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Review

Targeting protein kinase C and "non-kinase" phorbol ester receptors: Emerging concepts and therapeutic implications

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Abstract

Phorbol esters, natural compounds that mimic the action of the lipid second messenger diacylglycerol (DAG), are known to exert their biological actions through the activation of classical and novel protein kinase C (PKC) isozymes. Phorbol esters, via binding to the PKC C1 domains, cause major effects on mitogenesis by controlling the activity of cyclin–cdk complexes and the expression of cdk inhibitors. In the last years it became clear that phorbol esters activate other molecules having a C1 domain in addition to PKCs. One of the most interesting families of "non-kinase" phorbol ester receptors is represented by the chimaerins, lipid-regulated Rac-GAPs that modulate actin cytoskeleton reorganization, migration, and proliferation. The discovery of the chimaerins and other "non-kinase" phorbol ester receptors has major implications in the design of agents for cancer therapy.
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1. Introduction

Diacylglycerol (DAG), a lipid second messenger generated by phospholipase C (PLC) upon activation of seven-transmembrane and tyrosine kinase receptors, is an important endogenous activator of signaling kinases. Protein kinase C (PKC) isozymes, a family of 10 serine-threonine kinases, sense the elevations in DAG levels and redistribute to the membrane where they become activated and phosphorylate cellular substrates, leading to a plethora of effects on mitogenesis, cell death, differentiation, morphology and transformation [1–4]. In this review, we will focus on the biological actions of phorbol esters via PKC activation and the characterization of novel phorbol ester receptors.

2. PKC isozymes: the prototype targets for the phorbol esters and diacylglycerol

The PKC isozymes have been extensively studied as intracellular receptors for the phorbol esters, natural DAG-mimetics with tumor promoter activity. Phorbol esters have been used for decades as PKC activators, both in vitro and in animal models. Structurally distinct natural products mimic the actions of phorbol esters and are capable of binding to PKC with high affinity. These compounds include the daphnane derivatives mezerein and thymeleatoxin, ingenol esters, aplysiatoxins, teleocidines such as indolactam V (ILV), ingenol esters, and bryostatins [5]. PMA or TPA (phorbol 12-myrystate 13-acetate) represents one of the most valuable pharmacological tools to study multistage carcinogenesis in vivo, as they promote the clonal expansion of "initiated" cells previously treated with carcinogens such as DMBA. Some of the more hydrophilic tumor promoters, such as 12-deoxy-phorbol 13-phenylacetate (dPP) or 12-deoxyphorbol 13-acetate (prostratin), and bryostatin 1, lack tumor promoter activity. Moreover, these compounds block PMA-induced skin tumor promotion [6,7], although the mechanisms

Abbreviations: DAG, diaclyclycerol; DGK, diacylglycerol kinase; ILV, indolactam V; FRET, Florescence Resonance Energy Transfer; PAH, polycyclic aromatic hydrocarbon; PDBu, phorbol 12, 13-dibutyrate; PDK1, phosphatidylinositide-dependent kinase 1; PKC, protein kinase C; PLC, phospholipase; PMA or TPA, phorbol 12-myristate 13-acetate or phorbol 12-tetradecanoyl 13-acetate; RACK, Receptor for activated C kinase

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involved are not fully understood. Substantial biochemical evidence has demonstrated that phorbol esters bind to PKC isozymes with high affinity, and they do so in a manner that is dependent on acidic phospholipids such as phosphatidylserine [8]. Extensive work from many laboratories, particularly the laboratory of Peter M. Blumberg (NIH), has defined the structural requirements and cofactor requirements for phorbol ester binding to PKC isozymes [9,10]. Despite the advances in molecular strategies, phorbol esters still represent the most used approach to investigate PKC function.

There is considerable homology among the PKC family members; however, it is clear that each PKC has unique properties and couples to the activation of different signaling cascades, suggesting a complex array of intracellular networks that could be regulated by each member of the family. Based on their structural and biochemical properties the PKC isozymes have been classified into 3 subclasses: "classical" or cPKCs, "novel" or nPKCs, and "atypical" or aPKCs. The cPKCs comprise 4 calcium-dependent and phorbol ester/DAG-responsive isozymes: PKCα, βI, βII, and γ. The nPKCs comprise 4 calcium-insensitive, phorbol ester/DAG-responsive isozymes: PKCδ, ε, η, and θ. The aPKCs (PKCζ and ι/λ) do not bind phorbol esters or DAG. Each PKC isozyme is the product of a separate gene, with the exception of PKCβI and βII, which are alternative spliced variants from the PKCβ gene. As phorbol esters mimic the action of the second messenger DAG, only the cPKCs and nPKCs respond to the activation of receptors coupled to PLC and DAG generation. aPKCs are regulated through other mechanisms, such as transphosphorylation by phosphoinositide-dependent kinase-1 (PDK1) [11]. There are kinases related to PKCs, such as the PKDs, that bind phorbol esters and DAG. However, their substrate specificity is unrelated to that of PKCs. PKDs also have unique modes of regulation by phosphorylation mechanisms [2–4,12,13].

At the structural level, all PKC isozymes are related and have 4 well defined domains (conserved domains) located in two regions, the N-terminal or regulatory region, and the C-terminal or catalytic region. The regulatory region possesses the motifs involved in the binding of the phospholipid cofactors, phorbol esters/DAG and calcium, and it also participates in the associations with interacting proteins that control localization. The C-terminal region is the catalytic (kinase) domain, and it includes motifs involved in ATP and substrate binding. The regulatory and the catalytic domains are connected through a hinge region that is highly flexible and possesses sites for protease cleavage. There are 4 conserved (C1–C4) and 5 variable (V1–V5) domains in PKC isozymes. The C1 domain in cPKCs and nPKCs is responsible for the binding of phorbol ester and DAG. The C2 domain is responsible for phospholipid binding, and it confers calcium responsiveness to the cPKCs. The C3 domain is the ATP-binding site, and it is the target of the majority of PKC inhibitors. The C4 domain in the catalytic region has the substrate-binding site. The C-terminal region also possesses regulatory auto- and trans-phosphorylation sites. PKCs are autoinhibited by an N-terminal pseudosubstrate motif that binds to the catalytic region and keeps the enzyme in an inactive conformation. Upon binding of phorbol esters or DAG to the C1 domain, a conformational change relieves this autoinhibition and the enzyme becomes activated [2,12,13].

3. Phorbol esters as pharmacological probes for PKC activation

Phorbol esters and related compounds not only constitute essential tools to assess PKC function, but they also represent the most valuable ligands for the evaluation of binding properties in PKCs. Binding of phorbol esters in PKC occurs through specific interactions within the C1 domains. Phorbol 12,13-dibutyrate ([^{3}H]-PDBu) has been widely used in in vitro binding assays to determine the number of phorbol ester receptors (PKC and others, see below) in cells or tissues. Competition assays have established relatively little differences in the affinities of phorbol esters and related analogs for PKC isozymes. 12-deoxyphorbol esters and mezerein have some preference for cPKCs, while indole alakaloids such as ILV and octyl-indolactam V (octyl-ILV) have almost identical affinities for cPKCs and nPKCs. Thymeleatoxin has been reported to preferentially bind to PKCα, PKCβI, and PKCγ [8]. Phorbol esters are also widely used to assess PKC activation in cellular models due to their ability to translocate cPKCs and nPKCs to the plasma membrane and internal membrane compartments. The pattern of translocation may differ depending on the cell types, PKC isozymes and the analogs used. Translocation of PKCs is entirely dependent on the C1 domain, and mutation in residues that are essential for phorbol ester binding abolishes translocation to membranes [14].

4. PKC isozymes, cell cycle control, and cancer

In addition to the tumor promotion studies, numerous lines of evidence have implicated PKC isozymes in cancer progression. Changes in the expression of individual PKC isozymes and their effectors have been observed in various types of human cancers, suggesting that dysregulation of PKC signaling may be a factor in uncontrolled cell proliferation and neoplastic transformation. However, research in the last years has challenged the view that PKC mediates only mitogenic signaling. Indeed, phorbol esters can also inhibit cell proliferation or induce apoptosis in a large number of cell lines (see below) (Fig. 1). This heterogeneity may reflect the differential roles of PKC isozymes, which could act either as positive or negative regulators of cell proliferation [15–17a]. Early studies in fibroblasts have established opposite roles for different nPKCs, as PKCδ overexpressors grow very slowly and PKCε overexpressors have uncontrolled growth and form tumors in nude mice [18]. The specialized roles of each PKC relate to their differential localization and access to substrates upon activation, which ultimately confers pathway selectivity.

The specialization of PKC isozymes in the context of cancer can also be observed in vivo. In that regards, skin transgenic mice models overexpressing PKCs have provided valuable information. For example, transgenic mice overexpressing PKCε in the skin develop carcinomas irrespective of prior

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