




Review

The Role of Tyrosine Phosphorylation of Protein Kinase C Delta in Infection and Inflammation

Qingliang Yang ^{1,†}, Jordan C. Langston ^{2,†}, Yuan Tang ¹, Mohammad F. Kiani ^{1,3} and Laurie E. Kilpatrick ^{4,*} 

¹ Department of Mechanical Engineering, College of Engineering, Temple University, Philadelphia, PA 19122, USA; tug44932@temple.edu (Q.Y.); tud19329@temple.edu (Y.T.); mkiani@temple.edu (M.F.K.)

² Department of Bioengineering, College of Engineering, Temple University, Philadelphia, PA 19122, USA; tuj27061@temple.edu

³ Department of Radiation Oncology, Lewis Katz School of Medicine, Temple University, Philadelphia, PA 19140, USA

⁴ Center for Inflammation, Clinical and Translational Lung Research, Department of Thoracic Medicine and Surgery, Lewis Katz School of Medicine, Temple University, Philadelphia, PA 19140, USA

* Correspondence: laurie.kilpatrick@temple.edu; Tel.: +1-(215)-707-8420

† These authors contributed equally to this work.

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Abstract: Protein Kinase C (PKC) is a family composed of phospholipid-dependent serine/threonine kinases that are master regulators of inflammatory signaling. The activity of different PKCs is context-sensitive and these kinases can be positive or negative regulators of signaling pathways. The delta isoform (PKC δ) is a critical regulator of the inflammatory response in cancer, diabetes, ischemic heart disease, and neurodegenerative diseases. Recent studies implicate PKC δ as an important regulator of the inflammatory response in sepsis. PKC δ , unlike other members of the PKC family, is unique in its regulation by tyrosine phosphorylation, activation mechanisms, and multiple subcellular targets. Inhibition of PKC δ may offer a unique therapeutic approach in sepsis by targeting neutrophil-endothelial cell interactions. In this review, we will describe the overall structure and function of PKCs, with a focus on the specific phosphorylation sites of PKC δ that determine its critical role in cell signaling in inflammatory diseases such as sepsis. Current genetic and pharmacological tools, as well as in vivo models, that are used to examine the role of PKC δ in inflammation and sepsis are presented and the current state of emerging tools such as microfluidic assays in these studies is described.

Keywords: PKC; PKC δ ; phosphorylation; microfluidics; inflammation; sepsis

1. Protein Kinase C (PKC) Superfamily

Protein Kinase C (PKC) was first identified by Nishizuka and coworkers in 1977 and is now known to be composed of a family of phospholipid-dependent serine/threonine kinases [1]. PKC isoforms (PKCs) are involved in numerous signal transduction pathways and are implicated in the regulation of numerous cellular functions [2–4]. These kinases are composed of a highly conserved catalytic domain (C-terminus) and a regulatory domain (N-terminus) that demonstrates considerable variability across family members [2]. Based on structural elements and cofactor requirements, mammalian PKCs are classified into four broad categories comprising classical PKCs (cPKCs: α , β -I, β -II, and γ isoforms), novel PKCs (nPKCs: δ , ϵ , η , and θ isoforms), atypical PKCs (aPKCs: ι and ζ isoforms), and PKC-related kinases (PRKs 1–3) [2–7]. Calcium (Ca²⁺) and the lipid second messenger diacylglycerol (DAG) are required for cPKCs activation. DAG, but not (Ca²⁺), activates the nPKCs. The aPKCs do not require Ca²⁺ or DAG for activation, but are sensitive to other lipid second messengers such as

phosphatidylserine (PS) [8]. The activity of different PKCs is context-sensitive and these kinases can be positive or negative regulators of signaling pathways. This contextual dependency of the PKC function often makes it difficult to determine the precise roles of PKCs in normal and aberrant cellular processes [8,9]. Increased activity of several PKCs has been implicated in multiple diseases, including inflammation, sepsis, and cancer [5,9].

PKC δ is a unique nPKC that plays a significant role in several diseases, including cancer, diabetes, ischemic heart disease, and neurodegenerative diseases [10–19]. Recent studies from our research group and others have shown that PKC δ is also a critical regulator of the inflammatory response in sepsis [8,19–26]. While a role for PKC δ in sepsis is established, less is known about how PKC δ is activated during the inflammatory response. PKC δ , unlike other members of the PKC family, is unique in its regulation by tyrosine phosphorylation on multiple sites that determine activation, localization, and substrate specificity [2,27–30]. The goals of this review are to (1) review the overall structures and subfamilies of the PKCs and general activation mechanisms, (2) present an overview of the structure and unique regulation of PKC δ , and (3) describe the especially unique and critical roles of PKC δ in sepsis. We will focus on the specific phosphorylation sites of PKC δ that determine its critical role in cell signaling in inflammation. Finally, we will present genetic and pharmacological tools, as well as in vivo models, that can be used to examine the role of PKC δ in inflammation and sepsis, and how emerging tools such as microfluidics can be useful in such explorations.

The different PKCs share several common structural features (Figure 1). The catalytic domain, located at the C-terminus, contains the ATP binding site, as well as the substrate binding sites [2,5,31]. A hinge region connects the catalytic domain to the regulatory domain, which is a domain that regulates the activation state of the kinase through a pseudosubstrate region. The pseudosubstrate region is a substrate-mimicking short amino acid sequence that binds the substrate-binding cavity in the catalytic domain, rendering the enzyme inactive (Figure 2). PKCs contain several conserved membrane-targeting modules that are located in the regulatory domain (C1 and C2) and the catalytic domain (C3 and C4) [2].

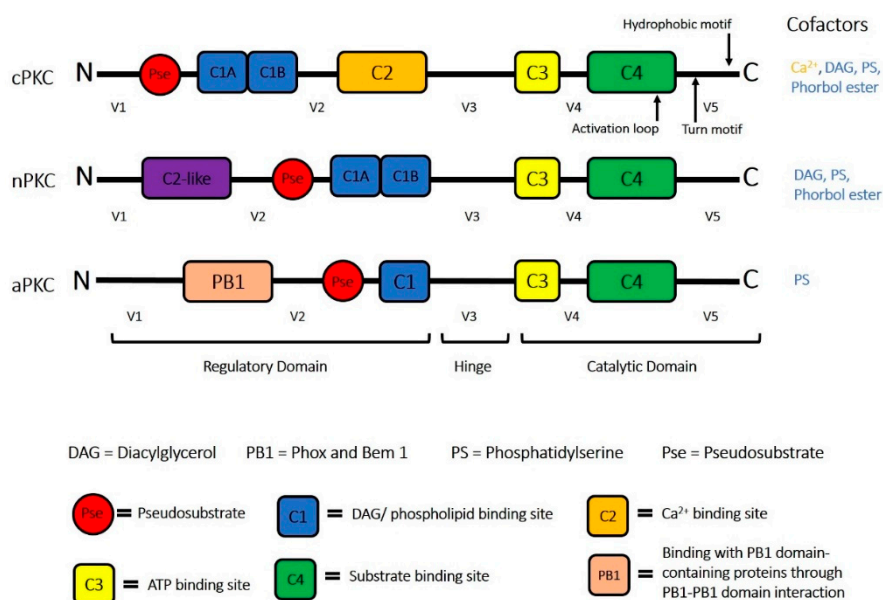


Figure 1. Structure of the three main classes of Protein Kinase C (PKC)s along with their respective activators. The hinge domain separates the regulatory domain and the catalytic domain. The regulatory domain contains: the pseudosubstrate (binds to C4 when not activated) for keeping the enzyme inactive; the C1 domain (including C1A and C1B) for DAG/PS/phorbol ester binding for cPKCs and nPKCs; the C2 domain for Ca²⁺ binding; the C2-like domain for nPKC spatial distribution; and the C1 domain (in aPKCs) for PS binding. The catalytic domain contains the C3 domain for ATP binding and C4 domain for substrate/pseudosubstrate binding.

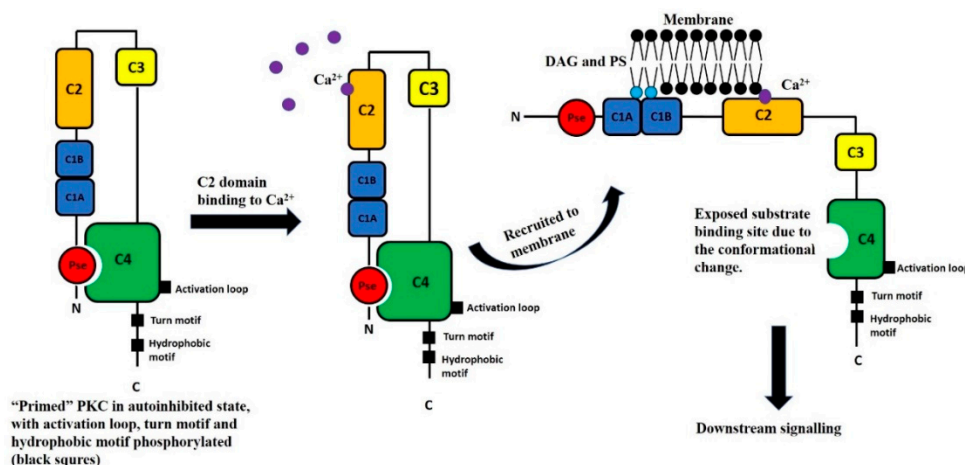


Figure 2. Schematic drawing of the activation steps of cPKCs. Following the three distinct phosphorylations at the activation loop, the turn motif, and the hydrophobic motif (for example, in human PKC β -II, corresponding to threonine 500, serine 641, and threonine 6601, respectively), PKCs are released into the cytosol, but with the pseudosubstrate occupying the substrate-binding site. Binding to Ca^{2+} , PS, and DAG results in membrane translocation and subsequent conformational change, which releases the pseudosubstrate from the substrate-binding site.

The C1 region in the regulatory domain also contains the pseudosubstrate region that controls PKC activity. The C1 domain is also the binding site for DAG and PS, critical cofactors in cPKC and nPKC activation [5,32–34], as well as the non-hydrolysable, non-physiological analogues, phorbol esters. DAG is the product of the hydrolysis of the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (PLC), which yields inositol triphosphate (IP_3) and DAG [8,27,35]. IP_3 in turn activates signaling pathways that elevate intracellular Ca^{2+} levels and thereby activate cPKCs [27]. For cPKCs and nPKCs, the DAG-mediated activation is initiated by the docking of DAG/PS to the two cysteine-rich regions (C1A and C1B) in the C1 domain. This docking event weakens the interaction of an inhibitory pseudo-substrate domain with the C-terminus catalytic core and recruits cPKCs and nPKCs to the membrane compartment [36]. cPKCs are also regulated by changes in cytosolic Ca^{2+} concentrations. The C2 domain is a critical Ca^{2+} -sensing membrane-targeting module in cPKCs [5]. The C2 domain in cPKCs binds two or three calcium ions [37–39] and facilitates the docking of cPKCs to the plasma membrane. In nPKCs, the C2-like domain lacks one or more of the conserved aspartate residues required for Ca^{2+} binding, and these isoforms are activated by DAG/PS in the absence of Ca^{2+} [5,29]. The function of the C2-like domain in nPKCs remains unclear. It is speculated that the C2-like domain is involved in the control of the nPKC spatial distribution via protein-protein interactions [31]. aPKCs, on the other hand, lack the C2 domain and have an incomplete C1 domain. Thus, aPKCs are Ca^{2+} -insensitive, and do not respond to DAG. aPKCs are activated through the Phox and Bem 1 (PB1) domain, which is a protein interaction module that mediates aPKCs interactions with other PB1 domain-containing scaffolding proteins and phospholipid co-factors such as PS [4,5,8,40,41]. The C3 and C4 domains form the ATP- and substrate-binding components, respectively, of the kinase core [34].

PKCs also contain five variable regions, which are poorly conserved across the different PKCs [5]. For example, in PKC δ , the V1 region contains the translocation inhibitor site; V2 contains the translocation activation site; V3 (at the hinge region) contains serine phosphorylation sites at 299, 302, 304, and tyrosine 311 and 322 phosphorylation sites; V4 contains the ATP binding sequence; and V5 contains the turn and hydrophobic motifs, as well as serine 643 and 662 phosphorylation sites (Figure 3) [5].

In order for these allosteric interactions to occur, however, PKCs must first be properly folded and in the correct conformation permissive for catalytic action (Figure 2). This is contingent upon phosphorylation of the catalytic region, at the activation loop, the turn motif, and the hydrophobic

motif [42]. First, PKCs are phosphorylated on the activation loop by phosphoinositide-dependent kinase, PDK-1, which functions as a switch to elicit the other two phosphorylations. Next, the turn motif and hydrophobic motif are autophosphorylated. After the three “priming” phosphorylation steps, the kinase is mature and released to the cytosol and is thus ready to respond to second messengers. It is worth noting that the activation loop phosphorylation is not required for the entire regulation process. Once the first step of phosphorylation is completed, the activation loop may be dephosphorylated [42].

Though the binding of DAG does not lead to a significant conformational change, it dramatically alters the surface properties of the kinase to create a hydrophobic surface for tight membrane binding. After binding to the membrane, the interaction of the C1 domain and the membrane leads to a conformational change that releases the pseudosubstrate from the substrate-binding site (Figure 2). This process readies the kinase to phosphorylate other proteins for downstream signaling. In the resting state, the pseudosubstrate of the regulatory domain occupies the substrate-binding site in the catalytic domain and maintains the enzyme in an inactive conformation.

2. PKC δ and Its Unique Role in Health and Disease

We have identified PKC δ as an import regulator of the inflammatory response in sepsis [8,19,22,43–47]. Multiple cell types express PKC δ and proinflammatory mediators involved in the septic response activate this kinase [44,48]. Importantly, PKC δ regulates neutrophil and endothelial proinflammatory signaling [22,46,47]. In neutrophils, PKC δ regulates inflammatory signaling, activation of the transcription factor NF- κ B and proinflammatory gene expression, secretion of cytokines/chemokines, and reactive oxygen species (ROS) production [22,46]. In endothelial cells, PKC δ is involved in NF- κ B activation, adhesion molecule expression, the release of inflammatory mediators important in neutrophil transmigration, and regulation of endothelial cell permeability [23,47]. Thus, PKC δ is an important signaling element in the regulation of neutrophil-endothelial crosstalk, neutrophil adherence/rolling/migration, and vascular endothelial damage [8,19,21–23,46,47].

2.1. PKC δ Activation

PKC δ , unlike other members of the PKC family, is unique in its regulation by tyrosine phosphorylation, activation mechanisms, and multiple subcellular targets [2,27].

2.1.1. PKC δ Phosphorylation

PKC δ activity is regulated by phosphorylation patterns, subcellular translocation, and cleavage in a context-dependent manner [2,29]. The three main conserved threonine and serine phosphorylation sites for PKC δ are Threonine-505 (Thr-505, activation loop), Serine-643 (Ser-643, turn motif), and Serine-662 (Ser-662, hydrophobic motif) [27]. However, PKC δ retains little phosphorylation in the activation loop (Thr-505) in many cell types [27]. Unlike other PKCs, mutations of Thr-505 to Alanine in PKC δ do not affect catalytic activity, but may be important for enzyme stability [27,49]. In general, phosphorylation of Ser-643 and Ser-662 is necessary for PKC δ catalytic activation and Thr-505 phosphorylation can enhance the catalytic activity of PKC δ [27,50–52].

Unlike serine and threonine phosphorylation, tyrosine phosphorylation is not conserved among the different PKCs and PKC δ activation is uniquely regulated by tyrosine phosphorylation patterns (Figure 3) [27,29,53]. Human PKC δ contains 20 tyrosine residues (19 for mice and 21 for rat) [29], and includes phosphorylation sites in the regulatory domain (Tyr-52, Tyr-64, Tyr-155, and Tyr-187), the hinge region (Tyr-311 and Tyr-332), and the catalytic domain (Tyr-505, Tyr-512, and Tyr-523) [27]. Tyrosine phosphorylation of the catalytic domain increases PKC δ activity, while tyrosine phosphorylation in the regulatory domain influences cellular actions rather than catalytic competence [2,12].

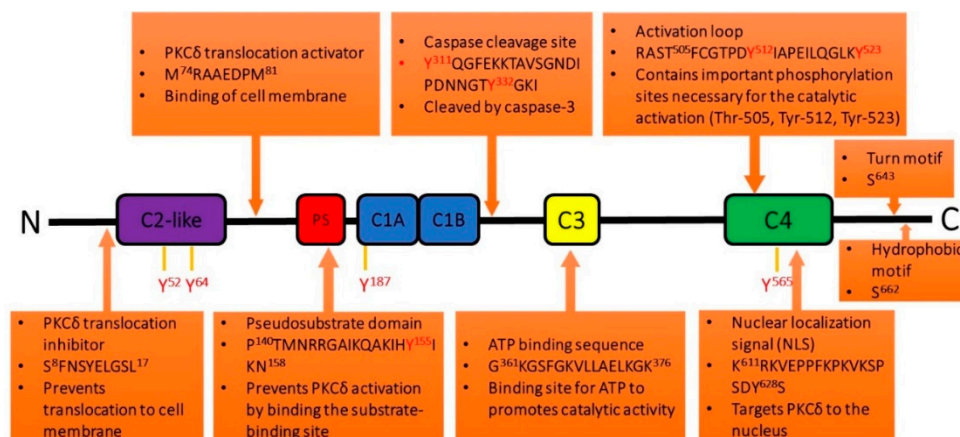


Figure 3. Important amino acid sequences (activators, inhibitors, regulatory signals) and tyrosine phosphorylation sites on PKC δ . Adapted from Malavez et al., 2009 [27].

Two important tyrosine phosphorylation sites are PKC δ Tyr-155 and PKC δ Tyr-311, which are critical phosphorylation sites associated with PKC δ -mediated proinflammatory signaling and the initiation of cytotoxic/apoptotic pathways [54–56]. Phosphorylation of PKC δ at Tyr-155 and Tyr-311 is required for nuclear translocation and enzyme cleavage [24,54,55]. Tyr-155 is located between the regulatory domain pseudo-substrate motif and the C1A domain and regulates apoptosis and gene expression [29,30,57]. PKC δ phosphorylation at Tyr-311, located in the hinge region, causes a conformational change that reveals the caspase cleavage site [29]. Our recent studies demonstrate that PKC δ Tyr-155 and PKC δ Tyr-311 are phosphorylated during sepsis and play key roles in sepsis-induced lung injury, the regulation of microvascular endothelium barrier function, and neutrophil-endothelial cell interactions (See Sections 2.2.3 and 2.2.4) [21,24]. Tyr-155 phosphorylation is also significant in cell apoptosis; mutations of this site increase cell proliferation in response to PMA [27,30]. Tyr-187 is a major phosphorylation site in response to PMA, PDGF, and etoposide, but does not appear to affect PKC δ kinase activity [12,58]. Tyr-187 and Tyr-64 are important phosphorylation sites for etoposide-induced apoptosis [58]. Tyr-52 is unique to PKC δ and located at the C2 domain [29,59]. Lyn, a member of the Src family kinases, phosphorylates PKC δ on Tyr-52, and this phosphorylated tyrosine residue then serves as a docking site for the SH2 (Src homology 2) domain of Lyn and reciprocal phosphorylation [60–62]. Tyr-52 is also phosphorylated in response to IgE in leukemia cells, and IgE-stimulated PKC δ phosphorylation reduces its activity to certain substrates, suggesting that PKC δ tyrosine phosphorylation may be important in substrate recognition [58]. Tyr-311, Tyr-332, and Tyr-512 are important phosphorylation sites for kinase activation and subcellular localization [12,27,58]. In addition, PKC δ Tyr-332 phosphorylation creates a docking site for Shc [12].

In addition to identification of the different functions and mechanisms of the individual tyrosine phosphorylation sites of PKC δ , the identification of PKC δ -specific substrates is also important to understand how this kinase regulates cellular function. Table 1 summarizes proteins identified as PKC δ substrates. For example, PKC δ preserves homeostasis by phosphorylating plasma membrane calcium ATPase (PMCA) that helps regulate calcium levels in the skin [27,63,64]. PKC δ phosphorylates caspase-3 in human monocytes, which promotes the apoptotic activity of caspase-3 both in vitro and in vivo [65]. PKC δ also phosphorylates the p52Shc protein at Ser-29 (when under oxidative stress), p66Shc at Ser-138 (ERK activation), and Heat Shock Protein 25 (HSP25) through binding at the V5 region, leading to the inhibition of apoptosis [29,66–68]. Additional substrates of PKC δ have been discovered with the aid of PKC δ inhibitors and activators, such as cytoskeleton proteins [28], the myristoylated alanine-rich C-kinase substrate (MARCKS) [28,69], troponin [28,70], the nuclear protein DNA-dependent protein kinase [28,71], and pyruvate dehydrogenase (a mitochondrial enzyme) [28,72]. Thus, PKC δ has a myriad of phosphorylation targets, and further studies are required to determine

the targets of PKC δ phosphorylation in specific cells and in various disease conditions, particularly in sepsis.

2.1.2. PKC δ Translocation and Subcellular Localization

PKC δ has been classically known to move from the cytosol to the plasma membrane upon activation into a mature, catalytically competent form. However, recent investigations have revealed that PKC δ can move to several subcellular compartments, including mitochondria, endoplasmic reticulum (ER), Golgi apparatus, nuclei, and caveolae [48,73–76]. This translocation of PKC δ is mediated by tyrosine phosphorylation [29]. In cardiomyocytes, PKC δ moves from the nucleus to focal contacts and cytoskeleton and around the nucleus [48]. PMA can enhance the movement of PKC δ to caveolae, leading to increased ERK activity [73]. PKC δ in its tyrosine phosphorylated form can also accumulate in the soluble portion of hydrogen peroxide-treated cardiomyocytes and, in itself, can act as a lipid-independent kinase [29]. PKC δ can transiently translocate to the ER following ER stress and binds to Abl (a tyrosine kinase) [74]. After briefly translocating to the ER, PKC δ then accumulates in the mitochondria, inducing apoptosis [74]. In human leukemia cells, ceramide release is caused by TNF- α -initiated apoptosis and the translocation of PKC δ from the plasma membrane to Golgi apparatus [75]. In glioma cells, PKC δ was found to induce apoptosis when targeted to the cytoplasm, nucleus, and mitochondria, whereas the ER translocation protected the cells from TNF-ligand-induced cell death [76]. Overall, there is no uniform pattern of PKC δ tyrosine phosphorylation and it is becoming more evident that the precise configuration of tyrosine phosphorylation depends on the stimulus that dictates the functional properties of the enzyme and its subcellular location. For example, in platelets, thrombin-induced Tyr-311 phosphorylation on PKC δ occurs subsequent to Thr-505 phosphorylation, while ADP-induced Tyr-311 phosphorylation does not appear to require the threonine phosphorylation [77]. In a rodent model of sepsis, pulmonary PKC δ is phosphorylated on both Tyr-155 and Tyr-311, resulting in PKC δ nuclear translocation and PKC δ cleavage [24]. Thus, PKC δ activation is stimulus-dependent and cell type-specific.

Table 1. PKC δ substrates and functions. Adapted from Steinberg 2004 [29].

Substrate	Effects
c-Abl	Increased activity [78,79]
SFKs	Variable [80]
SHPTP1 (protein tyrosine phosphatase) (SHP1)	Decreased phosphatase activity [81]
RasGRP	Uncertain [82]
Protein tyrosine phosphatase PTP α	Increased phosphatase activity [83]
PKC ϵ (hydrophobic motif)	Yields release from membranes [50]
STAT1 (Ser-727)	Interferon gene expression [84]
STAT3 (Ser-727)	Reduced DNA binding and transcription [85]
p300	HAT activity lowered, decreased transcriptional function [86]
14-3-3	Interfere with 14-3-3 polymerization and interactions with partners [87]
gp130	Increased gp130-STAT3 interaction [88]
p47(pbox) unit of NADPH	Activity enhancement [89]
β 4-integrin	Cell-laminin attachment decreases [90]
Caspase-3	Promote the apoptotic activity of caspase-3 in monocytes both in vitro and in vivo [65]
MARCKS	Cell attachment and spreading in skeletal muscle cells [69]
M2 Pyruvate Kinase	Tumor metabolism; uncertain [91]
Heat Shock Protein 27 (HSP27)	Protein chaperone, antioxidant, apoptosis inhibition [92]
Plasma membrane calcium ATPase (PMCA)	Regulate calcium levels in skin [30,65]
Heat Shock Protein 25	Inhibition of apoptosis [92]

Table 1. Cont.

Substrate	Effects
p52Shc protein p66Shc protein	Positively regulates H2O2-induced ERK activation [67] Negatively regulates H2O2-induced ERK activation [67]
Troponin	Decreased Calcium sensitivity of actomyosin [70]
Pyruvate Dehydrogenase Kinase	Inhibition of PDH resulting in necrosis and blocking ATP regeneration [71]
DNA-dependent protein kinase	Inhibition of p53 phosphorylation [93]
Bcl-2-associated death promoter (BAD)	Promotes apoptosis post-reperfusion after cardiac ischemia [94]
Dynammin-related protein 1 (Drp1)	Induction of mitochondrial fission and dysfunction following cardiac ischemia [17]
Glyceraldehyde-3-phosphoate dehydrogenase (GADPH)	Removal of injured mitochondria following ischemic damage [95]
PLS3	Higher phospholipid movement [96]
DNA-PK	Increase apoptosis due to malfunctional DNA [93]
Lamin B	Apoptosis [97]
hRad4	Increased hRad9-Bcl-2 interactions/apoptosis [98]
p73 β (Ser-289)	p73 β activation; apoptosis [66]

2.2. PKC δ in Inflammatory Diseases

We identified PKC δ as a critical regulator of the inflammatory response in sepsis and an important signal transducer of multiple signaling pathways [8,19–23,25,43–47]. PKC δ is activated by inflammatory mediators involved in sepsis, including pathogen associated molecular patterns (PAMPs) such as LPS and the bacterial peptide fMLP, as well as the proinflammatory cytokines TNF and IL-1 [44,48,99]. Moreover, PKC δ is activated in multiple cell types and organs in animal models of sepsis [19,47]. Key to sepsis-induced tissue damage is the excessive migration of activated neutrophils across the vascular endothelium [100–103]. Studies with PKC $\delta^{-/-}$ mice and PKC δ inhibitors indicate a role for PKC δ in regulating neutrophil trafficking to the lung in response to inflammation triggered by bacterial sepsis, asbestos, stroke/reperfusion injury, LPS, or pancreatitis [19,20,24,47,104–107].

2.2.1. Role of PKC δ in Sepsis—Animal Studies

During sepsis, systemic inflammation leads to increased adhesion molecule expression on neutrophils and endothelial cells, resulting in increased neutrophil-endothelial cell interaction, vascular endothelial damage, and organ dysfunction [108–110]. While neutrophils are critical to host defense, neutrophil dysregulation has a critical role in the early course of organ damage through the release of proteases, neutrophil extracellular traps (NETs), and oxygen radicals. Increased neutrophil recruitment in sepsis is associated with tissue damage, multiple organ dysfunction syndrome (MODS), and increased mortality [100–102,111].

Using a clinically relevant rodent model of polymicrobial sepsis induced by cecal ligation and puncture (CLP), we found significant lung injury within 24 hrs post CLP surgery, including increased neutrophil accumulation in lung tissue, pulmonary permeability, tissue edema, altered lung mechanics, and disrupted lung architecture [19,20,24,47]. In this sepsis model, we found PKC δ activation and phosphorylation on multiple sites, including Ser-643/676, Thr-505, Tyr-155, and Tyr-311 [19,24,25].

To examine the regulatory role of PKC δ in sepsis, we employed a selective peptide inhibitor developed by Mochly-Rosen's group [13]. This inhibitory peptide is derived from the first unique region (V1) of PKC δ (SFNSYELGSL; amino acid residues 8 to 17, see Figure 3), targets docking domains, and prevents translocation and substrate interaction [13]. This inhibitor targets the regulatory domain of PKC δ , but not the ATP binding site, so it is more specific than previously described PKC δ inhibitors such as rottlerin. Rottlerin has been shown to be a mitochondria uncoupler and, in recent years, has been shown to modulate biological and biochemical events in a PKC δ -independent manner [112,113]. This PKC δ peptide inhibitor is coupled to a membrane permeant TAT peptide (YGRKKRRQRRR) that

allows safe and effective intracellular delivery into target cells *in vitro* and *in vivo* [13,19,22,46,114,115]. Administration of the PKC δ peptide inhibitor in our animal model of sepsis decreased pulmonary PKC δ phosphorylation, attenuated lung injury, and improved gas exchange, indicating that PKC δ inhibition is lung protective in sepsis [19,20,24,47].

Further studies demonstrated that PKC δ inhibition reduced neutrophil influx into multiple organs, including the lung, kidney, and brain [20,24,25,47]. The vascular endothelium is involved in the pathogenesis of sepsis and is an active participant in the dynamic process of recruitment and activation of neutrophils through the production of chemokines/cytokines and expression of adhesion molecules [100,116–119]. ICAM-1 and VCAM-1 are crucial vascular endothelial cell adhesion molecules involved in neutrophil recruitment and are up-regulated by proinflammatory cytokines released during sepsis [120]; their expression was, however, attenuated by the administration of the PKC δ peptide inhibitor [47]. These studies suggest a link among PKC δ activity, the upregulation of adhesion molecules, and increased neutrophil migration in the injured lung. PKC δ was also activated in the brain in this sepsis model, resulting in increased PKC δ Ser-643 phosphorylation and membrane translocation [25]. PKC δ activation was associated with increased blood brain barrier (BBB) permeability that was attenuated by administration of the PKC δ peptide inhibitor [25].

2.2.2. Role of PKC δ in Neutrophil-Endothelial Cell Interactions—In Vitro Studies Using Microfluidics-Based Biomimetic Assays

Microfluidic systems provide a unique opportunity to explore *in vitro* the role of PKC δ in regulating neutrophil-endothelial cell interaction under physiologically realistic conditions [21,23,25,26]. Our group developed a novel microfluidic system (Figure 4) that resolves and facilitates the real-time assessment of individual steps, including the rolling, firm arrest, spreading, and migration of neutrophils into the extra-vascular tissue space in a single system. A Geographic Information System (GIS) approach [121] was used to digitize microvascular networks for the subsequent generation of synthetic microvascular networks using soft-lithography processes to develop a bioinspired microfluidic assay (bMFA). This bMFA was based on microvascular network morphologies obtained from *in vivo* animal data [122–126]. This microfluidic assay consists of vascular channels in communication with a tissue compartment filled with chemoattractants via a porous barrier. Neutrophils circulate in the vascular channels on a monolayer of activated endothelial cells under physiologic shear conditions.

In the bMFA, TNF- α activated human endothelial cells and upregulated the expression of the adhesion molecules and neutrophil adhesion to them [23]. Neutrophil adhesion was shear-rate dependent, with increased adhesion in vessels with lower shear rates and near bifurcations [23], and endothelial cells treated with the PKC δ inhibitor showed significantly decreased neutrophil adhesion and migration, consistent with our *in vivo* observations [21,23]. Mechanistic studies demonstrated that PKC δ regulates expression of the adhesion molecules E-selectin and ICAM-1. PKC δ is also an important regulator of endothelial cell permeability, and PKC δ inhibition attenuated TNF α -mediated endothelial cell permeability and decreased transendothelial electrical resistance (TEER) [25]. Similar changes in human brain microvascular endothelial cell permeability were obtained by employing a novel blood-brain-barrier (BBB) on-a-chip (B³C) microfluidic system [25] (Figure 4). Thus, PKC δ plays a key role in the regulation of proinflammatory signaling controlling the activation and recruitment of neutrophils and in regulating endothelial permeability, TEER, and tight junction protein expression [8,19–21,23–25,47].

PKC δ is also an important regulator of neutrophil-endothelial cell interactions in radiation-induced inflammation and vascular injury. Studies from our group and others have shown that the exposure of tissue to ionizing radiation (IR) causes an increase in leukocyte infiltration across endothelium and loss of barrier function [127–130]. Key to radiation-induced tissue damage is the excessive migration of activated neutrophils across the vascular endothelium [131,132]. In studies with human endothelial cells, we found that exposure to X-ray radiation (0.5–5 Gy) activated PKC δ

through phosphorylation (Ser-643) and translocation to membrane fraction [26]. Using our bMFA, we showed that PKC δ regulates radiation-induced neutrophil-endothelial cell interaction and endothelial cell function, and that PKC δ inhibition dramatically attenuated IR-induced endothelium permeability and significantly decreased neutrophil migration across IR treated endothelial cells [26]. Moreover, neutrophil adhesion to irradiated endothelial cells was significantly decreased after PKC δ inhibition in a flow-dependent manner. PKC δ inhibition downregulated the IR-induced overexpression of P-selectin, ICAM-1, and VCAM-1, but not of E-selectin. Thus, PKC δ is an important regulator of neutrophil-endothelial cell interaction post-IR exposure.

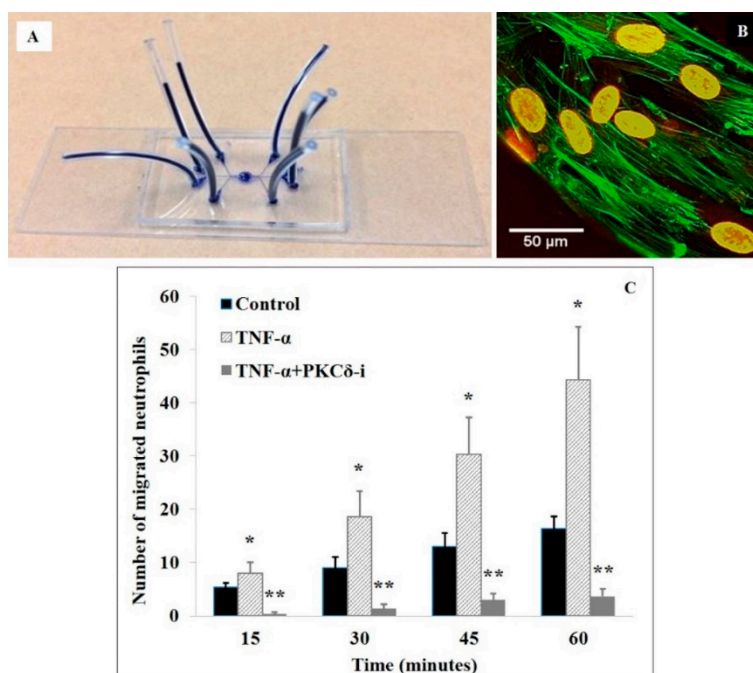


Figure 4. Microfluidic-based in vitro assay for studying the role of PKC δ in regulating neutrophil-endothelial cell interaction. (A) The assay is manufactured by soft lithography on polydimethylsiloxane (PDMS) and assembled on a microscope glass slide with plastic tubes (dark blue) allowing access to individual vascular channels and the tissue compartment. (B) 3D reconstruction of confocal images of human brain microvascular endothelial cells (HBMVEC) stained for F-actin with fluorescently labelled phalloidin (green) and for cell nuclei with Draq 5 (red) after 72 hrs of flow culture (0.1 μ L/min). (C) PKC δ inhibition with a PKC δ -TAT peptide inhibitor (PKC δ -i) reduces neutrophil migration across activated HBMVEC. Data are presented as mean \pm SEM ($n = 3$). ** $p < 0.01$, * $p < 0.05$ compared to the other two groups by ANOVA with Tukey-Kramer post-hoc. Reprinted with permission from Tang et al., 2018 [25].

2.2.3. PKC δ Phosphorylation in Sepsis and Inflammation—In Vivo Studies

Our in vivo studies demonstrated that sepsis triggered significant tyrosine phosphorylation of PKC δ [24]. Sepsis-induced lung injury was characterized by the phosphorylation of PKC δ at Tyr-311 throughout the distal lung, which is consistent with the finding that Tyr-311 is a critical phosphorylation site in the context of vascular inflammation [24,133]. Of particular interest, pulmonary endothelial cells, in contrast to pulmonary macrophages and epithelial cells, were the primary cell type exhibiting Tyr-155 phosphorylation in response to sepsis (Figure 5) [24]. This is a key observation, as in sepsis, pulmonary endothelium contains the first cells in the lung to encounter systemic proinflammatory mediators, making them the frontline inflammatory responders in systemic inflammation.

To interrogate the role of Tyr-155 phosphorylation in sepsis-induced lung injury and neutrophil recruitment to the lungs, PKC δ knock-in (KI) mice were produced where PKC δ Tyr-155 was mutated to phenylalanine (PKC δ Y155F KI mice) [21]. Compared to wild-type (WT) septic mice, there was a

significant decrease in neutrophil recruitment to the lungs in PKC δ Y155F KI septic mice, indicating an important role for Tyr-155 phosphorylation in regulating proinflammatory activity during sepsis [21].

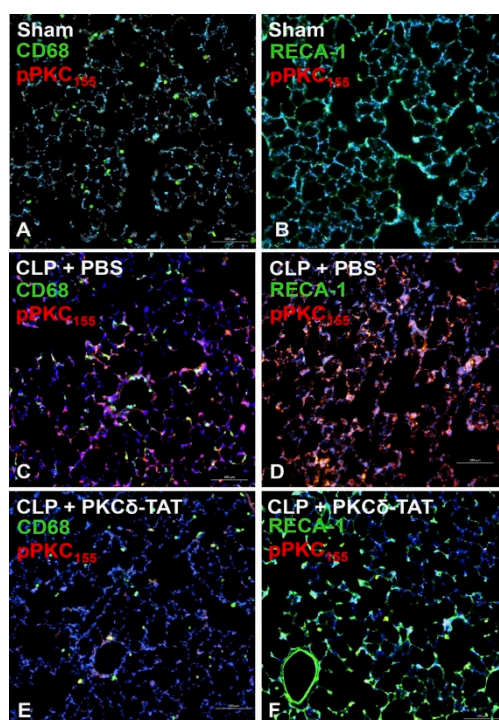


Figure 5. Immunohistochemical analysis of PKC δ phosphorylation at tyrosine 155 (pPKC δ ₁₅₅; red) in lung tissue sections at 24 h post-surgery of sham-operated animals (Sham) (A,B) and CLP-operated animals that received 200 μ g/kg PKC δ -TAT (CLP + PKC δ -TAT) (E,F) or a similar volume of PBS vehicle only (CLP + PBS) (C,D) immediately following surgery. (A,C,E) Tissue sections were also stained for CD68 (green), a marker for the cells of the macrophage lineage. Yellow/orange indicates co-localization of pPKC δ ₁₅₅ and CD68. (B,D,F) Tissue sections were also stained for rat endothelial cell antigen-1 (RECA-1; green), a marker for rat endothelial cells. Yellow/orange indicates co-localization of pPKC δ ₁₅₅ and RECA-1. All scale bars = 100 microns. Reprinted with permission from Mondrinos et al., 2015 [24].

2.2.4. PKC δ Phosphorylation in Sepsis and Inflammation—In Vitro Studies

To investigate the role of PKC δ Tyr-155 phosphorylation in neutrophil superoxide anion (O₂⁻) generation, bone marrow neutrophils were isolated from PKC δ Y155F KI mice [21]. PKC δ Y155F bone marrow neutrophil O₂⁻ production in response to fMLP or TNF α activation was significantly decreased compared to WT mice. Decreased O₂⁻ production was stimulus-dependent as PMA-mediated O₂⁻ generation was not affected. Formation of neutrophil extracellular traps (NETs) from PKC δ Y155F KI mice was also attenuated in response to IL-1 or TNF as compared to WT mice. Hence, PKC δ is an important regulator of O₂⁻ and NETs release, and PKC δ Tyr-155 is a key phosphorylation site regulating proinflammatory signaling controlling neutrophil activation [21].

To investigate further the role of PKC δ Tyr-155 phosphorylation in neutrophil-endothelial interaction in inflammation, we employed the bMFA to examine endothelial cell permeability and neutrophil migration [21]. Our studies demonstrate that the Tyr-155 phosphorylation site is a critical regulator of endothelium barrier function, neutrophil adhesion, and neutrophil transmigration. Consistent with our previous findings [21,23], PKC δ was found to play a more significant role in regulating the migration of neutrophils across endothelial cells as opposed to their adhesion to endothelial cells. Overall, these findings indicate that regulating PKC δ activity may provide novel therapeutic strategies for treating inflammation.

3. Concluding Remarks

The Protein Kinase C superfamily consists of multiple isoforms with separate and overlapping cellular and physiological functions that contribute to health and disease. Among them, PKC δ has a unique tyrosine phosphorylation pattern that diminishes or enhances biological processes such as neutrophil and platelet adhesion, migration, and adhesion molecule expression. Furthermore, the inhibition of PKC δ may offer a therapeutic pathway for reducing neutrophil-mediated organ damage in inflammatory diseases. Emerging in vitro methods (e.g., microfluidic platforms) provide unique perspectives for delineating biological mechanisms in a physiologically relevant environment prior to observation and study in animal models or clinical settings, reducing drug development costs and providing more precise and personalized diagnostic/treatment methods.

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