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Discovery and Prospect of Protein Kinase C Research: Epilogue

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A tale of protein kinases

Biological materials containing phosphorus are the subject of renewed interest for biochemists since late in the 19th century. Pheobus Levene, who had been a pupil of the great German chemist Emil Fischer, working at the Rockefeller Institute for Medical Research, New York, found a class of acidic macromolecules present in the cell nucleus. This material, containing a large amount of phosphorus and nitrogen, was not nucleic acid that had been previously described by Friedrich Miescher. He called this material vitellin or paranucleic acid which, in retrospect, was a mixture of highly phosphorylated transcription factors and other nuclear proteins. In 1932, he and Fritz Lipmann found that the phosphate was covalently attached to serine residues of the proteins (*1*). At this time they anticipated if the serine-phosphate could be a storage form of high energy phosphate bounds. Several decades later, enzymes capable of adding phosphate from ATP to phosvitin (egg york proteins) and casein (milk proteins) were found. However, the reaction was irreversible, and no obvious function could be assigned for the enzymes.

More physiologically relevant context of protein phosphorylation emerged from studies of hormone actions, initiated by Carl Cori and Gerti Cori in St. Louis who were interested in the control of blood glucose level. Work of their school members in the 1940s and early 1950s led to the conclusion that this was due to the conversion of glycogen phosphorylase from an inactive to an active form in response to epinephrine. In the mid 1950s it became apparent by Eddy Fischer and Ed Krebs that its activity was controlled by reversible phosphorylation (*2*).

In 1964, I had the opportunity to spend a year as a postdoctoral fellow in the laboratory of Fritz Lipmann in the Rockefeller Institute (today the Rockefeller University), and worked on the elongation factors of *Escherichia coli*. It was soon after Earl Sutherland proposed cyclic AMP as a second messenger of hormone action, and Marshall Nirenberg had recently announced that poly U encodes polyphenylalanine synthesis. One of the most debated topics at that time was how adaptive enzymes could be synthesized in bacteria. Cyclic AMP appeared to be needed for induction of adaptive enzymes. With Tom Langen, another postdoctoral fellow seated next to my bench, and with Vincent Allfrey, a pioneer of chromatin biochemistry, a possible relationship between the enzyme induction by cyclic AMP in prokaryotes and the phosphorylation of histone in eukaryotes was frequently discussed, and this discussion sparked my life-long interest in how protein phosphorylation mediates hormone actions. In the mid 1960s, however, no one could predict direct action of cyclic AMP on protein kinases.

Lessons from protein kinases A and G

At the end of the 1960s, when I moved to Kobe, Ed Krebs and his colleagues reported that cyclic AMP could activate glycogen phosphorylase kinase kinase, known as protein kinase A (PKA) today (*3*). Hirohei Yamamura in Kobe had isolated a functionally undefined kinase from rat liver using calf thymus histone as a phosphate acceptor, and confirmed that cyclic AMP greatly stimulated its catalytic activity. Soon he observed that, when the enzyme preparation was subjected to column chromatography, two enzyme peaks, cyclic AMP-dependent and independent, appeared, although the two peaks showed otherwise identical catalytic properties. It was noted that cyclic AMP converted the former form to the latter (*4*). Before long, in 1970, four individual laboratories of Krebs, Lipmann, Gordon Gill, and ours concurrently described that PKA consists of catalytic and regulatory subunits, and that cyclic AMP activates the enzyme by dissociating these subunits.

On the analogy of PKA, Jef Kuo and Paul Greengard in Yale at that time discovered cyclic GMP-dependent protein kinase (protein kinase G, PKG) first in arthropoda in 1970, and later in many other organisms. Masanori Inoue in our group in Kobe found that PKG, unlike PKA, was a single polypeptide chain, and was activated by binding cyclic GMP at a regulatory region within it. An inhibitory interaction between catalytic and regulatory regions of the PKG molecule was suggested since limited proteolysis with trypsin or calpain generated a constitutively active fragment of the enzyme which was no longer sensitive to cyclic GMP (*5*). This was a key observation which led to our subsequent finding of a new protein kinase, that is protein kinase C (PKC).

Discovery of protein kinase C

During analysis of mammalian PKG, we noticed that rat brains which had been stored frozen contained a very active protein kinase independent of any cyclic nucleotide. The only requirement for this enzyme was Mg^{2+} (we called it protein kinase M, PKM). Unexpectedly, this activity could not be detected in freshly sacrificed brain. Repeated freezing and thawing of fresh brain with dry ice resulted in the appearance of PKM, suggesting that it might be derived probably from PKG by limited proteolysis. Curiously, however, the activity of PKG in brain was extremely low. Before long, we found a large amount of previously unknown protein kinase which *per se* was inactive but could be converted to an active form by limited proteolysis (*6*). To explore the properties of the pro-

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tease responsible for this proteolysis, we soon found that a large amount of active principle which could activate the enzyme was associated with membranes, but all attempts to purify it were unsuccessful. Finally, we realized that this was because the principle was not a protein, but simply membrane phospholipids, especially phosphatidylserine. Even more curiously, our phospholipid preparation extracted from the brain membranes supported activation of the enzyme in the absence of added Ca^{2+}, whereas authentic pure phospholipids could not elicit any enzyme activation unless an unusually high concentration of Ca^{2+} was added to the reaction mixture. Analysis of the impurities in the brain phospholipid preparation by Yoshimi Takai led to the conclusion that diacylglycerol was an indispensable activator (*7*). Namely, the enzyme could be activated, without limited proteolysis, by diacylglycerol in the presence of phospholipids and very low concentrations of Ca^{2+}, thus we call it PKC (C stands for its requirement of Ca^{2+}). This finding definitively suggested a critical link of protein phosphorylation to the hormone-induced hydrolysis of inositol phospholipids, which was described by Mabel Hokin and Low Hokin with acetylcholine-stimulated pancreatic acinar cells in the early 1950s (*8*).

Diacylglycerol as signal mediator

To obtain the proof for diacylglycerol as the physiological mediator of hormone actions, a method was needed to activate PKC by introducing this neutral lipid into intact cells. Natural diacylglycerols that have two long fatty acid chains could not be readily intercalated into the cell membrane. We found, however, that if one of the fatty acid chains was replaced with acetyl group, then the resulting diacylglycerol such as 1-oleoyl-2-acetylglycerol (OAG) is sufficiently water-soluble to allow it to be dispersed into the membrane lipid bilayer, where it activates PKC directly. In the meantime, several reports in the literature had attracted our attention and suggested an excellent experimental cell system. Susan Rittenhouse-Simmons in Harvard, Boston showed that in thrombin-stimulated platelets, diacylglycerol accumulated transiently, possibly as a result of inositol phospholipid hydrolysis. On the other hand, Phil Majerus in St. Louis and Dick Haslam in Hamilton, Canada independently reported that upon stimulation of platelets with thrombin, two endogenous proteins with 20 and 47 kDa molecular size were heavily phosphorylated. It was recently known that the 20 kDa protein is myosin light chain, and is phosphorylated by a specific calmodulin-dependent kinase in response to the increase in the intracellular Ca^{2+} concentration. In contrast, the enzyme responsible for phosphorylation of the 47 kDa protein had not been identified. We soon found that this protein, but not 20 kDa protein, was heavily phosphorylated by adding the membrane-permeant diacylglycerol, OAG. With fingerprint analysis of the radioactive 47 kDa protein preparations that were phosphorylated in *in vivo* and *in vitro* systems, this protein was shown to be a specific PKC substrate, later called pleckstrin. Thus, the 20 and 47 kDa proteins served as excellent endogenous markers for the increase of Ca^{2+} and the diacylglycerol-dependent activation of PKC, respectively. In the spring of 1980, my colleagues, Yasuhiro Kawahara and others, were able to show that both Ca^{2+} increase and PKC activation were essential, and synergistically acted for full activation of platelets to release serotonin (*9*). Similarly, Kozo Kaibuchi showed unequivocally that PKC activation is indispensable for neutrophil-release reaction and T-cell activation (*10*). At this time we used a Ca^{2+} ionophore to increase intracellular concentrations of this cation, and did not know where Ca^{2+} comes from physiologically, although Bob Michell had postulated that inositol phospholipid hydrolysis could open a Ca^{2+} gate (*11*). On the way back from a discussion meeting at the Royal Society, London in December 1982, I stopped at Cambridge UK and met Mike Berridge. In a guest room in Trinity College we certainly discussed possible origins of Ca^{2+}, but this important question remained to be answered. In the fall of the next year, Mike and his colleagues presented a piece of evidence, at a small meeting in Zeist, Holland, that inositol 1,4,5-trisphosphate, the other product of the inositol phospholipid hydrolysis, could mobilize Ca^{2+} from its internal store (*12*). The traditional bifurcating pathway of signal flow from the cell surface into the cell interior through PKC activation and Ca^{2+} mobilization emerged in this way in the early 1980s.

Phorbol ester and cell signaling

In the first issue of *Cancer Research*, published in 1941, Isac Berenblum stated that "croton oil causes a marked augmentation of cartinogenesis when applied at weekly intervals to the skin of mice in conjunction with a very dilute solution of benzpyrene in acetone." The oil was obtained from the seeds of croton, and contained phorbol ester, a powerful tumor promoter. When applied to the cell, it elicited a wide variety of responses that were similar to those of hormones. A number of kinetic studies with various cell types had suggested that the primary site of its action could be its own receptor located on cell surface membranes.

In the summer of 1980, I attended the 4th International Conference on Cyclic Nucleotides in Brussels, and was invited to an evening garden party at Prof. H. de Wulf's home, where Monique Castanga, a French oncologist in Villeguif, Paris was also invited. She had spent the previous summer in the laboratory of Peter Blumberg in Harvard, Boston who had characterized the phorbol ester receptor. Monique and I discussed a possibility that phorbol ester could activate PKC through inositol phospholipid hydrolysis producing diacylglycerol. In the next summer, 1981, Monique came to Kobe for one month. We had already established the experimental system, platelets, that was required for testing if phorbol ester could produce diacylglycerol to activate PKC, and mimic the thrombin action.

The result, however, was very disappointing to us, because, in platelets, phorbol ester did not show any sign of producing diacylglycerol, nevertheless did produce remarkable phosphorylation of the endogenous 47 kDa protein and, in the presence of Ca^{2+} ionophore, it induced massive release of serotonin. This result meant to us that our already published idea that diacylglycerol is the messenger for the receptor-mediated PKC activation was not correct. I was, of course, extremely upset, particularly

because I had been invited to speak our idea at a meeting in Nottingham UK in the middle of the forthcoming September.

One sleepless night after this result, reading the review article of tumor promotion written by Blumberg (*13*), I noticed, to my surprise, that the phorbol ester contains a diacylglycerol-like structure in its molecule which is very similar to the membrane-permeant diacylglycerol, OAG, that we routinely used. What if phorbol ester could activate PKC directly. This revelation occurred at the end of the August. A series of subsequent experiments that fall was able to show that phorbol ester mimics diacylglycerol action and activates PKC directly, eventually leading to cellular responses (*14*). The results were confirmed immediately by scientific communities, and in the following year several groups of investigators showed that PKC and the phorbol ester receptor can be co-purified, and Ushio Kikkawa in Kobe found that the stoichiometric binding of phorbol ester to PKC using the enzyme in a pure form. As a result, the traditional concept of tumor promotion originally proposed by Berenblum from Oxford UK has been replaced by an explicit biochemical explanation that centered around understanding the role of PKC. Along this line of studies, phorbol esters and membrane-permeant diacylglycerol have been used as crucial tools for the manipulation of PKC in intact cells, and allowed the wide range of cellular processes regulated by this enzyme to be determined (*15*). It was realized much later that phorbol ester can bind also to other proteins such as chimerin and RasGRP, and potentially may affect cell functions through additional targets (*16*).

Molecular heterogeneity and mode of activation

Our discovery of PKC and phorbol ester action in the early 1980s definitively established a role of PKC in cellular signal transduction, and studies that focused on PKC rapidly became widespread in many research fields of physiology and medicine (*17*). However, we did not know how many isoforms might exist, and how many signaling events it might be involved in.

After the meeting at Nottingham UK in 1981 mentioned above, Phil Cohen invited me to Dundee, Scotland where he introduced me Peter Parker in his laboratory. Peter was interested very much in PKC action and tumor promotion. Since then, our paths crossed frequently, and in October 1985 after my seminar at the laboratory of Michael Waterfield in the Ludwig Institute, London, we agreed that PKC might not be a single entity, because the enzyme shows often double, occasionally triple bands upon gel electrophoresis. Complete sequences of several isotypes thus reported from both laboratories in the next year (*18*, *19*). Today, we know that the mammalian PKC is a large family of serine/threonine protein kinases consisting of 10 isotypes encoded by 9 genes (*20*). The PKC isotypes are conserved in various species including yeast, nematoda, fly, fish, and frog. The conserved region of the proteins is a serine/threonine protein kinase domain that is located in the C-terminal half of each isotype, and shows very similar, if not identical, catalytic properties. On the other hand, the N-terminal half of the enzyme molecule contains multiple characteristic functional and activation domains, as repeatedly described in detail in this series of reviews. In addition, in the last decade, several protein kinases which share kinase regions closely related to the PKC family have been isolated and characterized (*21*). These include protein kinase N (PKN of PRK), protein kinase B (PKB, Akt or rac-PK), and protein kinase D (PKD of PKCμ).

In parallel with these studies, the mechanism of activation of the PKC family has been investigated extensively, and it became far more complicated than was initially assumed. Alexandra Newton has shown elegantly that newly synthesized PKC molecules are catalytically inert, and seem to mature by phosphorylation by itself and by other protein kinases including PIP_2-dependent protein kinase 1, PDK-1 (*22*). Thus, a cross-talk has emerged between the signaling pathway starting from inositol phospholipid hydrolysis and the phosphatidylinositol 3-kinase pathway which was described first by Lew Cantley in the mid 1980s (*23*). Another cross-talk involving PKC activation through tyrosine phosphorylation in the enzyme molecule is becoming also clearer (*24*). Perhaps, covalent attachment and detachment of phosphate to the PKC molecule by various kinases and phosphatases may profoundly affect its intracellular localization as well as its targeting to distinct cellular compartment where it plays specific function.

Targeting and multiple lipid mediators

The specific function of individual PKC isotype has been the subject of great interest. For many years it had remained unclear whether the PKC isotypes show functionally redundancy or functional specialization, because the isotypes are catalytically indistinguishable from one another in *in vitro* enzymatic reactions. However, extensive studies in the last decade have shown that most of the PKC isotypes exhibit specific roles in cellular regulation as described in detail in this series of reviews.

Considerable evidence has accumulated supporting the concept that the protein components of signaling cascades are organized into complexes through their ordered association with scaffolding, adaptor or chaperone proteins. Such juxtaposition of signaling proteins and their substrates may facilitate their tight regulation, functional specialization, and their compartmentalization within the cell. As described earlier (*25*), the PKC isotypes exhibit distinct pattern of tissue expression and intracellular localization. It is becoming clearer that, upon stimulation of cell surface receptors, each PKC isotype is translocated or targeted to particular intracellular compartments, such as the plasma membrane, Golgi complex, mitochondria, cell nucleus, and cytoskeleton. Destination of the enzyme may be directed by lipid mediators, and perhaps also by phosphorylation. It depends on the structure of various functional domains in the N-terminal region of the PKC molecule. Some of the structural properties of such lipid-protein interaction are described by Jim Hurley (*26*).

Early studies in this laboratory and others have shown that several lipids transiently produced in membranes after receptor stimulation may take part in activating PKC (*27*). These lipids include free fatty acids, especially arachidonic acid, phosphatidic acid, lysophospholipids, phosphatidylinositol-polyphosphates, and ceramide in addi-

tion to diacylglycerol. Exogenous addition of each lipid alone or in combination exhibits different patterns of translocation of PKC isotypes with distinct destination. Curiously, such translocation sometimes shows oscillation, back and forth from the cytoplasm to the membrane. The dynamic behavior of the PKC isotypes was first visualized by Naoaki Saito in our group using the enzyme molecule fused to green fluorescent protein (*28*). It is attractive to surmise then that various enzymes which react with phospholipids and sphingolipids may all play roles in transmembrane control of cellular events.

Coda

Our knowledge of the PKC family has expanded enormously. Through a fine intracellular signaling network, the enzymes play pivotal roles in the control of diverse biological events, including release and exocytosis ranging up to cell growth and morphogenesis. Several decades ago, the membrane was generally viewed as a biologically inert entity that provides semipermeable barrier between exterior and interior compartments within and between cells. Signal-induced change of membrane lipids, however, seems extremely dynamic, spatially and temporally, and may profoundly affect cellular functions through interaction with various domains of the signaling molecule, such as PKC. Further exploration of the lipid-protein interaction at various intracellular compartments is an attractive research field to understand more the molecular basis of a wide range of biological regulation.

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