PKC θ is a Key Regulator of T-cell Behavior and a Drug Target for T cell-mediated Diseases

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Abstract

The protein kinase C-theta (PKCθ) isoform is a member of the calcium-independent novel PKC subfamily of serine/threonine kinases. It is an essential regulatory enzyme in mature T lymphocytes, where it plays a key role in coupling the activated TCR and the CD28 costimulatory receptor to their downstream signaling pathways. TCR/CD28 engagement induces the translocation of PKCθ to the center of the immunological synapse where it undergoes posttranslational modifications and becomes fully active. The activated PKCθ then initiates signaling pathways leading to the activation of transcription factors, including NF-kB, AP-1 and NF-AT that are essential for the survival, activation and differentiation of T cells. While PKCθ ablation was found to impair a wide range of in vitro responses of T cells, in vivo studies in Prkcq−/− mice revealed that distinct T cell subpopulations differ in their requirements for PKCθ and that PKCθ has a selective role in different immune responses. Thus, PKCθ participates in cellular mechanisms leading to excessive inflammatory responses, autoimmunity, and graft vs host (GvH) disease, but is dispensable for beneficial immune responses against viruses and during graft vs leukemia responses. These studies suggest that PKCθ may serve as a potential drug target for catalytic and allosteric inhibitors in selected T cell-mediated diseases, and that fine-tuning of PKCθ-dependent functions may help prevent autoimmunity and GvH, without impairing the ability of T cells to eradicate viral-infected and transformed cells.

Keywords: Protein kinase C theta; PKCθ; PKC; T cell receptor; TCR; Signal transduction

Introduction

Protein phosphorylation is a ubiquitous posttranslational process mediated by kinases and serves to regulate the activation state of numerous proteins. Many kinases possess the ability to integrate signals from specific surface receptors and play important roles in regulatory networks and signal transduction pathways that control cell activation and differentiation. One important family of kinases that transduce signals from a large number of cell surface receptors is the protein kinase C (PKC), originally discovered by Nishizuka and colleagues [1]. The first PKC family members identified were found to be sensitive to diacylglycerol (DAG) and calcium (Ca2+) ions, two products of phospholipase C-mediated hydrolysis of the membrane phospholipid, phosphatidylinositol 4,5-bisphosphatate (PIP2) [2-4]. These two second messengers transduce signals from a plethora of activated receptors where the hydrodynamic DAG associates with the cell membrane, while the second product of the PIP2 hydrolysis, the hydrophilic inositol 1,4,5-trisphosphate (IP3) binds IP3 receptors in the endoplasmic reticulum (ER) and triggers the release of free Ca2+ ions into the cytoplasm [5-7]. The utilization of phorbol esters, which mimic the activity of DAG, together with Ca2+ ionophores, demonstrated that PKC plays an essential role in the induction of T lymphocyte proliferation [8,9] and reactivation of effector cytotoxic T cells [10,11].

The PKC Family of Serine/Threonine Kinases

The PKC family includes nine structurally and functionally related isoforms that are encoded by distinct genes and are divided into three subfamilies based on the structure homology of their regulatory domains and their respective cofactor requirements [12,13]. Additional PKC isoforms are products of alternative splicing of the PKCθ [14] and PKCδ genes [15-17]. Members of the first subfamily, which include the conventional PKC (cPKC) isoforms, PKCα, β, and γ, are regulated via two DAG-binding C1 domains organized in tandem near the cPKC amino terminus [18-20], and an adjacent Ca2+ and phospholipid-binding C2 domain [19,21]. Members of the second subfamily include the novel PKC (nPKC) isoforms, PKCθ, ε, η, θ, which are DAG-dependent, but Ca2+- and phospholipid-independent for their activity. Members of the third subfamily include atypical PKC (aPKC), PKCζ and λ/ι that require neither Ca2+ nor DAG for their activity. The PKC enzymes are involved in metabolic processes in different cell types and are implicated in signal transduction networks that convert different environmental cues into cellular actions [22]. Individual PKC isoforms are differentially expressed in tissues and cell types, and six of these isoforms, including PKCα, δ, ε, η, θ and ζ are expressed at varying amounts in T cells [23]. Immunological studies using different genetic models and pharmacological drugs indicated that distinct PKC isoforms are required for different aspects of the activation and effector function of T cells. It is assumed therefore that distinct PKC isoforms may serve as drug targets in different T cell-mediated adaptive immune responses [24].

PKCθ

PKCθ is a member of the novel PKC subfamily, initially isolated from T cells, skeletal muscle and platelets [25-27]. Additional studies demonstrated that PKCθ exhibits a relatively restricted pattern of expression, with high levels in T lymphocytes [23,25], platelets [27-30]...
and skeletal muscle [26,27], and lower or undetectable levels in other tissues tested. For reasons that are not clear yet, it is highly expressed in gastrointestinal stromal tumors, but not in other mesenchymal or epithelial tumors [31-34].

Engagement of the TCR and the CD28 coreceptor on most T cells results in PKCθ translocation to the center of the immunological synapse (IS) [35,36]. Full activation of the enzyme requires the integration of TCR and CD28 costimulatory signals [37-39] and additional steps of posttranslational modification that increase the PKCθ catalytic activity [40]. These include the inducible phosphorylation of PKCθ at multiple sites on serine, threonine and tyrosine residues. Phosphorylation is mediated by several upstream kinases (including autophosphorylation) that affect the enzyme's topology, open the ATP binding site and activation loop, and convert PKCθ into a catalytically potent enzyme [41]. The most recent kinase described that phosphorylates the IS-residing PKCθ is the germinal center kinase (GSK)-like kinase (GLK) [42]. Similar to PKCθ, the GLK translocates to the IS of TCR-engaged T cells where it directly interacts with PKCθ and phosphorylates threonine 338 in its activation loop, thereby enabling better access of substrates to the enzyme's activation segment.

The IS-resident, enzymatically active PKCθ initiates a series of signaling events leading to activation of transcription factors, including nuclear factor-xB (NF-κB), activating protein-1 (AP-1) and nuclear factor of activated T cells (NF-AT), which are critical for T cell proliferation and differentiation [43-48]. These three types of transcription factors are primary physiological targets of PKCθ [43,49], which are essential for the induction of an optimal IL-2 response [50].

The translocation of PKCθ to the center of the IS is not a universal phenomenon in activated T cells, as demonstrated by Rustin and colleagues using an isolated population of regulatory T cells (Treg) [51,52]. Their findings indicated that although TCR/CD28 triggering of Treg resulted in IS formation, PKCθ translocates to the opposite cell pole, away from the IS, and negatively regulates their suppressive function, although the precise mechanism of action and mode of regulation of PKCθ in Treg is not yet clear.

Studies by Rao and colleagues further demonstrated that under certain activation conditions, PKCθ has the ability to translocate to the nucleus and physically interact with chromatin. Together with other nuclear enzymes, PKCθ molecules form active complexes that bind regulatory DNA regions and control the expression of selected microRNAs and specific T cell inducible genes [53].

The exact mechanisms by which the membrane-bound PKCθ delivers signals to the nucleus have not been fully resolved, but studies provided information on a number of effector molecules that operate along these pathways in activated T cells. These studies demonstrated that the regulation of NF-κB by PKCθ involves the multisubunit inhibitor of κB (IkB) kinase (IKK) complex [44,46,47,54,55]. Another important upstream regulator of the NF-κB signaling pathway is the IkBα, which can directly associate with the cytosolic NF-κB of resting T cells, and by masking its nuclear localization signal (NLS), prevent NF-κB translocation to the nucleus [56-58]. On the other hand, IKK-mediated phosphorylation of IkBα signals the protein for degradation [59], and by exposing the NF-κB NLS, it promotes NF-κB translocation to the nucleus where transcriptional upregulation of NF-κB-dependent genes occur. The current dogma, that PKCθ regulates NF-κB activity through its effect on IKK-IkBα, is further substantiated by in vivo studies demonstrating that T cells from PKCθ-deficient (Prkcq−/−) mice fail to respond to TCR stimulation with degradation of IkBα [45].

**PKCθ Binding Proteins and Substrates**

One of the most prominent target proteins that links PKCθ to IKK is the PKCθ substrate protein, caspase activation and recruitment domain (CARD) and membrane-associated guanylate kinase (MAGUK) domain-containing protein-1 (CARMA1) [60-63]. This scaffold protein is primarily expressed in lymphocytes [64,65] and its phosphorylation by PKCθ in TCR/CD28-stimulated T cells, promotes CARMA1 association with two additional effector molecules: the B-cell lymphoma/leukemia 10 (Bcl10), and the mucosa-associated lymphoid tissue 1 (MALT1) [66,67]. The resulting trimeric complex recruits to the IS [68-70] where the three partners cooperate in delivering signals leading to maximal activation of NF-κB [61,71].

Besides its effects on transcriptional regulation, PKCθ is also involved in the regulation of many other cellular functions. Some functions require the direct or indirect association of PKCθ with upstream kinases, which may affect the enzyme's conformation, activity or subcellular distribution (i.e., Lck [72]), while other functions are dependent on PKCθ interaction with downstream kinases, which serve as intermediate PKCθ-coupled signal transduces (i.e., Ste20-related upstream mitogen-activated protein kinase [80], AKT [54], PICOT [81] and the HIV nef protein [82], although the functional consequences of these interactions require further analyses.

A recent study suggests that the C2-like domain of PKCθ functions as a phosphotyrosine-binding (PTB) module that is also involved in regulating the PKCθ catalytic activity [83]. This study demonstrated that the PKCθ C2-like domain can interact with a CUB domain-containing protein 1 (CD2CPI; also termed CD318)-derived tyrosine phosphorylated peptide with a relatively high affinity, and that binding increases the potency of the PKCθ catalytic activity. Furthermore, mutating the PTB sequence in a way that prevented binding of the tyrosine phosphorylated CD2CPI, abrogated PKCθ activity and inhibited the TCR/CD28-mediated activation of a PKCθ reporter gene in T cells.

**PKCθ in distinct T cell subsets**

Following the discovery that PKCθ is expressed in T cells [50,84] studies have focused on the potential biological functions of this enzyme and provided substantial evidence to support a critical role in diverse intracellular signaling pathways that regulate T cell activation, proliferation, differentiation and apoptosis [45,48,85]. Despite its important functions in mature T cells, studies in Prkcq−/− mice demonstrated that PKCθ is dispensable for thymocyte maturation and differentiation [45], suggesting a redundancy with other thymocyte-residing PKC isoforms [86].
Additional in vivo studies performed in Prkcq−/− mice demonstrated that distinct T cell subpopulations differ in their requirements for PKCθ, a phenomenon that is dependent on the type of antigen and the immune response elicited. The current dogma suggests a requirement for PKCθ in Th2-type immune responses to allergens or helminth infection [87,88] and Th1-type immune responses leading to the development of experimental autoimmune encephalomyelitis (EAE), an inflammatory disease of the central nervous system that is widely used as a model of Multiple Sclerosis [85,89-92]. PKCθ is also required for the induction of experimental autoimmune myocarditis [91], Ag-induced arthritis [93], and systemic lupus erythematosus [42].

In contrast, ablation of PKCθ does not impair mouse resistance to Leishmania major infection, mediated primarily by Th1 cells [87,94], or CTL-mediated protection from viral infection, which may reflect compensatory activities mediated by innate immunity mechanisms [91,95-98].

Furthermore, PKCθ was found to be required for the induction of allograft rejection and graft-versus-host (GvH) and alloreactive T cell-mediated immune responses, [98,99], while being dispensable for graft-versus-leukemia responses in allogeneic bone marrow transplanted mice [98].

Although many of the effects induced by PKCθ ablation on the quality and intensity of immune responses are due to impaired TCR/CD28-coupled signaling pathways in the effector T cells (Teff), some of these effects could reflect changes in the activity of regulatory T cells (Treg) that under normal conditions downregulate specific functions of Teff. Recent findings supported this assumption by showing that PKCθ mediates a negative feedback on Treg functions [51]. In these studies activation of Treg led to PKCθ sequestration away from the IS, while inhibition of PKCθ increased the suppressive activity of Treg [51,52,100], although the activity of the mature PKCθ-deficient Treg was not affected [101].

The Immunological Synapse and PKCθ

The immunological synapse, also known as the supramolecular activation cluster (SMAC), is a temporally and spatially regulated plasma membrane structure formed at the interface between an antigen-presenting cell (APC) and a responding T cell, and is the site at which early T cell activation signaling events occur [102]. It is formed upon the recognition by the TCR of a cognate antigenic peptide-MHC on the surface of APC, when both cell types respond by redistributing their receptors, cytoskeletal proteins and intracellular signaling molecules to the contact area, which rearranges as a platform for effective signaling [103,104].

The IS is characterized by specific microclustering of selected receptors and effector molecules [105], and is composed of three concentric rings, each containing a relatively high concentration of a typical combination of molecules. The central-SMAC (c-SMAC) is characterized by a high content of TCR and PKCθ [35,36], in addition to costimulatory receptors (CD28, CD2, CD4, and CD8) and Src family tyrosine kinases (Lck and Fyn) [106]. Recent studies utilizing a high-resolution total internal reflection fluorescence (TIRF) microscopy observed two structurally and functionally distinct eSMAC compartments: 1. A central TCRhigh compartment, where TCR-associated signaling complexes are internalized and degraded and the entire signaling process is being terminated [107]. 2. An outer TCRlow compartment enriched in PKCθ and CD28 where the two proteins are colocalized [38]. Coinmuno precipitation studies confirmed that PKCθ and CD28 colocalization is a consequence of a physical interaction between the two proteins [38]. The second concentric ring of the IS, the peripheral-SMAC (pSMAC) possesses high concentrations of adhesion molecules (lymphocyte function-associated antigen-1 (LFA-1)) and the cytoskeletal elements (talin) [35], while the third outermost ring, the distal-SMAC (dSMAC), is characterized by its high content of CD43 and CD45 surface receptors [108,109].

Mechanism of Recruitment of PKCθ to the IS

Early investigations demonstrated that TCR engagement, which polarizes PKCθ and induces its recruitment to the IS, is greatly augmented by coligation of the CD28 stimulatory receptor [37-39]. More recent studies demonstrated a physical interaction between PKCθ and CD28 in PMA-stimulated T cells [38], findings that were further substantiated and set up the basis for the understanding of the mechanism by which the two protein interact [110]. These studies revealed that TCR/CD28 costimulation induces transient binding of PKCθ to the cytoplasmic tail of CD28.

Amino acid sequence comparison between PKCθ and PKCδ, the closest relative of PKCθ, indicated the existence of a lowest sequence homology between the two proteins in the V3 (hinge) domain (Figure 1), which corresponds to amino acids 291-378 of human PKCθ. Since PKCδ, in contrast to PKCθ, does not translocate to the IS of TCR/CD28 activated T cells [36], the findings suggested a potential role for the PKCθ-V3 domain in targeting the enzyme to the IS.

To analyze this hypothesis, a V3-deletion mutant of PKCθ (PKCθ-DV3) or a PKCθ exchange mutant, in which the native V3 domain was replaced by the PKCδ V3 domain, were ectopically expressed in T cells, and tested for their ability to associate with CD28. The results demonstrated that, in contrast to the wild type PKCθ, the two mutants failed to coinmunoprecipitate with CD28 [110]. Furthermore, the two genetically modified PKCθ proteins failed to translocate to the IS and to activate PKCθ-dependent reporter genes, such as the CD28 response element (RE/AP). On the other hand, an overexpressed isolated V3 domain of PKCθ localized to the center of the IS of TCR/CD28 stimulated T cells and associated with CD28. In a complementary line of studies, T cells recovered from mouse bone marrow (BM)

Figure 1: Structure of the human PKCθ protein.

Individual domains in the PKCθ regulatory and catalytic regions are indicated by rectangles with different colors. Molecules that bind PKCθ and are mentioned in the text are indicated above the enzyme (in light blue rectangles) and arrows point to specific PKCθ domains that mediate these interactions. PICOT is a representative of a growing number of proteins that were found to physically interact with PKCθ. Black lines represent phosphorylation sites and the type of amino acid residue that undergoes phosphorylation and its position is indicated below the black line. Kinases that are known to phosphorylate specific amino acids on PKCθ are indicated in a green box below the relevant phosphosite.

V: Variable domain; C: Constant domain; PTB: Phosphotyrosine binding motif; PR: Proline-rich motif; SB: Substrate binding region; DAG: Diacylglycerol; GLK: Germinal center kinase (GSK)-like kinase; PICOT: PKC-interacting Cosine of Thiorodoxin; Lck: Lymphoid cell kinase.
chimeras on a Prkco<sup>−/−</sup> background that were reconstituted with the PKCθ mutants described above failed to proliferate and produce IL-2 in response to CD3/CD28 costimulation, and exhibited a diminished capacity to upregulate CD69 or CD25 expression. Furthermore, ectopic expression of the isolated PKCθ V3 domain in T cells disrupted the activation-dependent association between the endogenous PKCθ and CD28, inhibited the recruitment of PKCθ to the IS, and severely impaired PKCθ-dependent functions, including T cell proliferation, IL-2 production and CD25 and CD69 upregulation. These results indicated that the isolated PKCθ V3 domain exhibits dominant negative effects, and possesses the potential for serving as a drug target for the selective inhibition of PKCθ.

Using an array of PKCθ V3 domain mutants, Kong et al. have identified in this region an evolutionarily conserved proline-rich (PR) motif (A<sup>328</sup>RPPCLPTP; corresponding to amino acid residues 328–336 of human PKCθ), which is essential and sufficient for PKCθ-CD28 association, as well as PKCθ localization to the IS, and induction of PKCθ-mediated functions. The two internal proline residues in this motif (Pro<sup>328</sup> and Pro<sup>336</sup>) were particularly critical in this regard [110].

Additional studies revealed that T cell activation-dependent association of PKCθ with CD28 and CD28 is mediated by Lck, which operate as an intermediate protein [110]. These studies demonstrated that upon TCR/CD28 crosslinking, the Lck-SH3 domain interacts with the PR motif in the PKCθ V3 domain, while the Lck-SH2 domain interacts with phospho-Tyr<sup>191</sup> in the P<sup>190</sup>YAP motif in the CD28 cytoplasmic tail.

Taken together, the above findings demonstrate a unique signaling mode of CD28 and establish the molecular basis for the specialized localization and function of PKCθ in antigen-stimulated T cells.

PKCθ as a Drug Target

Studies over the past two decades have demonstrated the critical role of PKCθ in the regulation of T cell proliferation and differentiation, and in the induction of specific types of T cell-mediated adaptive immune responses. While individual cPKC isoforms have been targeted for drug discovery for over 20 years [111], the interest in PKCθ as a potential drug target has become a focus of interest following the discoveries of its fundamental roles in the induction of harmful inflammatory responses mediated by Th2 (allergies) and Th17 (autoimmunity) cells, as well as in GVH and allograft rejection. The fact that PKCθ is dispensable for beneficial adaptive immune responses against virally infected cells and graft versus leukemia response following allogeneic bone marrow transplantation, supported the assumption that PKCθ may serve as a potential drug target, and that inhibitors of PKCθ may selectively suppress autoimmunity and allograft rejection, without affecting anti-viral and anti-cancer immunity.

The need for immunosuppressants that block T cell activation has led pharmaceutical companies to develop new small molecules capable of modulating the expression or biological activity of PKCθ. Among the most promising drugs, AE807 (ostatrazolin), is currently in the early phase of clinical trials [112] and although it inhibits several different PKC isoforms, in addition to PKCθ, this drug was found to downregulate NF-κB and NF-AT, but not AP-1, at nanomolar concentrations, and to inhibit IL-2 production and CD25 expression on the surface of primary human and mouse T cells. The mechanism by which AE807 affects PKC and inhibits T cell activation is different from that of the cyclosporine A (an inhibitor of the calcineurin protein phosphatase) and therefore the two drugs exhibit complementary inhibitory effects on T cell signaling pathways.
Most of the existing small molecule PKC inhibitors have toxic side effects because of their lack of absolute specificity, which reflects the relatively high conservation of catalytic domains within PKC family members. Furthermore, since most catalytic kinase inhibitors are ATP competitors, they need to be used at relatively high and potentially toxic concentrations in order to effectively compete with ATP, whose intracellular concentration is ~1 mM. As a result, current studies are aimed at the development of allosteric inhibitors that interact with regions on the kinase molecule, which are outside of the catalytic site, and are therefore likely to be more selective to individual PKC isoforms and exhibit less non-specific toxic effects [113]. The studies by Kong et al., revealed a new potential approach for attenuating PKCθ-dependent functions utilizing allosteric compounds based on the critical PR motif in the PKCθ-V3 domain that block PKCθ binding to CD28 [110]. Since this interaction is essential for PKCθ recruitment to the IS and for the induction of PKCθ-dependent downstream signaling, this new approach could serve as a basis for the development of new therapeutic agents that would selectively suppress undesired T cell-mediated inflammation and autoimmune processes or prevent allograft rejection, while preserving desired immunity, such as antiviral responses.

Concluding Remarks

Past studies have established the requirement for PKCθ in the regulation of many fundamental processes in T cell biology (Figure 2). More recent findings indicated that pharmacological inhibitors of PKCθ might represent beneficial therapeutic modalities for blocking pathological T cell mediates immune responses, without interfering with anti-viral and anti-cancer immunity. Furthermore, since PKCθ is a positive regulator of T cell surveillance, its selective inhibition may help eradicate leukemic T cells by increasing their sensitivity to apoptosis. The development of new small molecule inhibitors and allosteric inhibitors of PKCθ provides promises for future ability to manipulate PKCθ in different disease conditions. Precautions must be taken prior to the clinical application of such inhibitors, in order to evaluate their potential in vivo effects on other PKCθ-positive cell types, and potential iatrogenic effects on PKCθ-negative tissues.

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