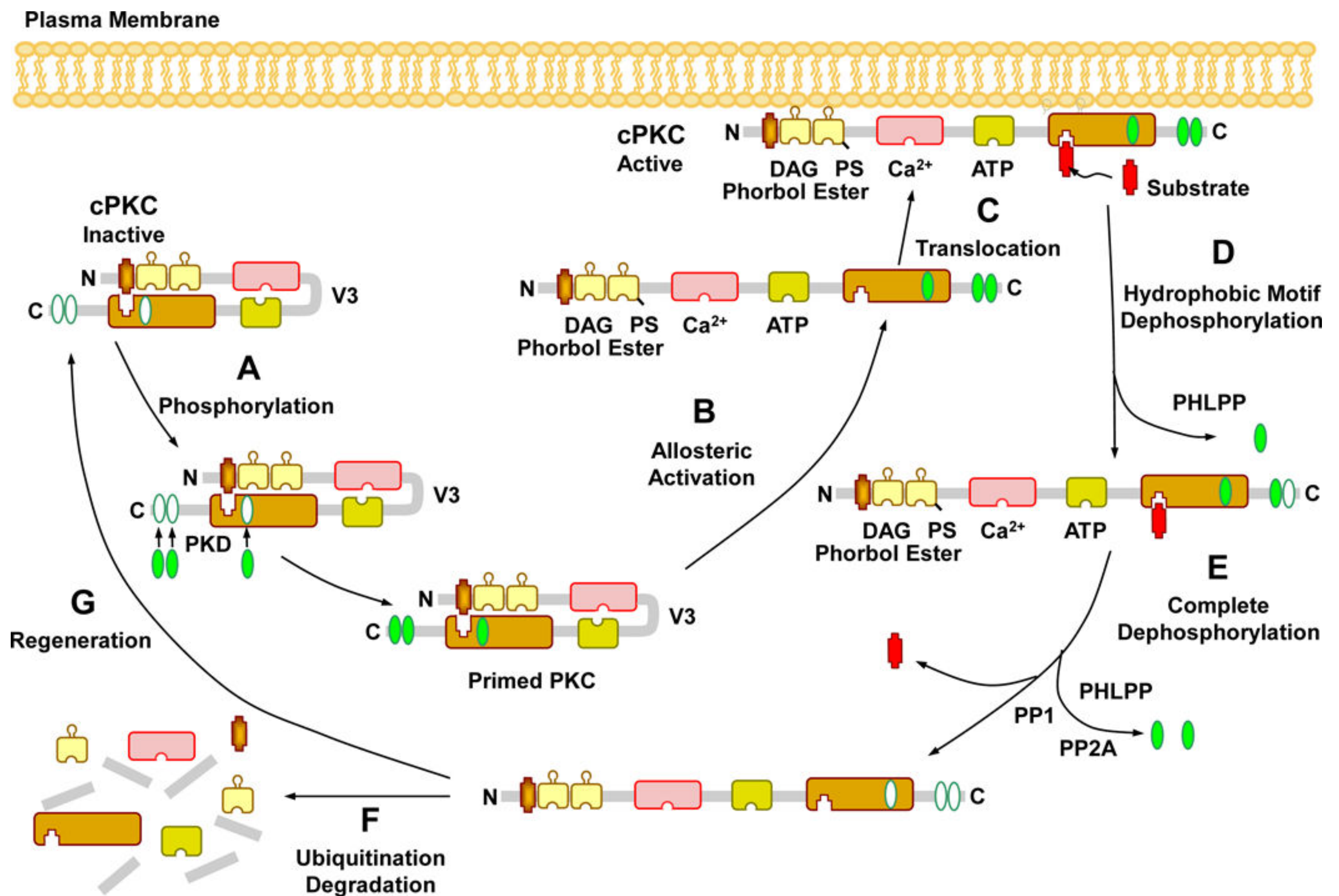

Fig. 2.

Activation, translocation, substrate interaction and deactivation of cPKCs. In the PKC cytosolic and inactive state, the pseudosubstrate binds the catalytic site in the C4 region, leading to folding of the regulatory and catalytic domain. Before it becomes catalytically competent, nascent PKC undergoes phosphorylation at three phosphorylation sites. The first and rate-limiting phosphorylation of the activation loop is catalyzed by phosphoinositide-dependent kinase (PDK). Consequently, a negative charge is introduced that properly aligns residues to form a competent catalytic domain and facilitate subsequent autophosphorylation at the turn motif and hydrophobic motif, a process that keeps PKC in a catalytically competent and protease resistant conformation. PKC activators such as PS, DAG, phorbol esters, and Ca^{2+} promote full allosteric activation and translocation of PKC to the plasma membrane. Allosteric activation also induces an open conformation state, making PKC susceptible to phosphatases and proteases and allows PKC to either enter an autophosphorylation/dephosphorylation cycle, or undergoes proteolytic degradation, PKC dephosphorylation terminates its kinase activity and is carried out by the PP2C member pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) at the hydrophobic motif, starting the process that consequently drives complete dephosphorylation of PKC by PP1/PP2A protein phosphatases at the turn motif. Dephosphorylation also predisposes “naked” PKC to ubiquitination and degradation, requiring new synthesis of the enzyme.

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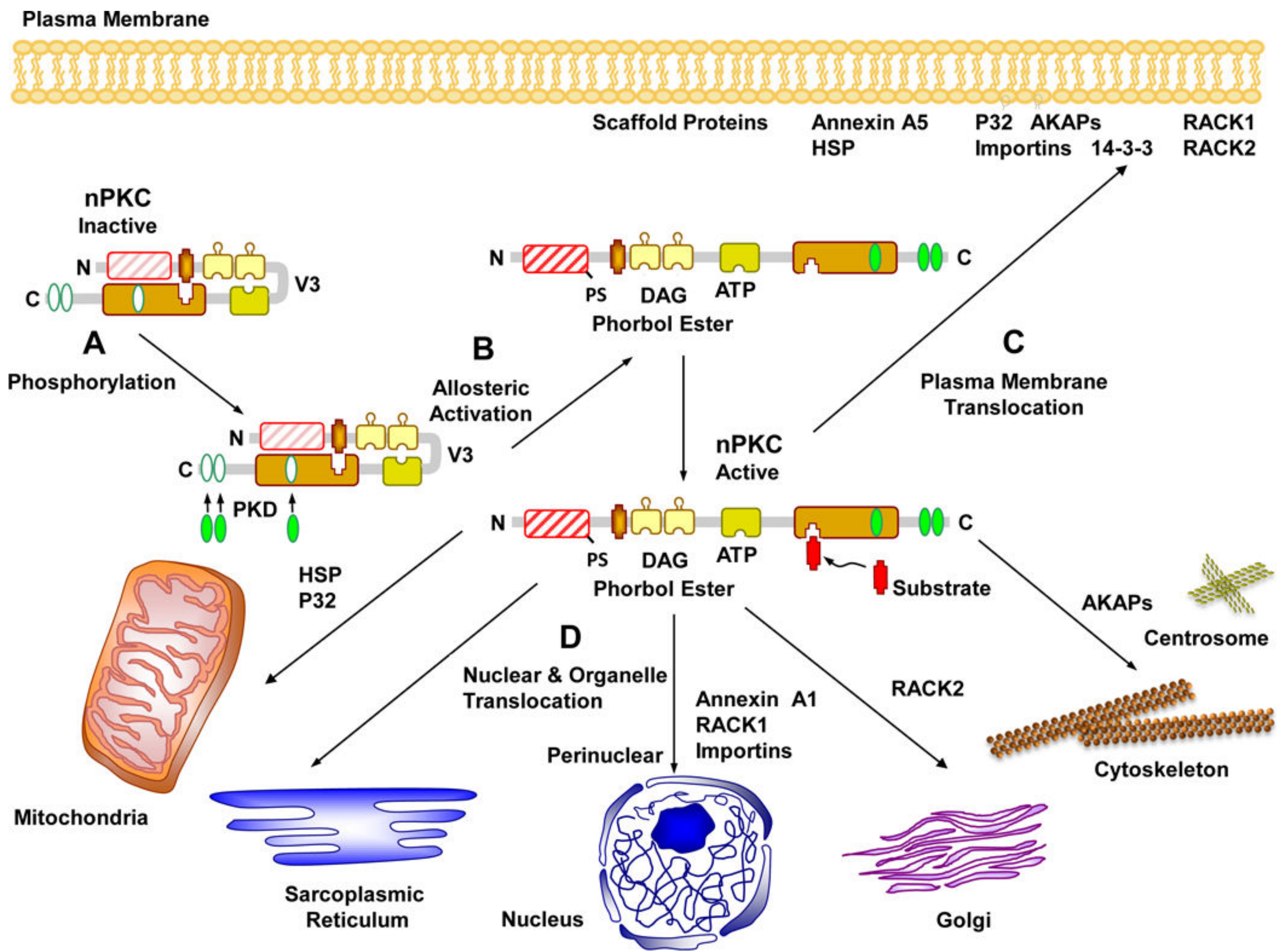

Fig. 3.

Activation and translocation of novel PKCs (nPKC). After undergoing phosphorylation (A) and allosteric activation (B), nPKCs may remain cytosolic or use scaffold proteins to translocate to the plasma membrane (C), or to the perinuclear region, the nucleus and cell organelles (D) such as the mitochondria, sarcoplasmic reticulum, Golgi, the cytoskeleton and centrosome.

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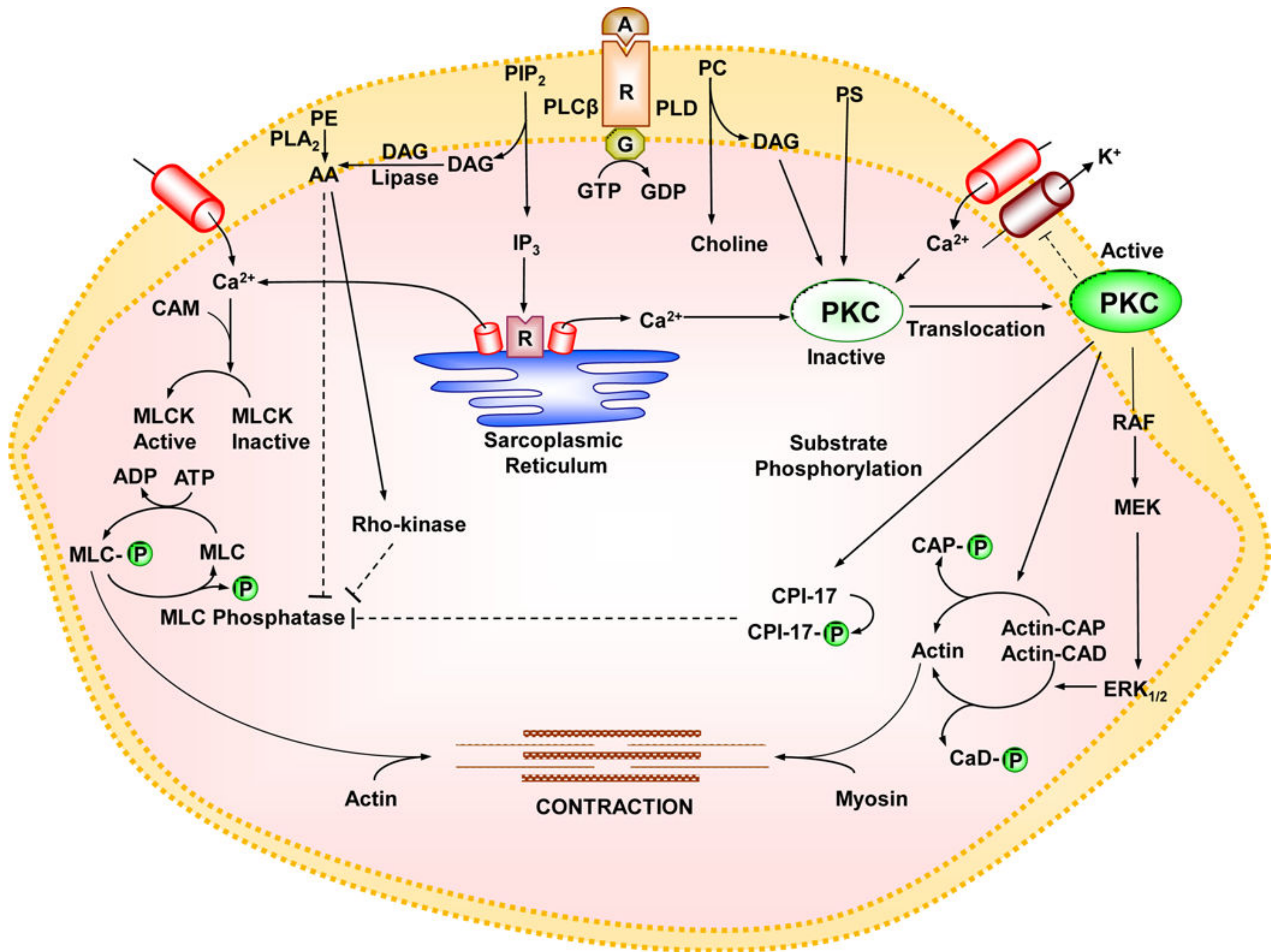

Fig. 4.

Pathways of VSM contraction. The interaction of an agonist (A) with its specific α-adrenergic receptor (R) and its coupled heterotrimeric GTP-binding protein (G) activates phospholipase C (PLCβ) which stimulates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) as well as phospholipase D (PLD) which stimulates the hydrolysis of phosphatidylcholine (PC) into choline and DAG. IP_3 stimulates Ca^{2+} release from the sarcoplasmic reticulum. Agonists also stimulate Ca^{2+} influx through Ca^{2+} channels. Ca^{2+} binds calmodulin (CAM), activates myosin light chain (MLC) kinase (MLCK), causes MLC phosphorylation, and initiates VSM contraction. DAG in the presence of PS, and in case of cPKCs Ca^{2+}, cause activation and translocation of PKC. PKC could inhibit K^+ channels leading to membrane depolarization and activation of voltage-gated Ca^{2+} channels, but could also inhibit Ca^{2+} entry through store-operated Ca^{2+} channels (SOCs) and transient receptor potential channels (TRPCs). PKC could cause phosphorylation of CPI-17, which in turn inhibits MLC phosphatase and increases MLC phosphorylation and VSM contraction. PKC-induced phosphorylation of the actin-binding protein calponin (CaP) allows more actin to bind myosin and enhances contraction. PKC may also activate a protein kinase cascade involving Raf, MAPK kinase

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(MEK) and MAPK ($ERK_{1/2}$) leading to phosphorylation of the actin-binding protein caldesmon (CaD) and enhanced contraction. DAG is transformed by DAG lipase into arachidonic acid (AA). Also, activation of phospholipase A2 (PLA2) increases the hydrolysis of phosphatidylethanolamine (PE) into AA. AA could activate RhoA/Rho-kinase, which in turn inhibits MLC phosphatase and further enhances the Ca^{2+} sensitivity of contractile proteins. Dashed line indicates inhibition.

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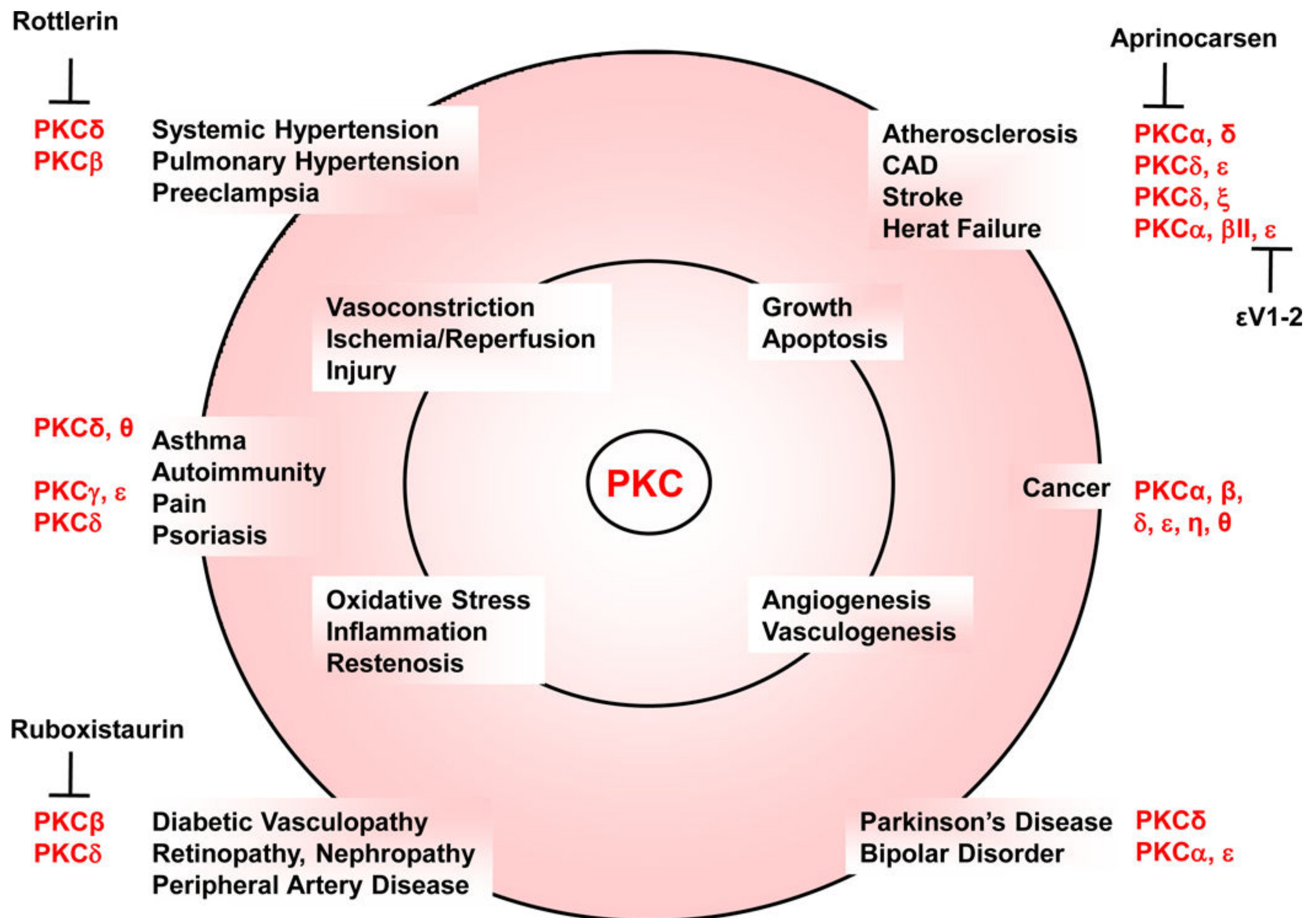

Fig. 5.

Implications for PKC in pathological processes and diseases. PKC mediates many pathological processes in the vascular system and other tissue and organs, which could contribute to vascular diseases such as hypertension and atherosclerosis, and other diseases such as cancer, asthma and autoimmune disease. The pathological changes in vascular PKC could also participate in diseases of other tissues and organs. For example, PKC through promoting angiogenesis could play a role in tumor growth. Also, PKC through promoting inflammation could play a role in asthma. PKC isoforms show varying contributions to different diseases, and isoform-specific PKC inhibitors such as the PKCα inhibitor aprinocarsen may be the key to combating their pathological effects while minimizing side effects. CAD, coronary artery disease.

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Table 1

Distribution of PKC isoforms and potential scaffold proteins in major tissues and representative blood vessels

PKC	MW (kDa)	Main Tissue Distribution	Blood vessel Examined	Location in Resting State	Location in Activated State	Potential Scaffold	Reference
cPKCs							
α	74–82	Universally expressed	Rat aorta	Cytosolic	Nuclear	RACK1	1(Watanabe et al., 1989; Wetsel et al., 1992; Haller et al., 1994; Khalil et al., 1994; Ohanian et al., 1996; Kanashiro et al., 2000a; Hoque et al., 2014)
			Rat mesenteric artery	Cytosolic/Membrane	Cytosolic/Membrane	p32	
			Rat carotid, porcine coronary artery, bovine aorta, ferret portal vein	Cytosolic	Plasma membrane	RACK1, AKAPs, HSP, p32	
β	80–82	Adipose tissue, liver, kidney, spleen, skeletal muscle, brain,	Rat aorta	Cytosolic	Nuclear	RACK1, p32	(Haller et al., 1994; Hoque et al., 2014; Mehta, 2014)
			Rat carotid	Cytosolic	Membrane	RACK1, AKAPs, HSP, p32, 14-3-3	
γ	70–82	Adrenal gland, brain	Rat mesenteric artery	Cytosolic	Cytosolic	RACK1, AKAPs, HSP Importins, 14-3-3	(Wetsel et al., 1992; Ohanian et al., 1996; Hoque et al., 2014)
nPKCs							
δ	76–82	Universally expressed	Rat aorta	Cytoskeleton/Organelle	Cytoskeleton/Organelle	RACK1, p32	(Liou and Morgan, 1994; Ohanian et al., 1996; Zhao et al., 2012; Hoque et al., 2014)
			Rat mesenteric artery	Membrane	Membrane	AKAPs, HSP, p32, 14-3-3	
ε	90–97	Pancreas, kidney, brain	Rat mesenteric artery, porcine coronary artery	Cytosolic/Membrane	Cytosolic/Membrane	AKAPs, p32	(Khalil et al., 1992; Wetsel et al., 1992; Ohanian et al., 1996; Kanashiro et al., 2000a; Hoque et al., 2014)
			Ferret aorta	Cytosolic	Surface membrane	RACK1, RACK2 AKAPs, HSP, p32, 14-3-3	
η	80	Lung, skin, brain	NIH 3T3 fibroblasts	Cytosolic/Membrane	Membrane		(Goodnight et al., 1995; Suzuki et al., 2009; Hoque et al., 2014)
θ	79	Lymphoid and hematopoetic cells, skeletal muscle				AKAPs, HSP, Importins, p32, 14-3-3	(Hoque et al., 2014; Hage-Sleiman et al., 2015)
aPKCs							
ζ	64–82	Universally expressed	Rat aorta, ferret aorta and portal vein	Perinuclear	Intranuclear	AKAPs, HSP, Importins, p32, 14-3-3	(Khalil et al., 1992; Wetsel et al., 1992; Liou and Morgan, 1994; Ohanian et al., 1996; Hoque et al., 2014)
			Rat mesenteric artery	Cytosolic	Cytosolic		

PKC	MW (kDa)	Main Tissue Distribution	Blood vessel Examined	Location in Resting State	Location in Activated State	Potential Scaffold	Reference
ι/λ	70	Testis, ovary, kidney, brain	Rabbit femoral artery and portal vein	Cytosolic	Cytosolic	AKAPs, HSP, Importins, 14-3-3	(Akimoto et al., 1994; Gailly et al., 1997; Hoque et al., 2014)

MW, molecular weight

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Table 2

Representative PKC substrates and the effect of their phosphorylation

Substrate	Effect of Substrate Phosphorylation	Reference
Histones		
H3T45	DNA fragmentation, apoptosis	(Hurd et al., 2009)
H3T6	Prevents LSD1 from demethylating H3K4 during androgen receptor-dependent gene activation. Promotes cell proliferation	(Metzger et al., 2010)
Membrane-bound proteins		
MARCKS (myristoylated, alanine-rich C kinase substrate)	MARCKS is bound to F-actin. Functions as cross-bridge between cytoskeletal actin and plasma membrane	(Hartwig et al., 1992)
The inhibitory GTP-binding protein Gi	Facilitates the dissociation of the αi subunit from adenylyl cyclase and thereby relieves it from inhibition.	(Kanashiro and Khalil, 1998)
Ion Channels		
BK_{Ca} channels	Inhibition, leading to membrane depolarization, activation of L-type voltage-gated Ca^{2+} channels, and increased $[Ca^{2+}]_i$ and vascular tone, e.g. in pulmonary artery and porcine coronary artery.	(Minami et al., 1993; Lange et al., 1997; Taguchi et al., 2000; Barman et al., 2004; Crozatier, 2006; Ledoux et al., 2006; Zhu et al., 2013)
Voltage-gated K^+ channel	Inhibition. Increases vascular tone	(Cogolludo et al., 2003; Novokhatska et al., 2013; Zhu et al., 2013; Brueggemann et al., 2014)
K_{ATP} channels	Inhibition. Alters the channel properties by modifying kinetics and/or the number of channels at the cell membrane, e.g. in mesenteric artery	(Levitan, 1994; Bonev and Nelson, 1996; Light, 1996; Zhu et al., 2013)
Store-operated Ca^{2+} channel	HEK293 cells. Inhibition.	(Shi et al., 2004)
Ion Pumps & Exchangers		
Ca^{2+}-ATPase activation	Activation. Promotes Ca^{2+} extrusion. Explains transient nature of agonist-induced increase in VSM $[Ca^{2+}]_i$.	(Salamanca and Khalil, 2005)
α1 subunit of Na^+/K^+-ATPase	Inhibition. Alters membrane potential and intracellular concentrations of Na^+ and K^+	(Bertorello et al., 1991)
Na^+/H^+ antiport exchanger	Activation. Increases cytoplasmic pH, which increases contraction	(Aviv, 1994; Austin and Wray, 2000; Wray and Smith, 2004)
Cytoskeletal & Regulatory Proteins		
Vinculin	Controls cell shape and adhesion	(Perez-Moreno et al., 1998)
Vimentin	Recycles β1-integrins to plasma membrane	(Ivaska et al., 2005)
CPI-17	Enhances myofilament force sensitivity to Ca^{2+}. Inhibits MLC phosphatase, increases MLC phosphorylation and enhances VSM contraction, e.g. in rabbit femoral artery	(Woodsome et al., 2001)
Calponin	Allows actin-myosin interaction and enhances VSM contraction	(Parker et al., 1994)
Raf	Initiates a cascade involving MAPK kinase (MEK) and MAPK, and phosphorylation of the actin-binding protein caldesmon (CaD) which reverses its inhibition of MgATPase activity and thus increases actin–myosin interaction and VSM contraction	(Khalil et al., 1995; Kim et al., 2008)
20-kDa MLC and MLCK	Counteracts Ca^{2+}-induced actin–myosin interaction and force development, e.g. in In rabbit mesenteric artery	(Inagaki et al., 1987)
Ribosomal Protein Kinases		
S6KβII	Nucleocytoplasmic shuttling of S6KβII. Regulates protein synthesis and the G1/S transition in the cell cycle	(Valovka et al., 2003)

Substrate	Effect of Substrate Phosphorylation	Reference
Other		
Arginine-rich protein substrates	Neutralizes the acidic patch in the substrate binding site. Displaces PKC pseudosubstrate from the kinase core	(House and Kemp, 1987; Newton, 1995)

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Table 3

PKC Inhibitors

Class/ Inhibitor	Chemistry	Site of Action	Isoform Selectivity	Kd or IC50	Reference
Isoquinolines H-7	1-(5-isoquinolinesulfonyl)-2-methylpiperazines	ATP-binding site		PKCβI 3.5 μM PKCζ 6 μM	(Howcroft and Lindquist, 1991)
Benzophenones Chelerythrine	1,2-dimethoxy-12-methyl[1,3]benzodioxolo[5,6-c]phenanthridin-12-ium	ATP-binding site	Pan-PKCs	0.66 μm	(Ding et al., 2011b)
Balanol	2-{[2,6-dihydroxy-4-({[(3S,4R)-3-[(4-hydroxybenzene)amido]azepan-4-yl]oxy}carbonyl)phenyl]carbonyl}-3-hydroxybenzoic acid	ATP-binding site	Pan-PKCs PKCβII> βI> η> δ> α> ε	4–9 μm	(Pande et al., 2008; Mochly-Rosen et al., 2012)
Indolocarbazoles Gö6976	5,6,7,13-tetrahydro-13-methyl-5-oxo-12H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-12-propanenitrile	Catalytic domain	PKCα, β1	PKCα 2.3, βI 6.2 nM	(Martiny-Baron et al., 1993; Grandage et al., 2006)
Gö6983	1H-Pyrrole-2,5-dione, 3-[1-[3-(dimethylamino)propyl]-5-methoxy-1H-indol-3-yl]-4-(1H-indol-3-yl)-	ATP-binding site Suppresses PKCμ auto-phosphorylation	Pan-PKC inhibitor Potent: PKCα, β, γ, δ Less potent: PKCζ	PKCα 7, β 7, γ 6, δ 10, ζ 60 nM	(Gschwendt et al., 1996; Peterman et al., 2004)
Enzastaurin (LY317615)	3-(1-methyl-1H-indol-3-yl)-4-(1-(1-(pyridin-2-ylmethyl)piperidin-4-yl)-1H-indol-3-yl)-1H-pyrrole-2,5-dione	ATP-binding site	Potent: PKCβ Less potent: PKCα, γ, ε	PKCα 39, β 6, γ 83, ε 110 nM	(Graff et al., 2005; Rovedo et al., 2011)
LY379196		ATP-binding site	PKCβ	3–6 μM	(Slosberg et al., 2000)
Staurosporine (CGP41251)	9,13-Epoxy-1H,9H-diindolo[1,2,3-gh:3′,2′,1′-lm]pyrrolo[3,4-j][1,7]benzodiazonin-1-one, 2,3,10,11,12,13-hexahydro-10-methoxy-9-methyl-11-(methylamino)-, [9S-(9α,10β,11β,13α)]-	ATP-binding site	Pan-PKCs Potent: PKCα, γ, η Less potent: PKCδ, ε	PKCα 2, γ 5, δ 20, η 4 nM	(Tamaoki et al., 1986; Meggio et al., 1995)
CGP53353	5,6-bis[(4-Fluorophenyl)amino]-1H-isoindole-1,3(2H)-dione	ATP-binding site	PKCβ	PKCβI 3.8, βII 0.41 μM	(Deng et al., 2012)
UCN-01	7-hydroxystaurosporine	ATP-binding site	cPKCs	25–50 nM	(Tamaoki, 1991)
Sotrastaurin (AEB071)	3-(1H-indol-3-yl)-4-(2-(4-methylpiperazin-1-yl)quinazolin-4-yl)-1H-pyrrole-2,5-dione	ATP-binding site	Pan-PKC, especially PKCθ	PKCα 0.95, βI 0.64, δ 2.1, ε 3.2, η 1.8, θ 0.22 nM (Ki)	(Evenou et al., 2009; Naylor et al., 2011)
Staurosporine Analogs Ruboxistaurin (LY333531)	(9S)-9-[[(Dimethyl-d6)amino]methyl]-6,7,10,11-tetrahydro-9H,18H-5,21:12,17-Dimethenodibenzo[e,k]pyrrolo[3,4-h][1,4,13]oxadiazacyclohexadecine-18,20(19H)-dione Hydrochloride	ATP-binding site	PKCβI, βII.	PKCβI 4.7, βII: 5.9 nM	(Aiello et al., 2011)
Midostaurin (PKC412, CGP41251)	(9S,10R,11R,13R)-2,3,10,11,12,13-Hexahydro-10-methoxy-9-methyl-11-(methylamino)-9,13-epoxy-1H,9H-diindolo[1,2,3-gh:3′,2′,1′-lm]pyrrolo[3,4-j][1,7]benzodiamzonine-1-one	ATP-binding site	Pan-PKCs	12 nM	(Millward et al., 2006)
Bisindolylmaleimide (GF 109203X, Gö 6850)	3-(1-(3-(Dimethylamino)propyl)-1H-indol-3-yl)-4-(1H-indol-3-yl)-1H-pyrrole-2,5-dione	ATP-binding site	Pan-PKC, especially PKCα, βI	PKCα 8.4, βI 18, βII 16, γ 20, δ 210, ε 132, ζ 5800 nM	(Toullec et al., 1991; Gekeler et al., 1996)
Ro 31-8220	Carbamimidothioic acid, 3-[3-[2,5-dihydro-4-(1-methyl-1H-indol-3-yl)-2,5-dioxo-1H-pyrrol-3-yl]-1H-indol-1-yl]propyl ester, methanesulfonate	Catalytic domain	Pan-PKC: PKCα, βI, βII, γ, ε	PKCα 5, βI 24, βII: 14, γ 27, ε 24 nM	(Wilkinson et al., 1993; Davies et al., 2000)
SCH47112		ATP-binding site			(Reynolds et al., 1997)
Dicationic, lipophilic compounds Dequalinium Cl	Quinolinium, 1,1′-(1,10-decanediyl)bis[4-amino-2-methyl-, chloride (1:2)	Covalently modifies the C2-domain	All PKC	7 μM-18 μM	(Castle et al., 1993; Manetta et al., 1993; Roffey et al., 2009)
Flavonoid Myricitrin	4H-1-Benzopyran-4-one, 3-[(6-deoxy-α-L-mannopyranosyl)oxy]-5,7-dihydroxy-2-(3,4,5-trihydroxyphenyl)-	Prevents PKCα and PKCε activation by phorbol esters	PKCα, ε		(Meotti et al., 2006)
Quercetin	4H-1-Benzopyran-4-one, 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-		Slight PKC inhibitor		(Navarro-Nunez et al., 2010)
Benzothiazole Riluzole	6-(trifluoromethoxy)benzothiazol-2-amine	ATP-binding site	PKCα		(Noh et al., 2000)
Perylenequinone Calphostin C (UCN-1028C)	1-[3,10-dihydroxy-12-[2-(4-hydroxyphenoxy)carbonyloxypropyl]-2,6,7,11-tetramethoxy-4,9-dioxoperylen-1-yl]propan-2-yl benzoate	Regulatory domain: Competes at the binding site for DAG and phorbol esters.	cPKCs, nPKCs	50 nM	(Ogiwara et al., 1998)
Phenolic ketone Rottlerin (Mallotoxin)	5,7-dihydroxy-2,2-dimethyl-6-(2,4,6-trihydroxy-3-methyl-5-acetylbenzyl)-8-cinnamoyl-1,2-chromene)	ATP-binding site	PKCδ Other nPKCs	PKCδ 5 μM Other PKCs 30 μM	(Gschwendt et al., 1994)
Macrolactone Bryostatin 1 (NSC 339555)	(1S,3S,5Z,7R,8E,11S,12S,13E,15S,17R,21R,23R,25S)-25-(Acetyloxy)-1,11,21-trihydroxy-17-[(1R)-1-hydroxyethyl]-5,13-bis(2-methoxy-2-oxoethylidene)-10,10,26,26-tetramethyl-19-oxo-18,27,28,29-tetraoxatetracyclo[21.3.1.1^{3,7}.1^{11,15}]nonacos-8-en-12-yl (2*E*,4*E*)-2,4-octadienoate	C1 domain of PKC: competes with phorbol ester and diacylglycerol binding	Twofold selectivity for PKCε over PKCα and PKCδ (short term administration activates PKC, long term inhibits)		(Kraft et al., 1986; Roffey et al., 2009; Mochly-Rosen et al., 2012)
Membrane lipids Sphingosine (D-erythro-Sphingosine)	2-Amino-4-octadecene-1,3-diol; trans-4-Sphingenine	Regulatory domain: Competitive inhibitor with phosphatidylserine		2.8 μM	(Khan et al., 1990)
N,N-Dimethyl-D-erythro-sphingosine	(E,2S,3R)-2-(Dimethylamino)octadec-4-ene-1,3-dio			12 μM	(Kim and Im, 2008)
Taxol Tamoxifen	2-[4-[(Z)-1,2-diphenylbut-1-enyl]phenoxy]-N,N-dimethylethanamine	Regulatory domain	cPKCs		(Zarate et al., 2007)
Purine nucleoside Sangivamycin	4-amino-5-carboxamide-7-(D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine	ATP-binding site		10 μM	(Osada et al., 1989)
Carbonitrile 5-vinyl-3-pyridinecarbonitriles		Catalytic domain	PKCθ	PKCθ 4.7 nM	(Tumey et al., 2009)

Class/ Inhibitor	Chemistry	Site of Action	Isoform Selectivity	K_d or IC_{50}	Reference
Pyrimidine 2,4-Diamino-5-nitropyrimidine		Catalytic domain	PKCθ		(Cywin et al., 2007)
Sterols Spheciosterol sulfate A		Catalytic domain	PKCζ	PKCζ 1.59 μM	(Whitson et al., 2009)
Spheciosterol sulfate B		Catalytic domain	PKCζ	PKCζ 0.53 μM	
Spheciosterol sulfate C		Catalytic domain	PKCζ	PKCζ 0.11 μM	
Antisense oligonucleotides Isis3521 (CGP64128A, Aprinocarsen)	20-mer phosphorothioate oligodeoxynucleotide	Inhibits PKCα mRNAexpression	PKCα	–	(Lahn et al., 2003)
Isis9606	19-mer phophorothioate oligodeoxynucleotide	Inhibits PKCα mRNA	PKCα	–	(Levesque et al., 1997)
Short peptides Myristoylated-pseudosubstrate peptide inhibitor	Peptide sequence: myr-FARKGALRQ	Substrate-binding site	cPKCs	–	(Eichholtz et al., 1993)
αV5-3	Peptide sequence: QLVIAN	Site: aa 642–647	PKCα	–	(Kim et al., 2011b)
βIV5-3	Peptide sequence: KLFIMN	Inhibits PKC translocation Site: aa 646–651	PKCβI	–	(Ferreira et al., 2011)
βIIV5-3	Peptide sequence: QEVIRN	Inhibits PKC translocation Site: aa 645–650	PKCβII	–	(Stebbins and Mochly-Rosen, 2001)
βC2-4	Peptide sequence: SLNPEWNET	Site: aa 218–226	All cPKCs	–	(Ron et al., 1995)
δV1-1 (KAI-9803, Delcasertib)	Peptide sequence: SFNSYELGSL	RACK-binding site Inhibits translocation Site: aa 8–17	PKCδ	–	(Chen et al., 2001)
εV1-2 (KAI-1678)	Peptide sequence: EAVSLKPT	RACK-binding site Inhibits translocation Site: aa 14–21	PKCε	–	(Gray et al., 1997)
KCε-12 and KCε-16		Substrate-binding site	PKCε	–	(Yonezawa et al., 2009)
ZIP	Peptide sequence: SIYRRGARRWRKL	ζ-pseudo substrate	PKCζ and aPKCs	–	(Braun and Mochly-Rosen, 2003)
γV5-3	Peptide sequence: RLVLAS	Site: aa 659–664	PKCγ	–	(Sweitzer et al., 2004)
Other α-tocopherol, adriamycin, aminoacridine, apigenin, cercosporin, chlorpromazine, dexniguldipine, polymixin B, trifluoperazine, UCN-02					

aa, amino acid

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Table 4

PKC knockout mouse models, their prominent phenotype, and major implications

PKC Knockout	Prominent Phenotype	Implications	Reference
PKCα$^{-/-}$	Increased BP in knockout mice fed a high-salt diet. Principal cells of renal cortical collecting ducts show increased number of epithelial Na channel (ENaC) per cell-attached patch clamp, increased membrane localization of α-, β-, and γ-subunits of ENaC, and increased open probability of ENaC channel.	PKCα reduces ENaC membrane accumulation and open probability	(Bao et al., 2014)
	In skeletal muscles and adipocytes, enhanced insulin signaling to insulin receptor substrate (IRS) 1-dependent PI_3K, PKB, and PKCλ, and downstream processes, glucose transport and activation of ERK	PKCα serves as a tonic endogenous inhibitor of IRS-1-dependent PI_3K, PKB, and PKCλ during insulin stimulation of glucose transport and ERK	(Leitges et al., 2002)
	Peripheral CD3(+)T cells show impaired CD3/CD28 Ab- and MHC alloantigen-induced T cell proliferation and IFN-γ production. PKCα$^{-/-}$ mice give diminished OVA-specific IgG2a and IgG2b responses following OVA immunization experiments	PKCα is necessary for T cell-dependent IFN-γ production and IgG2a/2b Ab responses	(Pfeifhofer et al., 2006)
PKCβ$^{-/--}$	ApoE$^{-/-}$ and PKCβ$^{-/-}$/ApoE$^{-/-}$ mice rendered diabetic with streptozotocin, Diabetes accelerated atherosclerosis in the aorta, increased the level of phosphorylated ERK1/2 and Jun-N-terminus kinase MAPK and augmented vascular expression of inflammatory mediators, and monocyte/macrophage infiltration and CD11c(+) cells accumulation, and processes were diminished by pharmacological inhibition of PKCβ and in diabetic PKCβ(−/−)/ApoE(−/−) mice.	PKCβ is linked to diabetic atherosclerosis through modulation of gene transcription, cell signaling and inflammation in the vascular wall. PKCβ could be a potential therapeutic target for prevention and treatment of diabetic atherosclerosis.	(Kong et al., 2013)
PKCγ$^{-/-}$	Exposure to hyperbaric oxygen was associated with increased thicknesses of the inner nuclear and ganglion cell layers of the retina. Destruction of the outer plexiform layer. Significant degradation of the retina Damage to the outer segments of the photoreceptor layer and ganglion cell layer	PKCγ may protect retina from damage by hyperbaric oxygen. Hyperbaric oxygen, should be used with care particularly in patients with a genetic disease such as spinocerebellar ataxia type 14 with nonfunctional PKCγ.	(Yevseyenkov et al., 2009)
PKCδ$^{-/-}$	Thickening of the articular cartilage and calcified bone-cartilage interface. Increased number of hypertrophic chondrocytes in the articular cartilage. -Loss of demarcation between articular cartilage and bone was concomitant with irregular chondrocyte morphology and arrangement. -Increased intensity of calcein labeling in the interface of the growth plate and metaphysis. -Reduced level of glycosaminoglycan production.	PKCδ plays a role in the osteochondral plasticity of the interface between articular cartilage and the osteochondral junction.	(Yang et al., 2015)
	Increased white blood cells and platelet counts, and bone marrow and splenic megakaryocytes. Increased megakaryocyte number and DNA content. Altered thrombopoietin-induced signaling and increased ERK and Akt308 phosphorylation in megakaryocytes. -Faster recovery and heightened rebound thrombocytosis after thrombocytopenic challenge.	PKCδ is important for megakaryopoiesis by regulating thrombopoietin-induced signaling.	(Kostyak et al., 2014)
	Fertility analysis has shown that mating pairs produce fewer pups per litter than wild-type pairs. Reduced number of total implantations in females. Sperms showed decreased capacity to penetrate the zona pellucida. Pregnant females exhibit a high incidence of embryonic loss post-implantation.	PKCδ is important for key reproductive functions and fertility in both males and females	(Ma et al., 2015)
PKCε$^{-/-}$	Embryonic fibroblasts exhibit reduced insulin uptake which was associated with decreased insulin receptor phosphorylation. Changed localization of insulin receptor with colocalization with membrane microdomains marker flotillin-1. Reduced redistribution of insulin receptor by insulin stimulation -Reduced expression of CEACAM1, a receptor substrate which modulates insulin clearance.	PKCε affects insulin uptake through promotion of receptor-mediated endocytosis, and that this may be mediated by regulation of CEACAM1 expression.	(Pedersen et al., 2013)

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PKC Knockout	Prominent Phenotype	Implications	Reference
PKCη$^{-/-}$	-Poor proliferation of T cells in response to stimulation by antigen -Defective homeostatic proliferation, a function requiring recognition of self antigens. -Higher ratio of CD4+ to CD8+ T cells compared to that of wild-type mice.	PKCη performs functions that are important for homeostasis and activation of T cells.	(Fu et al., 2011)
PKCθ$^{-/-}$	-The thymus contains less mature single positive T cells than wildtype. Thymocytes show defective activation of transcription factors AP-1, NFAT and NFκB and impaired phosphorylation of ERK after T cell receptor stimulation in vitro.	PKCθ plays a role in positive selection of thymocytes in a pathway leading to the activation of ERK, AP-1, NFAT, and NFκB.	(Gruber et al., 2010)
PKCζ$^{-/-}$	-Impaired secretion of T helper 2 (Th2) cytokines, as well as the nuclear translocation and tyrosine phosphorylation of Stat6 and Jak1 activation, essential downstream targets of IL-4 signaling. -Dramatic inhibition of ovalbumin-induced allergic airway disease.	PKCζ is critical for IL-4 signaling and Th2 differentiation. Asthma is a disease of chronic airway inflammation in which T helper (Th) 2 cells play a critical role, and PKCζ can be a therapeutic target in asthma.	(Martin et al., 2005)
PKCλ$^{-/-}$	Tissue-specific knockout in muscle shows impaired insulin-stimulated glucose transport (M) and insulin resistance. Knockout in liver shows impaired insulin-stimulated lipid synthesis and insulin-hypersensitivity. Knockout in adipocytes shows diminished Insulin-stimulated activity and glucose transport, ERK levels and activity. -Diminished adiposity and serum leptin levels.	PKCλ plays a role in insulin-stimulated glucose transport and ERK signaling in muscle, liver and adipocytes.	(Sajan et al., 2014)

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Table 5

PKC Isoforms Involved in Specific Vascular Diseases and PKC Modulators Used as Potential Therapeutic Tools in Clinical Trials

PKC	Vascular Disease	Role of PKC	Drug	Effect on PKC	Outcome	Comments	Reference
PKCβ	Diabetic Retinopathy	Detrimental. Cytokine activation and inhibition, vascular alterations, cell cycle and transcriptional factor dysregulation, abnormal angiogenesis, increased matrix protein synthesis	Ruboxistaurin	Inhibit	Reduced sustained moderate vision loss in large studies	Under review by FDA for diabetic retinopathy	(Tuttle et al., 2005; Vinik et al., 2005; Geraldes and King, 2010; Aiello et al., 2011; Mochly-Rosen et al., 2012)
			Ruboxistaurin	Inhibit	Failed to improve kidney outcomes	Studied as secondary outcome in large retinopathy trials	
			Ruboxistaurin	Inhibit	Mild decrease in symptoms	Requires validation in larger study	
PKCδ	Ischemic Heart Disease	Detrimental. Increases ROS production, decreases ATP generation, increases apoptosis and necrosis	Delcasertib for acute myocardial infarction (MI)	Inhibit	No benefit when given intravenously	Positive biomarker trend when given to patients with TIMI 0/1 flow	(Inagaki et al., 2003; Churchill and Mochly-Rosen, 2007; Bates et al., 2008; Mochly-Rosen et al., 2012)
			KAI-9803: Phase I clinical trial, intracoronary injection during primary percutaneous coronary intervention	Selective PKCδ RACK antagonist	Signs of potential drug activity (not dose-dependent)	Acceptable safety and tolerability	
PKCε	Ischemic Heart Disease	Protective. Protection of mitochondrial functions & proteasomal activity, activation of ALDH2 and reduction of aldehydic load	Adenosine for acute MI	↑PKCε	Reduced infarct size from 27% to 11% when given at 70 mcg/kg/min	No reduction in composite endpoint of death and CHF	(Mochly-Rosen and Kauvar, 2000; Ross et al., 2005; Budas et al., 2007; Chen et al., 2008; Mochly-Rosen et al., 2012)
			Adenosine for coronary bypass grafting	↑PKCε	Reduction in composite AMI, mortality, need for pressors postoperatively.	Requires validation in larger study	
			Acadesine for coronary bypass grafting	↑PKCε	Reduced two year mortality in the small group of patients who had a post-operative acute MI.	No reduction in death, acute MI, or stroke	