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Protein kinase C inhibitors for immune disorders

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Abstract

Protein kinase C (PKC) proteins are a group of well-conserved, intracellular signaling enzymes expressed in all cells and tissues, including immune cells. Much of the molecular insight into PKC immunobiology has been gleaned from studies using PKC gene (*Prkc*) knockout mice and the analysis of different disease models in these animals. More-recent studies have revealed that PKCs also have crucial roles in the pathogenesis of human immune disorders. Therefore, strategies to modulate the functions of PKC enzymes could have a major impact on the treatment and therapies of autoimmune diseases and other immune disorders.

Introduction

Signal transduction is a cellular process constituting the sensing of external stimuli by membrane receptors, biochemical modifications of receptors and membrane-associated proteins, relay of the information through a cascade of second messengers and intracellular proteins, and ultimately generation of physiological changes in response to the corresponding stimuli. At the intersection of second messenger and the activation of intracellular proteins lies protein kinase C (PKC), a family of serine/threonine kinases [1]. Upon the generation and accumulation of second messengers, such as diacylglycerol and calcium ions, at the site of stimulation, PKC enzymes translocate to cellular membranes, usually the plasma membrane. Binding to these second messengers, or some small compounds such as phorbol esters, is necessary to induce a conformational change and expose the kinase domain for substrate phosphorylation. Based on their protein architecture that defines cofactor requirements, members of this kinase family are classified into conventional PKC (cPKC: PKC α , PKC β I, PKC β II and PKC γ), novel PKC (nPKC: PKC δ , PKC ϵ , PKC η and PKC θ) and atypical PKC (aPKC: PKC ζ and PKC ι/λ). The cPKC isoforms can bind diacylglycerol and calcium ions, whereas activation of the nPKC isoforms requires diacylglycerol. The activity of the aPKC members is independent of these cofactors [1], and other cofactors required for their activation (if such cofactors exist) are not currently known.

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Conflicts of interest

The authors have no conflicts of interest to declare.

PKC in the immune system

The PKC family has been studied extensively in the immune system. To date, every member of the PKC family has been genetically disrupted in mice and their respective immune-related phenotypes examined (Table 1). With the exception of PKC γ , the expression of which is restricted to the central nervous system, and PKC ι/λ , whose deletion results in embryonic lethality [2], immune-associated defects were reported when each of the other PKC family members was deleted (Table 1). Intriguingly, these immunological perturbations are distinguishable from each other, indicating that these PKC isoforms have some nonredundant roles in the immune system.

Among the cPKC isoforms, the transcripts of *Prkca* and *Prkcb* genes are ubiquitously detected in immune cells. Nevertheless, T cells and plasmacytoid dendritic cells express higher levels of *Prkca*, whereas B cells and mast cells are the predominant cell populations that express high levels of *Prkcb* transcript (<http://www.immgen.org/>). Accordingly, the expression pattern of these genes correlates with the observed phenotype in the corresponding gene knockout animals. T cells from PKC α -deficient (*Prkca*^{-/-}) mice are severely impaired in proliferation and the production of interferon (IFN)- γ upon anti-CD3 plus anti-CD28 stimulation or following stimulation with allogeneic cells in a mixed lymphocyte reaction. However, *Prkca*^{-/-} B cells proliferate efficiently in response to anti-IgM stimulation, which triggers the antigen-specific B cell receptor [3]. By contrast, genetic disruption of the *Prkcb* gene results in defective B cell responses because PKC β -deficient B cells fail to activate the nuclear factor (NF)- κ B signaling pathway upon B cell receptor engagement [4].

The expression pattern of nPKC isoforms is more immune-restricted than that of the cPKCs or aPKCs (Table 1). Genetic disruption of the *Prkcd* gene causes an autoimmune phenotype with autonomous B cell hyperproliferation [5]. *Prkcd*^{-/-} mice display circulating autoantibodies, immune-complex-associated glomerulonephritis and lymphocyte infiltration into many organs, suggesting that PKC δ is indispensable for negative regulation of B cell homeostasis and the establishment of B cell tolerance [5]. By sharp contrast, we found recently that PKC η -deficient (*Prkch*^{-/-}) mice exhibit a moderately immune hyperreactive phenotype indicative of an important function in T cell homeostasis (unpublished data). *Prkch*^{-/-} mice that develop mild lymphadenopathy contain circulating autoantibodies, and their memory T cells display enhanced production of cytokines such as interleukin (IL)-2, IL-4 and IL-17A upon CD3/CD28 stimulation [6]. We further demonstrated that the regulatory T (Treg) cells of *Prkch*^{-/-} mice are impaired in their contact-dependent suppressive activity *in vitro* and *in vivo* (unpublished data), indicating that PKC η plays a crucial part in at least one effector mechanism utilized by Treg cells and, hence, in the maintenance of T cell tolerance.

The intimate relationship between PKC θ and the immune system has been established early following its initial discovery [7]. PKC θ is highly expressed in T cells (and, to a lesser extent, in muscle tissue). It was subsequently shown that PKC θ is the only PKC family member to translocate selectively to the central region of the immunological synapse (IS) in T cells upon stimulation with antigen-presenting cells [8,9]. Despite the fact that PKC θ has a

minimal role in T cell development in the thymus [10], *Prkcq*^{-/-} peripheral T cells are severely impaired in proliferation and the production of IL-2 and some other cytokines upon anti-CD3/CD28 stimulation [11,12]. In addition, *Prkcq*^{-/-} mice are unable to mount effective Th2 and Th17 responses [13,14], but the production of IFN- γ , indicative of Th1 response, and antiviral immunity mediated by CD4⁺ Th1 cells and CD8⁺ cytotoxic lymphocytes remain relatively intact in *Prkcq*^{-/-} mice [15]. The molecular basis of PKC θ IS localization and downstream functions in T cells has been delineated by demonstrating that PKC θ physically associates with the major T cell co-stimulatory receptor CD28 [16,17]. This physical association accounts for its localization at the center of the T cell IS and its crucial signaling functions (e.g. NF- κ B activation, proliferation and IL-2 production) emanating from the IS, which are required for productive activation of T effector (Teff) cells [17]. Interestingly, PKC θ is sequestered from the IS of Treg cells and, moreover, in sharp contrast to Teff cells, it negatively regulates the suppressive functions imposed by this T cell subset [18,19].

In contrast to other nPKCs with crucial functions in the adaptive immune system, the function of PKC ϵ is important in innate immune responses. Thus, PKC ϵ -deficient (*Prkce*^{-/-}) macrophages demonstrate a severely attenuated response to lipopolysaccharide stimulation and IFN- γ activation, resulting in reduced generation of inflammatory cytokines, such as tumor necrosis factor (TNF)- α and IL-1 β . These defects result in higher susceptibility of the *Prkce*^{-/-} mice to bacterial infections [20].

In PKC ζ -deficient (*Prkcz*^{-/-}) mice, the architecture of the secondary lymphoid organs is significantly altered at a very young (~2 weeks) age. However, this deformity is less apparent in more mature *Prkcz*^{-/-} animals [21]. Although the percentage of T and B cells is normal, a consistent increase in the immature B cell population was observed in *Prkcz*^{-/-} mice. Subsequent analyses indicated that the unresponsiveness to BCR engagement and enhanced apoptosis, as a result of a defect in NF- κ B activation in *Prkcz*^{-/-} B cells, leads to an overall impaired humoral immune response [22]. By contrast, the activation of *Prkcz*^{-/-} T cells was not severely affected. In another study, PKC ζ was found to be required for activation of the Jak1/STAT6 signaling pathway, which controls Th2 effector function and IL-4 receptor signaling [23]. Hence, *Prkcz*^{-/-} mice were protected from allergic lung inflammation, a result of defective Th2 effector function.

Despite the systematic analyses of immunological abnormalities in PKC gene knockout mice, it has proven more challenging to establish the clinical relevance of PKC enzymes and alterations in their function in human diseases of the immune system. Hence, much additional work needs to be performed in this area. This lag reflects several difficulties. First, despite demonstrable phenotypic immune system alterations, all *Prkc*-deficient mice, with the exception of PKC ι/λ , live into adulthood with no obvious signs of early disease or mortality. This probably reflects some degree of redundancy and, hence, functional compensation among PKC family members. Therefore, humans with genetically or otherwise altered PKC expression and/or function can show only mild signs of immunological disorders without life-threatening complications. Second, there have been only scant reports of clinical manifestations that can result from the loss or abnormal function of certain PKC isoforms. As a result, patients with PKC-associated diseases can go

undiagnosed. This situation is exemplified by a recent report, which characterized a patient from a consanguineous family suffering from recurrent infections and severe lupus-like autoimmunity [24]. Combined homozygosity mapping and exome sequencing identified a biallelic splice site mutation in the *Prkcd* locus, resulting in the absence of PKC δ protein [24]. Interestingly, this patient demonstrated immunopathology that is highly similar to the *Prkcd*^{-/-} mice [5]. This study has several implications: (i) it validates findings in mouse studies; and (ii) it points out the need for extensive future studies aimed at comprehensively characterizing the clinical and immunological phenotypes of patients who display altered patterns of PKC expression and/or function.

In addition to their primary contribution to disease, PKC enzymes could also function as indirect disease-modifying factors in immune disorders. Reports of genome-wide studies have provided reproducible and convincing evidence for the association of *Prkc* gene loci with human autoimmune diseases (Table 1). Genome-wide association studies (GWAS), which compare single nucleotide polymorphisms (SNPs) between thousands of diseased versus healthy individuals, followed by powerful statistical analyses, have identified specific SNPs within the *Prkc* loci that are significantly associated with various autoimmune diseases, including type I diabetes, rheumatoid arthritis (RA) and inflammatory bowel diseases. More extensive investigations accompanied by high power statistical analyses are required to examine the extent of PKC contributions toward the pathogenesis of these multifactorial disorders.

In summary, mouse studies and, to a lesser extent, human studies have consistently and reproducibly demonstrated a causal relationship between PKC isoforms and immune disorders. Hence, strategies designed to inhibit the functions of defined PKC enzymes could be beneficial for the treatment of autoimmune diseases and other immune disorders such as graft versus host disease (GvHD) and transplant rejection.

PKC inhibitors

The PKC family of kinases sits at the crossroad of multiple signaling pathways, making them as attractive targets for the treatment of a wide variety of human diseases. However, inhibiting the functions of PKC in T cells has gained the most traction in the treatment of autoimmune diseases because: (i) PKC has prominent roles in controlling T cell activation and the differentiation of certain T cell subsets; (ii) T cells display a unique PKC expression profile with higher level expression of a subset of PKC (i.e. PKC α , PKC η and PKC θ); and (iii) the loss or inhibition of PKC isoforms in T cells does not severely mitigate antiviral immunity. Therefore, T-cell-expressed PKCs, in particular PKC θ , represent potentially attractive therapeutic targets to achieve a selective suppression of the pathogenic T cell responses (e.g. autoimmunity) without compromising beneficial antiviral immunity.

Numerous structure-based and other approaches have been pursued in attempts to develop selective small molecule PKC inhibitors, with T cells representing a favored target tissue. Hitherto, only AEB071, also known as sotrastaurin, has made substantial progress in clinical trials with potential applications in several immune disorders [25,26]. AEB071 blocks the catalytic activity of PKC α , PKC β and PKC θ isoforms at a low picomolar concentration

range, as well as PKC δ , PKC ϵ and PKC η at higher, nanomolar, concentrations [25]. The broad selectivity of AEB071 toward PKC family members could underlie its relative efficacy because other PKCs, in addition to PKC θ , could have compensatory and redundant activities in T cell functions [6,27]. The use of AEB071 has been expanded to other disease settings. For example, AEB071 has been shown to be effective *in vivo* in inhibiting GvHD disease [28,29], and in a mouse xenograft model of transplanted diffuse large B cell lymphoma [30]. Phase I/II clinical trials are currently underway to determine the efficacy of AEB071 in metastatic uveal melanoma of the eyes (NCT01430416 and NCT01801358).

Besides AEB071, several other small molecule compounds have been reported to inhibit PKC functions in T cells, raising hopes that these compounds might potentially be valuable in the treatment of autoimmune diseases. As mentioned earlier, PKC θ acts as a negative regulator in Treg cells, and Treg cells from RA patients were reported to display defective suppressive activity [19]. A PKC θ -selective inhibitory compound, C-20, was shown to enhance the suppressive function of Treg cells from RA patients with accompanying attenuated production of IFN- γ by autologous Teff cells [19]. In addition, treatment of Treg cells with the C-20 compound was able to protect mice against the development of inflammatory bowel disease in a transfer model of colitis, suggesting the potential utility of this compound in several autoimmune settings.

In mouse T cells, PKC α cooperates with PKC θ to promote T cell activation [31]. Hence, dual inhibition of PKC α and PKC θ could potentially exert a more T cell immunosuppressive effect. Based on this notion, a novel small molecule PKC inhibitor, R524, was developed. R524 inhibits the kinase activity of PKC α and PKC θ at the nanomolar concentration range, with lower activity against other PKC isoforms [32]. In a mouse GvHD model, administration of R524 abrogated T cell proliferation and significantly reduced GvHD symptoms [32]. Despite the significant impairment of CD4⁺ T cell functions, R524-treated mice showed a beneficial reduced susceptibility to graft versus leukemia (GvL) response because CD8⁺ cytotoxic T cells (CTLs) were spared from R524 inhibition [32]. This study, along with other reports [31,33], demonstrates the utility of inhibiting PKC in GvHD responses without compromising the GvL response, which is crucial for inhibiting or preventing the development of leukemia in bone marrow transplant recipients.

Current efforts in the search for small molecule modulators of PKCs have focused on the kinase domain of PKCs. However, the kinase domain is well conserved among PKC family members as well as within other members of the larger protein kinase superfamily. Therefore, it has been a challenging task at the molecular level to obtain small molecule compounds that have a high degree of specificity and selectivity toward a specific PKC isoform. More recently, the notion of allosteric kinase inhibitors (i.e. small compounds that bind to sites other than the catalytic domain and are still able to inhibit enzyme activation through an induced conformational change) has emerged. In this regard, our recent observations regarding the molecular basis for the selective recruitment of PKC θ to the center of the IS and its downstream signaling functions bear special relevance. Early on, we found that the regulatory domain of PKC θ is required for its translocation to the IS and into membrane lipid rafts [34]. More recently, we reported that antigen stimulation of T cells leads to a physical association of PKC θ with T cell co-stimulatory molecule CD28, and that

this association accounted for the unique IS localization of the enzyme [17]. We identified a proline-rich motif within the V3 (hinge) domain of PKC θ that was necessary and sufficient for the interaction with CD28 and demonstrated that deletion or mutation of this motif resulted in defective T cell activation, including a defect in Th2 and Th17 (but not Th1) differentiation. More importantly, the signaling functions of endogenous PKC θ could be blocked by ectopic expression of the V3 domain alone, which functioned as a dominant-negative ‘decoy’ to disrupt the PKC θ –CD28 interaction [17]. This result points toward the possibility of targeting the V3 region for allosteric inhibition. In comparison to the catalytic site, the V3 domain is potentially more attractive as a drug target owing to its high variability among members of the PKC family. Therefore, allosteric inhibition of PKC (and other) kinases represents a potential alternative approach to develop highly selective, and possibly less toxic, kinase inhibitors for the treatment of immune diseases.

Concluding remarks

During the past couple of decades, there has been considerable progress along the winding road toward understanding the immunobiology of PKCs. The availability of PKC gene knockout mice and the preclinical analysis of disease models of these animals have revealed the important roles of PKC family members in various manifestations of the immune system. These include the activation of innate immune cells upon pathogen infection, the bridge between innate and adaptive immune responses, the activation and differentiation of adaptive immune cells and the maintenance of self-tolerance and immune homeostasis. Despite these advances, our understanding of the functions of PKC in the human immune system is still limited. The application of recent powerful research technologies, such as next-generation sequencing and mass cytometry, to PKC research will certainly expand our horizons in this regard. More importantly, current and future knowledge should serve as a rational platform for generating improved understanding of the contribution of different PKCs to human pathology, as well as to develop clinically useful PKC-specific treatment modalities for human diseases in general, and immune system diseases in particular.

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Table 1

Expression of PKC family members and immune alterations associated with their deletion

Isoform	Predominant immune cell expression^a	Immune phenotypes in knockout animals	GWAS on immune-related diseases
PKCα	T cells and pDC	Defects in T cell activation [3]	Multiple sclerosis [35]
PKCβ	B cells and mast cells	Defects in B cell activation [4]	Inflammatory bowel disease [36], rheumatoid arthritis [37]
PKCγ	Below detectable level	None reported	None reported
PKCδ	B cells, mast cells, macrophages	Defects in B cell homeostasis [5]	Inflammatory bowel disease [36]
PKCϵ	Ubiquitous (low)	Defects in macrophage activation [20]	Red blood cell traits [38]
PKCη	T cells and macrophages	Defects in T cell homeostasis [Kong <i>et al.</i> , manuscript submitted]	Rheumatoid arthritis [37]
PKCθ	T cells and mast cells	Defects in T cell activation [11,12]	Type I diabetes [39], rheumatoid arthritis [40,41], celiac disease [42]
PKCζ	Ubiquitous (low)	Defects in T and B cells [22]	None reported
PKCι/λ	Ubiquitous (low)	Embryonic lethal [2]	None reported

^aTranscript expression data were derived from BioGPS (<http://biogps.org/>) and Immunological Genome Project (<http://www.immgen.org/>).