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What came first: PKCθ or the immune synapse?

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Summary

Stimulation of T cells by peptide-presenting antigen-presenting cells (APCs) induces formation of a highly organized complex of receptors, signaling molecules and cytoskeleton components at the immune synapse (IS), the contact site between T cells and APCs. Conjugate formation between T cells and APCs initiates the formation of the IS. After this event, micrometer-scale molecular movements occur in the T cell plasma membrane and the actin cytoskeleton undergoes reorganization. Our current knowledge suggests that formation of the IS is an essential step during T cell activation. This is probably related to the proper localization of certain proteins in specific compartments. One of these proteins is protein kinase Cθ (PKCθ), which is absolutely required for T cell activation. During the last years we have made great advances in understanding the function and targets of this kinase, and recent reviews have summarized these findings. In contrast, we do not know the exact mechanism that activates PKCθ after TCR engagement and the role of PKCθ activation in the formation of the IS. In this review I analyze the mechanism of the translocation of PKCθ and discuss the function of PKCθ in the formation of the IS and, vice versa, the role of the IS in the translocation of PKCθ.

Key words: **T cell • PKCθ • immune synapse • WASP • PLC**

Abbreviations: **TCR** – T cell receptor, **PKC** – protein kinase C, **aPKC** – atypical PKC, **cPKC** – conventional PKC, **nPKC** – novel PKC, **NF-AT –** nuclear factor of activated T cells, **WASP** – Wiscott-Aldrich syndrome protein, **IS** – immune synapse, **MHC** – major histocompatibility complex, **APC** – antigen-presenting cell, **SMAC** – supramolecular activation cluster, **cSMAC** – central SMAC, **pSMAC** – peripheral SMAC, JNK – c-Jun NH2-terminal kinase, **AP-1** – activating protein, **NF-κB** – nuclear factor κB, **IL-2** – interleukin 2, **BisVIII** – bisindolylmaleimide VIII, **DAG** – diacylglycerol, **PDK1** – 3-phosphoinositide-dependent kinase-1, **PI3K** – phosphatidylinositol 3-kinase, **PLC** – phospholipase C, **WIP** – WASP interaction protein, **LCMV** – lymphocytic choriomeningitis virus, **PIP3** – phosphatidylinositol (3,4,5)-trisphosphate, **PH** – Pleckstrin homology, **PMA** – phorbol myristate acetate.

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T CELL RECEPTOR ACTIVATION

An effective immune response depends on the ability of specialized immunocytes to identify foreign molecules or antigens and respond by differentiation into mature effector cells. This tightly regulated process is mediated by a cell-surface antigen-recognition apparatus and complex intracellular receptor-coupled signal-transducing machinery, which operate at high fidelity to discriminate a self from a non-self antigen.

The T cell antigen-specific receptor (TCR) is composed of two covalently bound polymorphic subunits, which provide antigen specificity, in association with at least four different types of invariant chains, which are essential for signal transduction. Activation of T lymphocytes requires sustained physical interaction of the TCR with a major histocompatibility complex (MHC)-presented peptide antigen. Stimulation of T cells by peptide-presenting antigen-presenting cells (APCs) induces formation of a highly organized complex of receptors, intracellular signaling molecules, and F-actin at the contact site between T cells and APCs: the immunological synapse (IS) or supramolecular activation cluster (SMAC)[22, 31].

Formation of the IS is a multistep process that is initiated by conjugate formation between T cells and APCs. After this event, micrometer-scale molecular movements occur in the T cell plasma membrane and the actin cytoskeleton undergoes reorganization[18]. It also involves the assembly of signaling complexes consisting of TCRs, costimulatory accessory receptors (CD28, LFA-1, CD4/CD8, etc.), intracellular signaling effector proteins, and the reorganization of the actin cytoskeleton and clustering of specialized membrane microdomains or lipid rafts[12, 22, 31, 36, 53, 59, 60]. The IS is divided into at least two identifiable areas[31], the central SMAC (cSMAC) and peripheral SMAC (pSMAC). While the cSMAC contains the TCR, the pSMAC is enriched with LFA-1 integrins. The spatial organization and stability of the IS determine the functional outcome of TCR engagement[17].

As described above, certain signaling proteins that play an essential role in TCR signaling are also translocated to the cSMAC. For example, the translocation of protein kinase C (PKC)θ is so specific that this protein is used for labeling the IS[13, 31, 32, 53]. The structure, function and regulation of the T cell synapse currently represent an intensive area of research among T cell biologists. T cells depend on actin cytoskeleton reorganization to induce lipid raft clustering[21, 40, 53]. The guanidine exchange factor Vav and its main target, the GTPase Rac, mediate this reorganization[53]. The very same pathway is respon sible for the translocation of PKCθ. Therefore, an open question is the role of PKCθ in the formation of the IS. Does PKCθ play a role in the formation of the IS? Or is it simply translocated after TCR stimulation? In other words, what came first, the chicken or the egg, PKCθ activation or IS formation?

PKCθ, THE PROTEIN

The discovery of PKC as a lipid- and Ca^{2+}-dependent serine/threonine kinase which serves as a cellular receptor for tumor-promoting phorbol ester[33] implicated PKC in the activation and mitogenesis of T cells. This was further substantiated by demonstrating that cellular depletion of PKC by prolonged treatment with phorbol esters or PKC inhibitory drugs significantly inhibited T cell activation[4]. During the same period, progress has been made in isolating the cDNAs encoding different PKC enzymes. This work revealed that PKC constitutes a family of multiple enzymes encoded by different genes which have distinct biochemical properties, expression profiles and physiological functions. The PKC family consists of three subfamilies: cPKC (or conventional PKCs: α, β1, β2 and γ), aPKC (or atypical PKC: ζ and ι/λ), and nPKC (or novel PKCs: δ, ε, θ and μ). However, the specific contribution of distinct PKC isoforms to TCR-coupled signaling pathways has not been resolved until very recently. This gap in our knowledge was largely due to the relatively slow progress in identifying specific physiological substrates and functions of distinct PKC isoenzymes in T cells.

PKCθ was isolated as a Ca^{2+}-independent PKC isoform expressed selectively in T cells and muscle cells[9]. It displays several unique properties attesting to its important role in T cell activation[2, 7, 26]. First, it selectively activates the transcription factors activating protein (AP)-1 and nuclear facktor (NF)-κB[10, 14, 27, 47] and synergizes with calcineurin to activate nuclear factor of activated T cells (NF-AT), the interleukin (IL)-2 gene[20, 58] and Fas ligand[56, 57]. Furthermore, PKCθ integrates TCR and CD28 signals that lead to activation of the CD28 response element in the IL-2 gene promoter[14]. Lastly, PKCθ also activates the c-Jun NH_2-terminal kinase (JNK) in T cells[8, 20, 55, 58]. However, the biological significance of this pathway is unclear, since JNK is not required for IL-2 production by naive activated T cells[16], and JNK activation is intact in T cells from PKCθ knockout mice[47]. The importance of PKCθ in T cell activation was confirmed by the analysis of $PKC\theta^{-/-}$ mice, which display a selective T cell activation defect at the level of AP-1, NF-κB, and IL-2 induction[47]. Importantly, PKCθ does not play any known role in thymic development[47].

Two recent reports have unexpectedly shown that PKCθ also controls the activation of the transcription factor NF-AT, the main target of intracellular calcium increase[3, 37]. The TCR-induced calcium signal is also impaired in T cells derived from PKCθ$^{-/-}$ mice. Altman et al.[3] have shown that PKCθ activates the tyrosine kinase Tec, which in turn activates phospholipase C (PLC)γ1, the enzyme responsible for the production of IP3 and thereby calcium release from the intracellular stores.

In addition, PKC enzymes play diverse roles in regulating cell death. PKC activation by phorbol ester treatment protects cells from apoptosis in various cell systems[19, 25, 42, 46]. T cells are sensitized by the PKC inhibitor, bisindolylmaleimide VIII (BisVIII), to Fas-mediated apoptosis[61]. PKCθ and, to a lesser degree, PKCε protect T cells from Fas-induced apoptosis[11, 54]. The mechanism involves BAD phosphorylation by PKCθ itself[54] and/or by a pathway activated by PKCθ[11]. This effect is probably specific for T cells, because PKCδ, another member of the nPKC subfamily that is not expressed in T cells[54, 56], plays a pro-apoptotic role in neuronal apoptosis following growth factor deprivation[50]. Conversely, BisVIII protects neurons from growth factor deprivation-induced cell death[49]. This anti-apoptotic function suggests that PKCθ could be a drug target for therapeutic intervention in human T cell leukemias (for review[51]).

THE CHICKEN OR THE EGG. ONE SIDE: THE ROLE OF THE IS IN PKCθ TRANSLOCATION

A major contribution to our understanding of the role of PKCθ in T cell biology was made when it was discovered that engagement of antigen-specific T cells by peptide-presenting APCs led to a rapid, stable and highly stoichiometric localization of PKCθ, but not other T cell-expressed PKCs, to the cSMAC[31, 32]. This localization occurs at a high stoichiometry and lasts for at least 2–4 h, suggesting that it could play an important role in propagating and extending activation signals that are required for T cell commitment to IL-2 production long after early tyrosine phosphorylation events have been extinguished. In both CD4$^+$ and CD8$^+$ T cells, PKCθ rapidly redistributes to the cSMAC[38, 45]. Whereas CD28 costimulation is not essential for accumulation of PKCθ at the IS, it is necessary for the localization of PKCθ at the cSMAC[24]. In addition, antigen stimulation induces translocation of PKCθ to specialized membrane microdomains or lipid rafts[13], which are known to play an important role in T cell activation[12, 60]. Furthermore, the lipid raft translocation of PKCθ is essential for its proper function during T cell activation[13]. Similar to PKCθ itself, lipid rafts also cluster at the IS in antigen–stimulated T cells. But, unlike PKCθ, the rafts are not restricted to the cSMAC. Thus, lipid raft translocation of PKCθ *per se* is unlikely to entirely account for the very specific concentration of the enzyme in the cSMAC during antigen stimulation. Nevertheless, receptor-induced clustering of lipid rafts may serve as an important driving force that promotes the initial translocation of PKCθ to the IS, where additional mechanism(s) may function to selectively recruit it into a specific subregion of the IS, i.e. the cSMAC.

Both lipid rafts and PKCθ are translocated to the IS by a Vav-dependent mechanism[53]. Unlike other T cell–expressed PKC enzymes, a fraction of cellular PKCθ associates with the cytoskeleton upon T cell activation[30, 55]. Vav promotes the translocation of PKCθ from the cytosol to the membrane and cytoskeleton[55]. It also induces PKCθ activation in a CD3/CD28 costimulation pathway that is dependent on Rac and actin cytoskeleton reorganization. These findings reveal that the Vav/Rac pathway promotes the recruitment of PKCθ to the T cell synapse and its activation. They also suggest that a unique mechanism, other than (or in addition to) the conventional diacylglycerol (DAG)- and PLC–dependent pathway for PKC activation, regulates PKCθ activation, since PLCγ1-induced activation of PKC would also be expected to activate other conventional and novel PKC isoforms. The nature of this putative mechanism was addressed in a recent study[52]. Using three independent approaches, i.e. a selective PLC inhibitor, a PLCγ1-deficient T cell line, or a dominant negative PLCγ1 mutant, we demonstrated that CD3/CD28-induced membrane recruitment and activation of PKCθ are PLCγ1-independent. In contrast, the same inhibitory strategies blocked the membrane translocation of PKCα. On the other hand, membrane or lipid raft recruitment of PKCθ was absent in T cells treated with phosphatidylinositol 3-kinase (PI3K) inhibitors or in Vav-deficient T cells and was enhanced by constitutively active PI3K[52]. These findings do not completely rule out a requirement for DAG binding to the PKCθ C1 domain as an important step in its membrane binding and activation. Some DAG may still be required to initiate PKCθ membrane binding. Although this event *per se* may not be sufficient to recruit PKCθ to specific membrane compartments such as the IS or lipid rafts, it may facilitate its interaction with membrane or cytoskeletal components required for translocation of PKCθ to the cSMAC and its full activation. Such an interacting partner could be a membrane-localized protein kinase that trans-phosphorylates PKCθ or an adaptor/scaffold protein that recruits and anchors it to specific membrane microdomains[31, 32] or lipid rafts[13]. For example, DAG production is not needed for PKCθ translocation[15, 52], but the adaptor function of PLCγ1

could be responsible for the translocation of PKCθ to the membrane[15].

The requirement for both Vav and PI3K in PKCθ membrane translocation and activation most likely reflects the fact that Vav is a critical target for PI3K in a single pathway regulating PKCθ activation in the IS. This putative mechanism is consistent with the finding that Vav is activated by PIP_3 binding to its PH domain[23], a process that promotes Vav recruitment to the membrane and facilitates its catalytic activation by regulatory tyrosine phosphorylation[1]. The requirement of both TCR and CD28 signals for stable activation and membrane or lipid raft translocation of PKCθ[13,14] could reflect this dual regulatory mechanism for Vav activation. The finding that Vav and constitutively active PI3K do not cooperate to enhance membrane translocation of PKCθ[52] is also consistent with the notion that PI3K and Vav function in a single pathway. However, the possibility that Vav and PI3K function in two independent pathways to promote PKCθ translocation and activation cannot be formally ruled out. Comparison of PKCθ with other PKC isoenzymes identifies a number of conserved phosphorylation sites implicated in regulation of its activity. 3-Phosphoinositide-dependent kinase-1 (PDK1) phosphorylates a critical threonine residue in the activation loop of PKCδ and PKCζ, increasing their activities[35]. PDK1-induced phosphorylation of T538 is essential for the catalytic activation of PKCθ. In contrast, the S676 and S695 may be sites of autophosphorylation that contribute to the regulation of its catalytic activity[28]. PDK1 overexpression induced PKCθ translocation to the membrane and to the cytoskeleton compartment[52], but overexpressed PDK1 did not localize in the membrane under resting conditions, and was not translocated by anti-CD3 + anti–CD28 stimulation or PMA treatment. Moreover, the PI3K inhibitor LY294002 only partially blocked the PDK1 effect, while the dominant negative construct VavΔPH blocked it completely. These data suggest that Vav function was still necessary for PDK1-induced PKCθ translocation and that PDK1 overexpression could overcome the requirement of endogenous PDK1 for PI3K products. Overexpression of PDK1 with PKCθ did not increase the transcriptional activation of NF-κB or AP-1 when compared to PKCθ alone[52]. Taken together, these data suggest that PDK1 binding is not the mechanism for PKCθ translocation. We suspect that PDK1 is involved in the maturation of PKCθ, a prerequisite process for PKCθ translocation, but it is not involved in TCR-induced PKCθ translocation. In summary, the pathway responsible of PKCθ translocation involves PI3K, Vav, Rac and changes in the cytoskeleton, but it is partially independent of PLCγ1 enzymatic activity[15, 52, 55].

THE CHICKEN OR THE EGG. THE OTHER SIDE: PKCθ IS REQUIRED FOR CYTOSKELETON REMODELING

The Wiskott-Aldrich syndrome protein (WASP) plays an essential role in controlling TCR-induced actin nucleation and polymerization and, more generally, in the regulation of cytoskeletal rearrangements important for hematopoietic cell function[48]. WASP interaction protein (WIP) controls actin polymerization in two ways: it keeps WASP in an inactive conformation that prevents it from binding to the Arp2/3 complex of nucleating proteins, but it also binds and stabilizes actin filaments[29]. Conversely, T cells from $WIP^{-/-}$ mice fail to proliferate, secrete IL-2, or increase their F-actin content after TCR ligation. Furthermore, $WIP^{-/-}$ T cells are deficient in conjugate formation with superantigen-presenting B cells and anti-CD3/ICAM-1-containing lipid bilayers and have a disorganized actin cytoskeleton[6]. WIP has a consensus PKC phosphorylation motif within its WASP binding domain[43]. PKCθ-dependent phosphorylation of this site negatively regulates WIP binding to WASP, allowing WASP activation by Cdc42-GTP and actin stabilization by WIP. Consistent with these findings, a point mutation of the WIP phosphorylation site showed markedly reduced WASP binding. Moreover, $PKC\theta^{-/-}$ T cells had a severely impaired F-actin increase upon anti-CD3 stimulation, supporting the notion that PKCθ-dependent phosphorylation of WIP is critical for its dissociation from WASP. Therefore, PKCθ could be needed for the formation of the IS.

In contrast, expression of a constitutively active mutant of PKCθ fails to induce F-actin polymerization in Jurkat T cells[55]. In addition, other studies suggest that PKCθ actually needs to reside within the cSMAC in order to function properly, and that CD28 costimulation plays a unique and non-redundant role in effecting this highly selective localization[5, 24]. Only the focused concentration of PKCθ in the cSMAC correlated with NF-κB activation, as revealed by confocal analysis of the nuclear translocation of this transcription factor[5]. Moreover, the isolated catalytic domain of PKCθ, which is fully active *in vitro*, failed to translocate into lipid rafts and activate NF-κB[13]. This defect was rescued when an Lck-derived raft-targeting signal was fused to the catalytic region.

Therefore, PKCθ is needed for the formation of the mature IS. But, at the same time, it needs to localize in the IS to be fully active. This reveals the paradox, because in order to activate PKCθ, the IS should already be formed.

There are some explanations for this apparent paradox. A possibility is that TCR engagement induces a short and transient activation of PKCθ. This would be enough

to trigger formation of the IS. In this first step, DAG would mainly mediate the recruitment of PKCθ. Later on, lipid raft clustering and actin polymerization would induce translocation of PKCθ to the IS. This second step will be performed by Vav/Rac activation, localizing PKCθ in the correct compartment and leading to NF-κB activation[3]. In fact, Riteau et al.[39] have proposed something similar for Vav translocation to the contact area between natural killer cell and target cells.

Another possible explanation is that Sasahara et al.[43] used antibodies to stimulate cells. The selective colocalization of PKCθ and TCR at the synapse can be induced by physiological TCR and CD28 ligands present on the surface of APCs, i.e. peptide/MHC complexes and B7, respectively, but not by anti-TCR/CD28 antibodies[32]. This observation suggests that anti-receptor antibodies do not precisely mimic the signals induced by physiological ligands. It is possible that the use of anti-CD28 antibodies (as opposed to B7) accounts for this difference, since B7 or an anti-CD28 antibody were found to induce distinct biochemical events in T cells in terms of Ras activation or the tyrosine phosphorylation of Vav[34]. Therefore, PKCθ would be essential for cytoskeleton remodeling induced by antibodies, by not by APCs.

AND NOW WHAT?

Obviously, the next step is to determine if T cells derived from PKCθ$^{-/-}$ mice show a defect in cytoskeleton remodeling after physiological stimulation by APCs. The problem of this experiment is the activation of a high proportion of T cells to perform biochemical studies. The best protocol would be to cross the PKCθ$^{-/-}$ mice on a TCR transgenic background, a time-consuming project. For the moment, there are alternative protocols. One possibility is to activate T cells with superantigen, but it is not clear if this activation will represent the same as antigen stimulation. The other is to use infected animals, i.e. with lymphocytic choriomeningitis virus (LCMV). In fact, we developed a new protocol to study PKCθ translocation to the membrane and cytoskeleton in CD8$^+$ and CD4$^+$ T cells[53]. C57BL6 (H-2^b) mice were infected with LCMV and 7–8 days later were sacrificed at the time of the peak of the immune response[41, 44]. Splenocytes from these infected animals were used as effector cells and were incubated in the presence of different relevant LCMV peptides to induce CD8$^+$ or CD4$^+$ activation. To induce specific CD8$^+$ T cell activation, splenocytes were incubated with 1 μg/ml of the LCMV immunodominant peptides GP_{33} (aa sequence: K_{33}AVYNFATCG) and NP_{396} (F_{396}QPQNGQFI). To stimulate the specific CD4$^+$ T cell activation, splenocytes were incubated with 5 μg/ml of the immunodominant peptides $GP_{61\text{-}80}$ (G_{61}LKGPDIYKGVYQFKSVEFD) and NP_{309} (S_{309}GEGWPYIACRTSIVGRAWE). Almost 50% of the PKCθ was translocated with the relevant CD8$^+$ peptides, while around 15% was translocated by the relevant CD4$^+$ peptides. These data are in concordance with the data obtained by intracellular cytokine staining. Therefore, this protocol gives an acceptable number of specific T cells for a specific antigen.

For the moment, it is going to take a while to know what came first: IS formation or PKCθ translocation; the chicken or the egg. But, sooner or later, we will figure it out.

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