1

The Ins and Outs of Protein Kinase C

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1. Protein Kinase C

The seminal discovery of protein kinase C (PKC) by Nishizuka and co-workers ***(1)*** in the late 1970s provided the first chapter in the story of one of the most studied enzymes in biology. The subsequent finding that PKC transduces signals that cause lipid hydrolysis, followed shortly thereafter by the discovery that PKC was the long sought-after receptor for the potent tumor-promoting phorbol esters, catapulted PKC to the forefront of research on signal transduction. More than 35,000 research articles have been published on PKC. An abundance of reviews describe the structure, regulation, and biological function of PKC and the interested reader is referred to these for a more detailed overview than the brief one provided below ***(1–11)***. The goal of this volume is to present a lab manual on classic and novel techniques that are currently used for studying PKC.

2. PKC Primer

PKC comprises a family of serine/threonine kinases whose members play a pivotal role in cell signaling. Specifically, PKC isozymes transduce the myriad of signals that produce lipid second messengers. Examples of physiological stimuli that activate PKC are mitogens, which operate through tyrosine kinase receptors, or catecholamines, which operate through G protein-coupled receptors. PKC can also be hyperactivated by treating cells with phorbol esters (*see* Chapter 34). These molecules bind PKC over two orders of magnitude more tightly than diacylglycerol, and coupled with their resistance to metabolism, cause essentially constitutive activation of PKC.

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There are three subclasses of PKCs, which have in common an amino-terminal regulatory domain linked to a carboxyl-terminal kinase domain (**Fig. 1**). The regulatory domain comprises at least one membrane-targeting module that directs PKC to the membrane in response to generation of lipid second messengers (*see* Chapter 8). Engaging these modules on the membrane activates PKC by providing the energy to release an autoinhibitory pseudosubstrate sequence from the substrate-binding cavity in the kinase domain, thus activating the enzyme (*see* Chapter 5). Conventional (α, βI, βII, γ) and novel (δ, ε, η/L, θ) PKCs are allosterically regulated by diacylglycerol, which binds to the C1 domain (this domain is a tandem repeat in most isozymes; *see* **Fig. 1**). Conventional isozymes are under additional control by Ca^{2+}, which binds to the C2 domain and promotes its interaction with anionic phospholipids. Although novel PKCs contain this domain, the Ca^{2+} binding pocket lacks essential aspartates involved in coordinating Ca^{2+} and thus does not bind Ca^{2+}. Atypical PKCs (ζ, ιλ) contain a single membrane-targeting module, the C1 domain, but the ligand-binding pocket is compromised so that it is unable to bind diacylglycerol. The function of this domain in atypical PKCs is not known. In addition to second messenger regulation, the activity of all isozymes of PKC is stimulated by phosphatidylserine: this lipid participates in anchoring the C1 domain to the membrane. The structures of the isolated C1 and C2 domains have been solved; that of the kinase domain has been modeled based on the crystal structure of the related protein kinase A (*see* Chapter 23).

Before PKC is competent to respond to second messengers, it must first be phosphorylated at three conserved positions in the kinase core (*see* Chapter 13). The first phosphorylation is catalyzed by the recently discovered phosphoinositide-dependent kinase, PDK-1 ***(12)***. For conventional and novel PKCs, this phosphorylation is constitutive and part of the maturation process of the enzyme. It serves to correctly position residues in the active site for catalysis without directly activating the enzyme. Activation requires removal of the pseudosubstrate from the active site, which depends on the second messenger-mediated membrane translocation. In contrast, the phosphorylation of atypical PKCs is under moderate regulation by 3′ phosphoinositides. These isozymes do not appear to be allosterically regulated by second messengers, and it may be that phosphorylation by PDK-1 serves as the direct on/off switch for enzymatic activity.

The function of PKC is exquisitely sensitive to its subcellular location (*see* Chapter 26). This location is not only dictated by the protein:lipid interactions described above but also by protein:protein interactions. A variety of anchoring proteins for PKC have been described. These proteins tether both inactive and active PKC at specific intracellular locations. A striking example of the importance of such scaffold proteins is in the *Drosophila* phototransductive

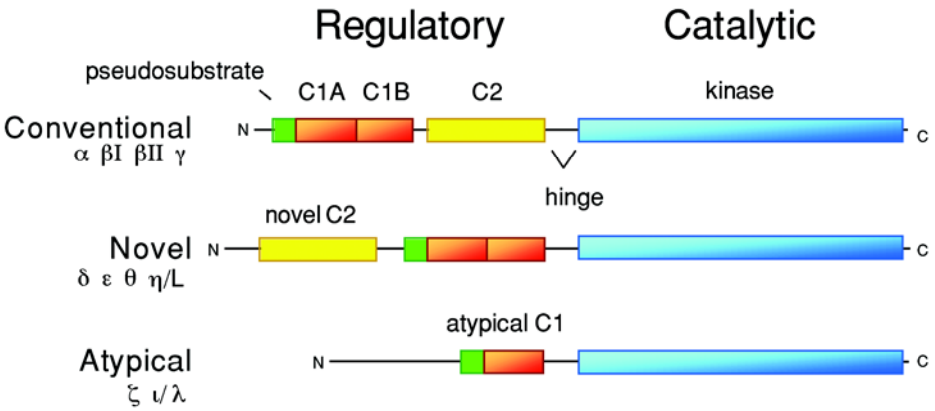

Fig. 1. Schematic showing the domain structure of the conventional, novel, and atypical subclasses of PKC. Indicated are the pseudosubstrate, C1 and C2 domains in the regulatory moiety, and the carboxyl-terminal kinase domain. Note that the kinase domain is sometimes subdivided into C3 and C4 domains representing the ATP-binding and substrate-binding lobes of the kinase (e.g., *see* **Fig. 3** in Chapter 32). Adapted from **ref. *9***.

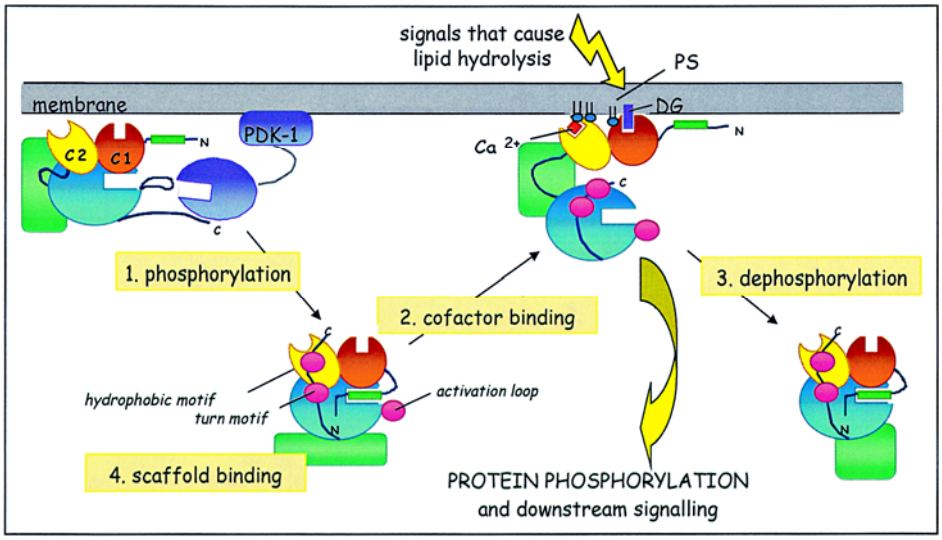

Fig. 2. Model illustrating how the function and subcellular location of protein kinase C is under the coordinated regulation of 1. phosphorylation mechanisms, 2. cofactor binding, 3. activation-dependent dephosphorylation mechanisms, and 4. binding to scaffold proteins. Pink circles represent phosphorylation sites. The upstream kinase, PDK-1, is shown in purple. Shaded green boxes represent scaffold proteins for the various activation states of protein kinase C. Note that engaging the C2 (yellow) and C1 (orange) domains on the membrane locks PKC in the active conformation. In this conformation, the pseudosubstrate (green rectangle) is removed from the substrate-binding cavity in the kinase domain (blue circle) allowing substrate phosphorylation and down-stream signaling.

cascade. In this system, components of the signaling cascade are coordinated on a single scaffold, the inaD protein. Disruption of the interaction of PKC with the scaffold abolishes signaling through this pathway.

Once activated, PKC phosphorylates an abundance of downstream substrate proteins that regulate many distinct cellular processes (*see* Chapter 20). It should be noted, however, that despite two decades of research on PKC, a unifying signaling mechanism centered on PKC has remained elusive. Nonetheless, some themes are emerging. For example, various PKC family members, in particular PKCε and PKCζ have been shown to regulate cell growth and gene transcription by activating the mitogen-activated protein kinase pathway. Similarly, PKC has been implicated in cellular differentiation, such as neurite extension in the rat pheochromocytoma cell line PC12. Activated PKC is very sensitive to dephosphorylation and prolonged activation results in dephosphorylation and eventual proteolysis, a process referred to as downregulation.

The study of PKC has been greatly helped by the use of pharmacological probes, most notably phorbol esters (*see* Chapter 32). Genetic approaches have also provided much insight into the function of PKC (*see* Chapter 36).

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2

Early Studies of Protein Kinase C

A Historical Perspective

Yasutomi Nishizuka and Ushio Kikkawa

1. Introduction

The covalent attachment of phosphate to either seryl or threonyl residues of proteins was identified first by F. Lipmann and P. A. Levene at the Rockefeller Institute for Medical Research (Rockefeller University, New York) in 1932; these researchers were interested in the chemical nature of acidic macromolecules present in the cell nucleus (paranucleic acid was the term used by Levene). This nuclear material was presumably a mixture of what we now call transcription factors. The enzyme responsible for this protein modification, casein kinase (phosvitin kinase), was subsequently found by M. Rabinowitz and Lipmann, but no obvious function was assigned for this enzyme. Another line of study focusing on glycogen metabolism initiated by C. F. Cori and G. T. Cori at St. Louis in the early 1940s, and by eminent investigators, such as E. W. Sutherland, T. W. Rall, E. G. Krebs, E. Fischer, and J. Larner, clarified the role of reversible phosphorylation in controlling the breakdown and resynthesis of glycogen.

In the mid-1960s, Y. Nishizuka spent 1 year as an NIH International Postdoctoral Research Fellow in the laboratory of Lipmann to work on the elongation factors of protein synthesis in *Escherichia coli*. Discussions there about a possible relationship between nuclear phosphoproteins and bacterial adaptive enzymes induced by cyclic AMP sparked Nishizuka's lifelong interest in protein kinases in hormone actions. At the end of the 1960s, when Nishizuka moved to Kobe, Krebs and his colleagues announced that cyclic AMP activates glycogen phosphorylase kinase kinase, known as protein kinase A (PKA) today

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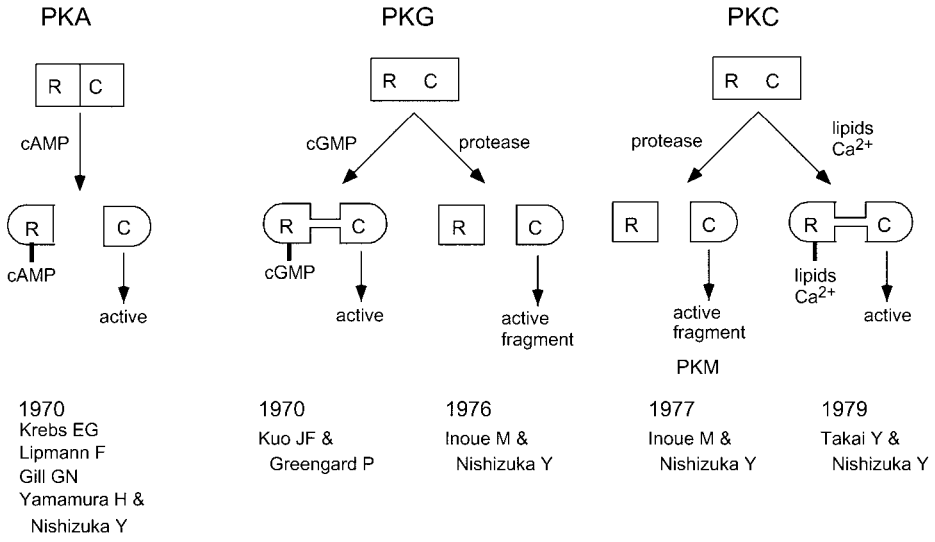

Fig. 1. Mode of activation of three protein kinases.

(1). H. Yamamura and Nishizuka at that time in Kobe isolated a functionally unidentified kinase from rat liver with histone as phosphate acceptor and confirmed that cyclic AMP greatly stimulated its catalytic activity. Soon, in 1970, four laboratories (Krebs, Lipmann, G. N. Gill, and Yamamura and Nishizuka) concurrently reported that PKA consists of catalytic and regulatory subunits and that cyclic AMP activates the enzyme by dissociating these subunits (**Fig. 1**).

The 1970s marked the initiation of several important studies of protein kinases (**Table 1**). In the case of cyclic GMP-dependent protein kinase (PKG), discovered by J. F. Kuo and P. Greengard in the brain in 1970, M. Inoue in the Kobe group found that this enzyme, unlike PKA, is a single polypeptide chain and is activated by cyclic GMP, which binds simply to its regulatory region to promote catalytic activity. They found that a constitutively active enzyme fragment insensitive to the cyclic nucleotide could be generated by limited proteolysis *(2)*. This enabled us to find a new enzyme, Ca^{2+}-activated, phospholipid-dependent protein kinase that is protein kinase C (PKC).

2. PKC and Link to Receptor

Higher levels of an active fragment named protein kinase M (PKM; M for its only known requirement, Mg^{2+}), which was assumed to be derived from PKG, were found in previously frozen rat brain compared with freshly obtained brain, where not much fragment was detected. Freezing and thawing resulted in