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Early Studies of Protein Kinase C

A Historical Perspective

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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
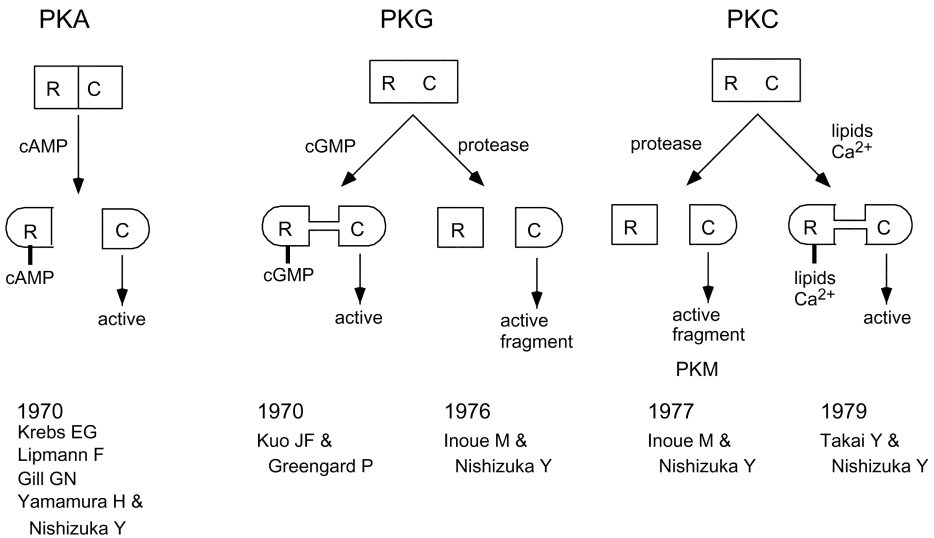
PKA   PKG   PKC

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

Table 1
Discovery of Protein Kinases (1955–1980)

Years	Researchers	Studies
1955	E. Fischer and E. G. Krebs	Glycogen phosphorylase kinase
1960	M. Rabinowitz and F. Lipmann	Casein kinase (phosvitin kinase)
1963	J. Larner	Glycogen synthetase kinase
1964	T. Langan and F. Lipmann	Histone kinase
1968	D. A. Walsh, J. P. Parkins, and E. G. Krebs	Protein kinase A
1970	J. F. Kuo and P. Greengard	Protein kinase G
1977	P. Greengard	Calmodulin-dependent protein kinase
	K. Yagi; D. G. Hartshorne	Myosin light chain kinase
	P. Cohen	Calmodulin in phosphorylase kinase
	Y. Nishizuka and colleagues	Protein kinase C
1978	R. L. Erikson	Src protein kinase
1979	H. Hunter	Protein tyrosine kinase
1980	S. Cohen	Receptor tyrosine kinase (epidermal growth factor receptor)

the appearance of the active fragment, suggesting the presence of a proenzyme susceptible to limited proteolysis, perhaps by a Ca^{2+}-dependent protease, later called calpain *(3)*. We soon noticed the existence of such a putative proenzyme in the brain; curiously, it was not sensitive to any cyclic nucleotide, but produced PKM upon limited proteolysis. Attempts to identify the protease responsible for this proteolysis led us to uncover large quantities of an activating substance associated with the membrane. It was not a protease, but simply anionic phospholipids, particularly phosphatidylserine. Even more curiously, crude phospholipids extracted from brain membranes could support activation of the enzyme in the absence of added Ca^{2+}, whereas pure phospholipids obtained from erythrocyte membranes could not produce any enzyme activation unless a higher concentration of Ca^{2+} was added to the reaction mixture (**Fig. 2**).

Analysis of the lipid impurities on a silicic acid column led us to conclude that diacylglycerol was an essential activator *(4)*. This observation suggested a critical link between protein phosphorylation and the signal-induced hydrolysis of inositol phospholipids that was described by M. R. Hokin and L. E. Hokin with acetylcholine-stimulated pancreatic acinar cells in the early 1950s *(5)*. A ubiquitous distribution of PKC in mammalian and other animal tissues was immediately confirmed by J. F. Kuo in Atlanta. Today, we know that the enzyme is distributed more widely, and it is also extensively studied for its role in yeast, Nematoda, and the fly.
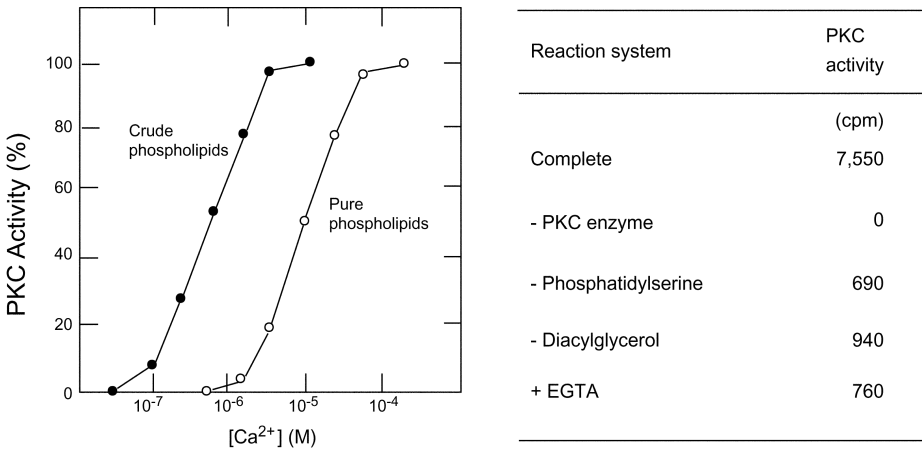
Fig. 2. Activation of PKC by phospholipids, diacylglycerol, and Ca^{2+}.

To obtain evidence that diacylglycerol is the intracellular mediator of hormone actions, we needed a method to activate PKC within intact cells. Diacylglycerols that have two long chain fatty acids could not be readily intercalated into the cell membrane. If, however, one of the fatty acids is replaced with a short chain, acetyl group, then the resulting diacylglycerol, such as 1-oleoyl-2-acetyl-glycerol, obtains some detergent-like properties and could be dispersed into the membrane lipid bilayer and could thus activate PKC directly. Incidentally, two observations reported in the literature attracted our attention. The first was the observation by S. Rittenhouse-Simmons in Boston that diacylglycerol accumulated transiently in thrombin-stimulated platelets, possibly as a result of inositol phospholipid hydrolysis. Second were the articles by P. W. Majerus in St. Louis and by R. J. Haslam in Hamilton both describing that upon stimulation of platelets with thrombin two endogenous proteins with 20- and 47-kDa molecular size became heavily phosphorylated. It was already known that the 20-kDa protein is myosin light chain and is phosphorylated by a specific calmodulin-dependent kinase. Using the fingerprint technique, we discovered that the 47-kDa protein, known as pleckstrin today, was a substrate specific to PKC both in vitro and in vivo. Thus, these two proteins served as excellent markers for the increase of Ca^{2+} and diacylglycerol-dependent activation of PKC, respectively. In the spring of 1980, we were able to show that both Ca^{2+} increase and PKC activation were essential and acted synergistically for the full activation of platelets to release serotonin *(6)*. Similarly, it was possible to show unequivocally that PKC activation is indispensable for neutrophil release reaction and T-cell activation *(7)*, establishing a link between PKC activation and receptor stimulation *(8)*. At

that time, we used Ca^{2+} ionophore to increase intracellular concentrations of this cation and did not know where Ca^{2+} may come from, although R. Michell had proposed that inositol phospholipid breakdown may open Ca^{2+} gates *(9)*. In September 1983, at a meeting in Zeist, M. Berridge and his colleagues in Cambridge first described the important inositol 1,4,5-trisphosphate (IP_3) story *(10)*.

3. Phorbol Ester and Exploration

On July 25, 1980 in Brussels, at a garden party in Prof. H. de Wulf's home on the occasion of the Fourth International Conference on Cyclic Nucleotides, Nishizuka had an exciting time discussing with M. Castagna in Villejuif a potential connection between PKC activation and phorbol ester that mimics a variety of hormone actions. Castagna had spent the previous summer in the laboratory of P. Blumberg, who was then at Harvard in Boston, where the phorbol ester receptor was characterized. In August 1981, Castagna joined us in Kobe to test whether PKC had any connection to phorbol ester action. In contrast to usual carcinogens that bind to DNA to produce mutations, phorbol ester appeared to bind to a membrane-associated receptor, eventually leading to gene expression, differentiation, and proliferation *(11,12)*. We had already established experimental systems that were needed for testing whether phorbol ester could cause inositol phospholipid hydrolysis to activate PKC. However, it was extremely disappointing to find that in platelets phorbol ester did not show any evidence of producing diacylglycerol. Instead, this tumor promoter induced remarkable phosphorylation of the endogenous 47-kDa protein. We interpreted this to mean that our already published idea that diacylglycerol is the mediator for PKC activation was not correct.

During a sleepless night, reading the review article written by Blumberg *(11,12)*, an idea occurred: what if phorbol ester could activate PKC directly because it contains a diacylglycerol-like structure very similar to the membrane-permeant lipid molecule that we had used (**Fig. 3**)? This revelation occurred at the end of August. A series of subsequent experiments performed that fall were able to show that phorbol ester mimicked diacylglycerol action by increasing the affinity of PKC for Ca^{2+} and phosphatidylserine, thereby activating the enzyme directly, eventually leading to cellular responses *(13)*. In March 1982, these results were presented at the UCLA-NCI Symposium, Evolution of Hormone Receptor System, organized by R. A. Bradshaw and G. N. Gill in Squaw Valley. This talk under the chair of G. Todaro from NCI obviously attracted great attention, and the results were confirmed immediately. In the following year, several groups of investigators showed that PKC and the phorbol ester receptor can be copurified (J. E. Niedel, P. Blumberg, J. J. Sando, and C. L. Ashendel), and U. Kikkawa described the stoichiometric binding of

$$CH_2-O-\overset{\overset{\displaystyle O}{\|}}{C}-(CH_2)_7-CH=CH-(CH_2)_7-CH_3$$

$$CH-O-\overset{\overset{\displaystyle O}{\|}}{C}-CH_3$$

$$CH_2OH$$

Diacylglycerol-like structure

1-Oleoyl-2-acetyl-glycerol

Membrane permeant Diacylglycerol
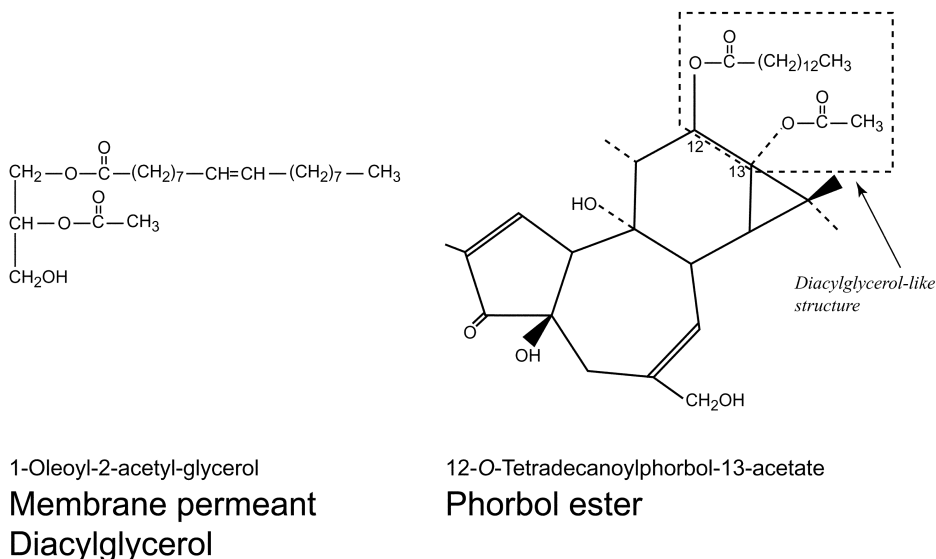
12-*O*-Tetradecanoylphorbol-13-acetate

Phorbol ester

Fig. 3. The structure of synthetic diacylglycerol and phorbol ester. The chemical structure of the tumor promoter was previously identified by E. Hecker and B. L. Van Duuren in the late 1960s.

phorbol ester to PKC using the enzyme in a pure form *(14)*. Before long it was shown that phorbol ester could cause translocation of PKC from the cytosol to the membrane (W. B. Anderson). As a result, the traditional concept of tumor promotion proposed originally by I. Berenblum from Oxford as early as 1941 had been replaced by an explicit biochemical explanation that centered on understanding the role of PKC. Phorbol esters and membrane-permeant diacylglycerols, including dioctanoylglycerol, later developed by R. M. Bell, have since been used as crucial tools for the manipulation of PKC in intact cells and have allowed the wide range of cellular processes regulated by this enzyme to be determined *(15)*. It was realized much later, however, that phorbol ester can bind to other cellular proteins, such as chimaerin *(16)* and RasGRP3 *(17)*, and potentially affect cell functions through additional targets.

4. Structural and Functional Diversity

A more detailed molecular understanding of PKC came after the cloning and sequencing of the enzyme in the mid-1980s. On the occasion of the J. Folch's Memorial Colloquium on Inositol Phospholipids, organized by J. N. Hawthorne at Nottingham in September 1981, P. Cohen introduced P. Parker in his laboratory at Dundee to Nishizuka. Since then, their paths crossed frequently and, in October 1985, after a seminar on PKC, at the laboratory

of M. Waterfield in the Ludwig Institute London, Parker and Nishizuka both agreed that PKC may not be a single entity because the enzyme often shows double, sometimes triple bands upon gel electrophoresis. Complete sequences of several isoforms thus appeared from both laboratories in the next year *(18,19)*. We now know that the PKC family consists of at least 10 isoforms encoded by nine genes. The structural feature and several functional domains of each isoform are well investigated as repeatedly documented in excellent reviews *(20–22)*. In addition, protein kinases that share kinase domains closely related to the PKC family have been isolated and characterized *(23)*.

It has also become clear in the last decade from numerous investigations in many places, especially those led by A. Newton in San Diego and by Parker in London, that the mechanism of activation of the PKC family enzymes is far more complicated than we initially thought. Newton has shown elegantly that the newly synthesized PKC appears inert and is matured by phosphorylation by itself and also by other kinases, including phosphoinositide-dependent kinase 1 *(22)*. Thus a cross-talk has emerged between the signal pathways of inositol phospholipid hydrolysis and phosphatidylinositol 3-kinase activation, which was described first by L. Cantley in Boston 10 years ago *(24)*. Another cross-talk for PKC activation through tyrosine phosphorylation of the enzyme molecule is becoming clearer *(25)*.

The catalytic and functional competence of the isoforms appears also to depend on the specific intracellular localization of each, namely their transloca-tion or targeting to particular compartments, such as plasma membrane, Golgi complex, and cell nucleus, as directed by lipid mediators. In other words, targeting appears to represent the activation and isoform-specific functions at the site of destination. Curiously, such targeting is sometimes oscillating. The dynamic behavior of the PKC isoforms was visualized first by N. Saito in our Kobe group with the enzymes fused to green fluorescent protein *(26)*. The structures of some of these targeting domains in the enzyme molecule have been clarified *(27)*.

Indeed, it has been a while since we proposed phospholipase A_2 as an additional player in signal transduction because free fatty acids, especially arachidonic acid, and lysophospholipids are frequently synergistic with diacylglycerol to activate PKC both in vitro as well as in vivo systems *(28)*. It is plausible then that, in addition to diacylglycerol, many of the lipid products produced transiently in membranes by the action of phospholipases A_2 and D, sphingomyelinase, and phosphatidylinositol 3-kinase also play key roles in the translocation and targeting of PKC family members and related protein kinases. These lipid products could direct PKC to distinct intracellular compartments to perform their specific biological functions, for example by producing so-called lipid rafts through lipid–protein and/or protein–protein interactions.

Such a fascinating idea was first proposed by D. Mochly-Rosen *(29)* and S. Jaken *(30)*.

5. Coda

For many years, the cell membrane was thought to be a biologically inert entity that splits the exterior and interior cellular compartments. Inositol was recognized as a constituent of plant tissues in the 19th century, but was found in the mammalian brain by D. W. Woolley in 1941. The following year, J. Folch and Woolley at Rockefeller University in New York described the chemical structure of inositol phospholipid. In the 60 years since then, our knowledge as to the biological role of membrane lipids as well as of protein kinases in cell-to-cell communication has expanded enormously, as briefly described above. More developments in the PKC story will certainly be unveiled in the future, especially for medical and therapeutic usage; for example, for treatment of cancer (A. Fields) and diabetes (G. King).

Acknowledgments

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