Current Cancer Research

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Protein Kinase C in Cancer Signaling and Therapy

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Part I
Regulation of PKC Isozyme Function: From Genes to Biochemistry

Chapter 1
Protein Kinase C in Cancer Signaling and Therapy: Introduction and Historical Perspective

Alex Toker

Keywords Protein Kinase C • Diacylglycerol • Phorbol ester • Signal transduction

The Protein Kinase C (PKC) signal relay pathway represents one of the best understood mechanisms by which extracellular signals elicit cellular responses through the generation of lipid second messengers. Thirty years have elapsed since the late Yasutomi Nishizuka discovered an enzymatic activity that was dependent on calcium and phosphatidylserine and activated by diacylglycerol (DG) (Takai et al. 1979a). During the 1980s, biochemical studies focused primarily on elucidating the mechanisms by which DG, calcium and cofactors such as phosphatidylserine control PKC activity. During this decade, it also became evident that multiple isoforms of PKCs exist in mammals and other organisms. The 1990s saw a flurry of research which identified the mechanisms of PKC phosphorylation by autophosphorylation and also by upstream kinases. Studies also revealed substrates of PKC that transduce the lipid signal, leading to the realization that PKCs control a multitude of cellular responses and phenotypes in response to virtually all cellular agonists. Toward the end of the millennium genetic studies using homologs recombination to knock out PKC isozymes in various organisms began to unravel the specific functions of PKCs in physiology. It was not until the third decade of PKC research that all of the information that had been collected over these years could be translated into therapeutic benefit, whereby small-molecule inhibitors were developed for specific therapeutic interventions in human pathophysiologies. Much of this work is ongoing and clinical trials using PKC antagonists are yielding exciting and potentially fruitful results.

This chapter is devoted to a review of the key mechanisms that control PKC activity through lipid second messengers, phorbol esters, phosphorylation, and protein interactions. When considering the key findings in the history of PKC, it is essential

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to recognize the landmark studies by Hokin and Hokin who in the mid-1950s were the first to recognize that extracellular agonists in the form of acetylcholine could stimulate the incorporation of radiolabeled phosphate into phospholipids (Hokin and Hokin 1953, 1954). These studies gave birth to an entirely new field of lipid signaling, and subsequent studies by Michell showed that the phospholipase-mediated hydrolysis of minor membrane phosphoinositides such as $PI45P_2$ is responsible for the release of two key second messengers, DG and inositol trisphosphate (IP_3) (Lapetina and Michell 1973a, b). At the same time, Berridge, Irvine, and Schulz revealed that one byproduct of this reaction, soluble inositol phosphates such as IP_3, elicit the release of calcium from intracellular stores (Streb et al. 1983). In the late 1970s, Nishizuka and colleagues had identified a highly active protein kinase activity from rat brains that was sensitive to magnesium, and so termed it PKM. Realizing that they were probably dealing with a proteolytic fragment of a protein kinase, they subsequently purified and characterized the holoenzyme and found that it was activated by phospholipids such as phosphatidylserine. Because they found that this activity was also enhanced by the calcium-activated protease calpain, they termed it PKC (Takai et al. 1979a, b). However, they also recognized that crude preparations of phospholipids were more effective at activating this new enzymatic activity than phosphatidylserine alone, and their quest to identify the molecular species within these crude preparations informed them that diacylglycerol serves to activate PKC. The field had at this point come full circle with the elegant mechanism we are so familiar with today, whereby extracellular signals stimulate phosphoinositide-specific phospholipases inducing hydrolysis of $PI45P_2$, leading to DG and IP_3 and calcium production, which represent the rate-limiting signals required for maximal PKC activation.

An equally important landmark finding in the field was the discovery that tumor-promoting substances collectively known as phorbol esters are potent activators of PKC. Phorbol esters such as PMA (phorbol 12-myristate 13-acetate) are potent tumor promoters that are the biologically active components of the phytotoxins in the sap of the *Euphorbiaceae* family of tropical plants. Nishizuka and colleagues reported that phorbol esters bind to and activate PKC (Castagna et al. 1982), and this was made possible by the synthesis of hydrophilic versions of these compounds such as phorbol dibutyrate by Blumberg and colleagues. This also led to the identification of high-affinity binding sites on PKC that bind to both DG and PMA, suggesting that despite an altogether very different structure, phorbol esters activate PKC by molecular mimicry of DG. Immediately thereafter, Sando and Anderson found that exposure of cells to phorbol esters resulted in the rapid redistribution of PKC to the plasma membrane, leading to enzymatic activation (Kraft et al. 1982). For decades, this membrane translocation mechanism served as an effective molecular readout for the activation state of PKC. Although still in use, this biochemical readout has given way to genetically encoded FRET-based biosensors that report on the activation of PKC in intact cells (Violin et al. 2003).

The next major landmark finding in the field was the sequencing and cloning of the first PKC isoform, termed the major phorbol ester receptor, made by Parker, Waterfield and colleagues in the mid-1980s (Parker et al. 1986). This coincided

with the identification and cloning of the PKCα, PKCβ, and PKCγ isoforms (Coussens et al. 1986), eventually leading to the realization that there exist 10 distinct mammalian PKC isoforms expressed with varied distribution and abundance in cells and tissues. The notion that multiple PKC isoforms exist was actually first realized by Huang and colleagues who detected multiple isozymes of the calcium- and phospholipid-activated PKC in rat brains (Huang et al. 1986). The cloning of PKC revealed a conserved catalytic kinase domain most similar to that of the protein kinase A, but a unique regulatory domain comprising two copies of the C1 domain (C1a and C1b) which represents the ligand (DG)-binding site, a C2 domain which coordinates calcium binding, and an amino-terminal region called the pseudosubstrate because it comprises the optimal PKC phosphorylation consensus sequence except that the phospho-acceptor is replaced by an alanine. The PKC family is now classified into: conventional isoforms PKCα, PKCβI and the alternative splice variant PKCβII, and PKCγ that are activated by DG, calcium and phosphatidylserine; novel PKC isoforms PKCδ, PKCε, PKCη, and PKCθ that are activated by DG and phosphatidylserine but are calcium-insensitive; and atypical PKC isoforms PKCζ and PKCι/λ that are both DG- and calcium-insensitive. Although an additional family member was identified and termed PKCμ, it was subsequently reclassified into the PKD family of kinases because the catalytic kinase core of PKD is more similar to calcium- and calmodulin-dependent protein kinases that it is to PKCs. A series of elegant biochemical and biophysical studies in the 1980s and into the early 1990s by Newton, Parker, Nishizuka, Blumberg and other laboratories provided exquisite detail into the mechanisms of the molecular activation of PKC isoforms by DG, calcium, and phosphatidylserine. These studies revealed that the two membrane targeting modules of PKCs, the C1 and C2 domains, serve to position PKC at the inner leaflet of the plasma membrane, whereby the C1 and DG interaction leads to an increase of the enzyme for anionic phospholipids such as phosphatidylserine, and in turn calcium facilitates the interaction of the C2 domain with the same anionic phospholipids. The chapters by Newton and also by Kazanietz and Merida review in detail the role of calcium, DG, and anionic phospholipids in PKC activation. Equally important was the finding that C1 domains also bind phorbol esters such as PMA and PDBU, but an important distinction exists between phorbol ester and DG binding to C1 domains. Phorbol esters bind to C1 domains with several orders higher affinity than does DG, and thus in cells exposed to PMA, PKC is recruited and retained on membranes with different temporal kinetics than is observed with DG as the physiological ligand that is transiently released following $PI45P_2$ hydrolysis. Blumberg reviews the mechanisms of phorbol ester activation of PKCs in his chapter.

In 1989, the Fabbro laboratory was the first to report the posttranslational modification of PKC in the form of phosphorylation (Borner et al. 1989). Subsequent studies by Newton and other laboratories revealed three key phosphorylation sites in the catalytic domain of PKCs that are highly conserved (Keranen et al. 1995). It was later found that phosphorylation of these three key sites, known as the activation loop residue, turn motif and hydrophobic sites, is required for maximal PKC activity in cells. Biochemical and cell-based assays showed that the two

carboxyl-terminal sites, the turn motif and hydrophobic sites, are regulated by an intermolecular autophosphorylation reaction in conventional PKCs, but it also became clear that the activation loop, whose phosphorylation is absolutely required for maximal PKC activity in cells, was catalyzed by an upstream kinase. It was not until Alessi and Cohen identified the PDK-1 (Phosphoinositide-Dependent Kinase-1) enzyme as the upstream kinase that phosphorylates the equivalent motif on the related AGC kinase family member Akt/PKB (Alessi et al. 1997), that the Toker, Parker, and Newton laboratories went on to show that PDK-1 also phosphorylates the activation loop residue in all PKC family members (Chou et al. 1998; Dutil et al. 1998; Le Good et al. 1998). Very recent studies by Sabatini and colleagues have added further complexity to the model of PKC phosphorylation, suggesting that in addition to autophosphorylation, the mTor protein kinase in the TORC2 complex can also catalyze phosphorylation of the carboxyl-terminal residues in PKCs (Sarbassov et al. 2004). The regulation of PKC phosphorylation is discussed in detail in the chapter by Newton.

Finally, the scaffolding of PKC isoforms in proximity to both substrates as well as upstream activators such as DG and phosphatidylserine is critical for efficient signal relay. In the early 1990s, Mochly-Rosen and colleagues were the first to identify a family of proteins that interact with the active conformation of PKC (Mochly-Rosen et al. 1991). For this reason, they termed them RACKS (Receptors for Activated C KinaseS) and, in a series of studies, revealed the mechanism by which activated PKCs directly bind with RACKS and position them to discrete locations, thus facilitating downstream signaling. Identification of the PKC:RACK binding sites also permitted the generation of specific peptides which could be introduced into cells, thus uncoupling the interaction and terminating PKC signaling. Subsequently, other PKC scaffolding proteins such as STICKS (Substrates That Interact with C Kinase) as well as 14-3-3 proteins were found to directly bind PKC isozymes, thus providing additional regulation in downstream signal relay. Similarly, Scott and colleagues found a separate family of proteins terms AKAPs (A Kinase Anchoring Proteins) that act as true scaffolds because they directly assemble signaling complexes comprising kinases such as PKA and PKC, as well as phosphatases such as calcineurin (Klauck et al. 1996). A number of AKAPs are now known to spatially and temporally coordinate the assembly of PKC isoforms in discrete cellular locations, thus ensuring both efficiency and specificity in signal transmission. The regulation of PKC activation and downstream signaling by adapter proteins is covered in the chapter by Mochly-Rosen.

In summary, a number of landmark findings in the 30 years since the discovery of PKC by Nishizuka have propelled this field into the signaling limelight time and time again. Ultimately, this made it possible to begin to investigate the relevance and importance of PKC isoforms in pathophysiology, which in turn facilitated the development of chemical inhibitors designed to attenuate PKC activity in clinical settings. The following chapters are authored by investigators who made seminal contributions in the field of PKC and lipid signaling and they discuss in detail the major mechanisms of PKC activation by lipid second messengers, phorbol esters, phosphorylation, and scaffolding proteins.