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JB Reflections and Perspectives

Yasutomi Nishizuka: Father of protein kinase C

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It is 6 years since we received the tragic news that our great mentor Dr Yasutomi Nishizuka passed away suddenly at the age of 72 years. Dr Nishizuka made several epoch-making discoveries in his life, *e.g.* the tryptophan metabolism, protein synthesis, ADP-ribosylation, regulation of cAMP-dependent protein kinase and discovery of protein kinase C (PKC). Among them his name will be remembered for a long time as a father of PKC, momentous discoveries in the twentieth century, which are still actively pursued by many laboratories worldwide.

Keywords: diacylglycerol/inositol turnover/lipid mediators/phorbol ester/protein kinase C.

Abbreviations: aPKC, atypical protein kinase C; cPKC, conventional protein kinase C; nPKC, novel protein kinase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; PKG, cGMP-dependent protein kinase; OAG, 1-oleoyl-2-acetylglycerol.

Dr Yasutomi Nishizuka (Fig. 1) studied a wide variety of research areas including, the tryptophan metabolism, protein synthesis, ADP-ribosylation and protein phosphorylation. He challenged the most important themes of the times and always gave strong impacts to many scientists. He evaluated scientific quality in terms of physiological relevance, *i.e.* he paid no heed to researches pursuing popularity. His great achievements were the result of his outstanding insights, his indomitable spirit and charisma that attracted followers all over the world.

Prologue—his research before the discovery of PKC

In April 1958, Dr Yasutomi Nishizuka joined, as a graduate student, the laboratory of Dr Osamu Hayaishi (Kyoto University School of Medicine) who had come back to Japan from USA with his great discovery of oxygenase. Under his guidance Dr Nishizuka started his career in science and wrote his first paper about the transaminase of alanine from pseudomonas aeruginosa (*1*). The paper that made him famous was a study which enzymatically clarified NAD synthesis from tryptophan in rat liver (*2*). This paper has been highly evaluated in the research of vitamin world.

In July 1964, he won an NIH fellowship and decided to visit Prof. F. Lipmann who discovered the importance of ATP in higher animals at Rockefeller Institute. It was a period when the mechanism of protein synthesis was being investigated. Dr Nishizuka succeeded in isolating two protein elongation factors (related to GTP) with Prof. Lipmann. They were named T factor (later separated to Tu and Ts) and G factor (possessing GTPase) (*3*). It was a very important finding not only for clarifying the mechanism of protein synthesis but also opening the world of GTP-binding proteins.

Then he returned to Dr Hayaishi's laboratory and continued with his colleagues the studies on NAD again. And they discovered a novel protein modification, *i.e.* ADP-ribosylation where NAD was a donor substrate for ADP-ribose moiety and showed that eukaryotic protein elongation factor was ADP-ribosylated by diphtheria toxin resulting in a loss of its function (*4*, *5*). This was an epoch-making discovery leading to a new research area of mono- and poly-ADP ribosylation.

In the fall of 1968, Dr Nishizuka began his studies on protein phosphorylation. The launch in this research area was a little earlier than the time Prof. E. G. Krebs and his colleagues found cyclic AMP-dependent protein kinase (PKA) (*6*). Those days it was a time of 'student unrest' all over Japan, and Kyoto University was not an exception. Students shouted for their slogan and universities were closed for months. At that time, Dr Nishizuka was chosen as the Professor of the new Department of Biochemistry, Kobe University School of Medicine. On January 1, 1969, he moved to Kobe and realized the strict fact with surprise that the laboratory was poorly equipped with biochemical instruments even such as a refrigerated centrifuge. But he made a fresh resolve again that he will continue the studies on protein phosphorylation here.

He started his research in Kobe on how protein phosphorylation mediated hormonal actions. Before long, his group was successful to resolve two enzyme fractions of PKA on column chromatography: one was cyclic AMP dependent and the other was independent while treatment of the former fractions with cAMP resulted in the conversion into the enzymatic properties of the latter (*7*). Independently and almost

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Fig. 1 Yasutomi Nishizuka. The photograph was taken in 1988 when he was awarded the Order of Culture, from the Emperor of Japan and is reproduced with permission from his wife Mrs Nishizuka.

simultaneously, four laboratories, *i.e.* Nishizuka's, Lipmann's, Gill's and Krebs' groups demonstrated that PKA consists of catalytic and regulatory subunits, and that cyclic AMP activates the enzyme by dissociating the catalytic subunit from the regulatory one (*8–11*). He devoted himself to the cyclic AMP actions in those days.

His lectures were very attractive to young students. He would tell his students, 'University of Tokyo or Kyoto may have won reputations as old imperial universities in Japan. Believe me, Kobe University can soon be a leading university in the world at least in science if you join us'. Many of those students believed him and came to his laboratory and Mr Y. Takai (now a Dean of Kobe University Graduate School of Medicine) was one among them.

Around that time, his group also challenged to reveal the mechanism underlying regulation of cGMP-dependent protein kinase (PKG) and found it an awkward enzyme because the activity was far less than that of PKA. However, they learned a good lesson from the studies on PKG, *i.e.* proteolysis of PKG resulted in a robust increase in the enzyme activity in the absence of cGMP (*12*), and this observation led to the hint of discovery of PKC before long.

Discovery of PKC

During analyses of mammalian PKG, it had been noticed that rat brains, which had been stored frozen, contained a very active protein kinase independent of any cyclic nucleotides for its activity. The requirement for this enzyme was high concentrations of Mg^{2+} (protein kinase M, PKM). Working long and hard in the cold room, the Nishizuka team found that much higher activities could be obtained from frozen brain stocks rather than from freshly prepared samples. In addition, yields improved even more when, for economy (?), protease inhibitor levels were reduced. Professor Nishizuka and his team then drew on their experience with PKG and hypothesized that they were studying a constitutively active proteolytic fragment, and set out to identify the 'pro-enzyme' (*13*).

Struggling with the activity loss during purification, they noticed that the activity recovered by the addition of membrane components, realizing that the pro-enzyme is latent in its activity and is activated in association with membranes. Finally, the Nishizuka group identified the pro-enzyme and reported that it was activated by membrane phospholipids. The group soon noticed that crude extracts of phospholipids from brains, rather than purified lipids, were the most effective for the enzyme activity and set out to uncover the nature of the 'impurity'. One of the active components was diacylglycerol (*14*), which led to the conceptual breakthrough that PKC might be activated in couple with the lipid hydrolysis pathway (*15*) discovered by Hokin and Hokin (*16*).

To obtain conclusive evidence for diacylglycerol as the physiological mediator of hormone actions, he searched for various techniques experimentally useful. Natural diacylglycerols that have two long fatty acid chains could not be readily intercalated into the cell membrane. If one of the fatty acid chains of diacylglycerol was replaced with an acetyl group such as 1-oleoyl-2-acetylglycerol (OAG), it was proven sufficiently membrane permeable and to activate PKC strongly.

In the meantime, several reports in the literature suggested an excellent experimental cell system. Susan Rittenhouse-Simmons and her group showed that in thrombin-stimulated platelets, diacylglycerol accumulated transiently, possibly as a result of inositol phospholipid hydrolysis (*17*). On the other hand, Majerus and Haslam independently reported that upon stimulation of platelets with thrombin, two endogenous proteins with 20- and 47-kDa molecular size were heavily phosphorylated (*18*, *19*). It was found that the 20-kDa protein was myosin light chain that was phosphorylated by one of calmodulin-dependent protein kinases in concert with increased intracellular Ca^{2+} concentrations. In contrast, the enzyme responsible for the phosphorylation of the 47-kDa protein remained unidentified.

The Nishizuka's group thought that PKC was the enzyme responsible for the 47 kDa protein phosphorylation upon stimulation of platelets by thrombin. Indeed, the addition of OAG to platelets induced the phosphorylation of the 47-kDa protein (*20*). His group soon showed that both an increase in Ca^{2+} and PKC activation were needed for the full activation of platelets (*21*). The 47-kDa protein was later named

pleckstrin. Thereafter, pleckstrin served as an excellent endogenous marker for PKC.

At that time, they experimentally used a Ca^{2+} ionophore to increase intracellular calcium concentrations; however, the origin of Ca^{2+} fluxes in physiological conditions was still ambiguous then. Michell postulated that inositol phospholipid hydrolysis could open a Ca^{2+} gate (*22*), although molecular mechanism was unknown. In 1983, Berridge and his colleagues, presented evidence that inositol 1,4,5-trisphosphate, the other product of phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis, could mobilize Ca^{2+} from internal stores (*23*). The traditional 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.

It has been well known that croton oil causes a marked augmentation of carcinogenesis 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 tumour 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. At first, Dr Nishizuka speculated that phorbol ester would produce diacylglycerol to activate PKC. However, it was not correct and he soon modified his idea from the hint that the phorbol ester contained a diacylglycerol-like structure in its molecule: the phorbol ester might activate PKC directly just like OAG. A series of experiments was able to show that the phorbol ester activated PKC directly (*24*). The results were immediately confirmed and they further concluded that PKC is a receptor for phorbol ester (*25*, *26*). Along this line of studies, phorbol esters and membrane-permeant diacylglycerol have been used as crucial tools for the manipulation of PKC activation in intact cells and have allowed the wide range of cellular processes regulated by this enzyme to be determined [(*27*), most frequently cited paper in the world in the 1980s]. PKC and phorbol ester action in the early 1980s definitely established a role for PKC in cellular signal transduction, and made one of the greatest contribution in physiology and medicine in these decades [(*28*), most frequently cited paper published in 1986].

The PKC field began to grow rapidly with the molecular cloning of PKCs: the calcium-dependent PKCs (or conventional, cPKCs) and subsequently the calcium-independent PKCs (or novel, nPKCs) followed by the atypical PKCs (aPKCs) (Fig. 2) [for a review, see Ohno and Nishizuka (*29*)]. In addition, several protein kinases which share kinase regions closely related to the PKC family have been isolated and characterized [for a review, see Mellor and Parker (*30*)]. These include protein kinase N (PKN or PRK), protein kinase B (PKB, Akt or rac-PK) and protein kinase D (PKD or 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. The first breakthrough on enzyme activation was made by the discovery of the pseudosubstrate sequence in the PKC regulatory region that is involved in intramolecular inhibitory interactions and then followed by the observations that newly synthesized PKC molecules are catalytically inert, and need to be primed competent by phosphorylation by PIP_2-dependent protein kinase 1 (PDK-1) with the aid of HSP90, followed by subsequent phosphorylation by the mammalian target of rapamycin

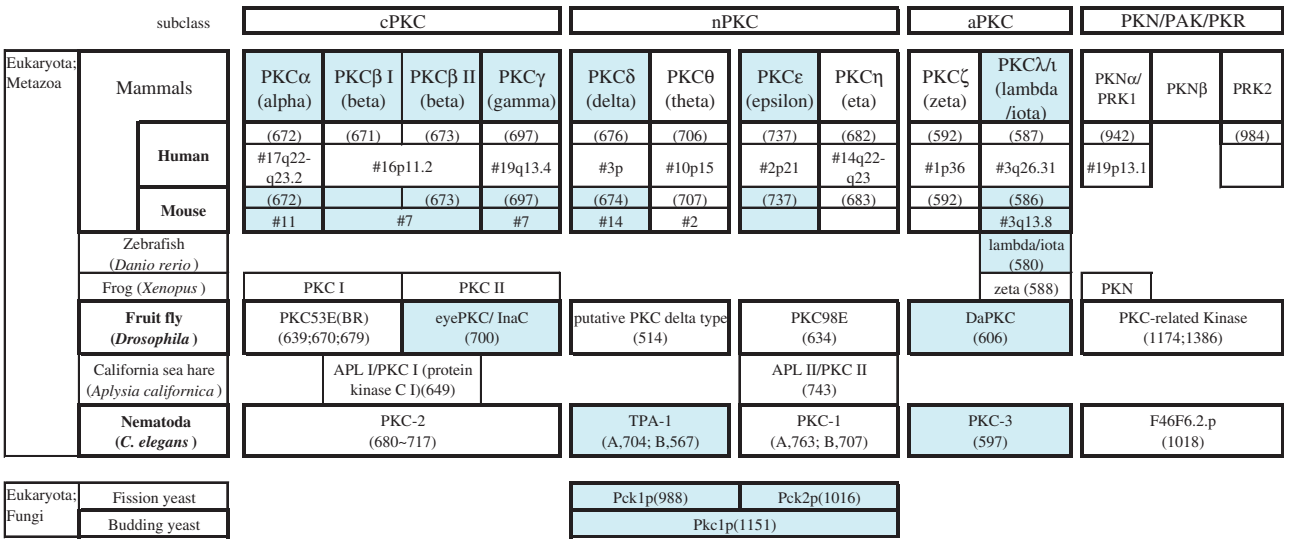

Fig. 2 PKC isotypes in mammals and lower eukaryotes. Mammalian PKC isotypes are classified into four groups, cPKC, nPKCδ/θ, nPKCε/η and aPKC. Each class has counterparts, orthologues, in two model genetic organisms, *Drosophila* and *C. elegans*. Yeast has only one (or two) PKC that shares structural features with cPKC, nPKC, aPKC and PKN/PAK/PKR. The blue boxes indicate that genetic evidence is available for the function of the isotype in the whole body. Numbers in the parentheses show amino acid residue numbers. Numbers starting with hash indicate chromosome number and locus. Adopted from Ohno and Nishizuka (*29*).

2 complex (mTORC2) [for a review, see Newton (*31*)]. This phosphorylated latent form, with the pseudosubstrate site binding to the substrate-binding pocket in the kinase domain, becomes full-active in a subtype-specific manner, *e.g.* when regulatory domain is recruited to plasma membranes through Ca^{2+}, phosphatidylserine and diacylglycerol, autoinhibition is relieved and the catalytic domain of cPKC is free to exert its action on target substrates. *In vivo* tyrosine phosphorylation, initially documented in 1993 for PKCδ (*32*), also regulates PKC activity in a positive or negative manner by a mechanism specific for each isozyme.

As the number of PKC expands, investigators have tried to assign specific functions to members of PKC subtypes. Although a degree of redundancy has been suggested through similar enzymatic characteristics among the subtypes, increasing evidence supports individual, non-redundant, albeit often subtle roles for many members of these subtypes. Such challenges include studies on visualization of PKC movement in living cells using green fluorescent protein (GFP)-fused PKC subtype (*33*), on tissue-specific localization of PKC subtypes using subtype-specific antibodies, and on characterization of PKC knockout or transgenic mice [for a review, see Choi and Messing (*34*)]. From these numerous studies, PKC subtype-mediated signaling is indispensable to understand molecular basis of wide variety of physiology and pathophysiology, *e.g.* role of aPKCs in T cell receptor signaling events (*35*), the PKC subtypes in cell migration [for a review, see Larsson (*36*)], and in memory [for a review, see Nelson and Alkon (*37*)].

Perspective

Discovery of PKC contributed greatly not only to understand PKC-mediated signaling as mentioned above but to establish the importance of lipids in signal transduction. Several decades ago, the membrane composed of lipids was generally viewed as a biologically inert entity that provides semipermeable barrier between exterior and interior compartments within and between cells. The phenomenon of receptor-mediated rapid hydrolysis of inositolphospholipids in the plasma membranes discovered in the middle of twentieth century (*16*) was a 'conundrum' to many scientists for a long time. Once the original paradigm 'one of the phospholipid metabolites functions as a second messenger, *i.e.* diacylglycerol binds to and activates PKC in concert with calcium and phosphatidylserine' has been verified, it is the beginning of new research field, namely lipid-mediated cell signalling. It has soon become clear that upon receptor stimulation various lipids in the membranes are metabolized to generate various lipid mediators such as free fatty acids, lysophosphatidylcholine and arachidonic acid in addition to diacylglycerol, which may participate in the activation and translocation of specific PKC subtypes [for review, see Nakamura and Nishizuka (*38*)]. These concepts have been extended drastically: similar to glycerophospholipids, sphingolipid metabolites such as ceramide, sphingosine and sphingosine 1-phosphate are now active research areas of interest (*39*). It is an attractive idea that almost all membrane lipids including phospholipids, sphingolipids and cholesterol may have specific signalling roles in transmembrane control of cellular events.

Epilogue

Kobe University suffered great damages from the Hanshin Awaji Earthquake in January 1995. Prof. Nishizuka was appointed the President of Kobe University just after the earthquake. He literally forwent his sleeping and eating time and led the Kobe University staffs for the restoration of the University. He successfully achieved this mission. He founded Biosignal Research Center in Kobe University and tried to make it a 'Mecca' for research on signal transduction. Besides a very demanding job as a President, he mentored young researchers and devoted himself to the development of the Center. Until he retired in February 2001 from the presidency, he dedicated six years to the development of Kobe University. Although Kobe University unfortunately experienced the worst earthquake, the only consolation was that Dr Nishizuka was the President of Kobe University at that time and the University was restored in such a better condition that nobody could have imagined. Dr Nishizuka's agenda as President was to educate good brains and to produce leading young scientists. He believed that youth is always a key to the development of science and also plays a leading role in the evolution of the University. He hoped that the students that he educated would have the confidence to compete with world authorities and leave influential footsteps in every field that will be succeeded by the next generation.

After he officially retired from the President of Kobe University, he enjoyed his life until his last moment, leading research at the Biosignal Research Center and at the Department of Biochemistry in Kobe University and conducting administrative works in Hyogo Prefecture. He also undertook the role of an Honorary Adviser in Sysmex Corporation, a health care testing company founded in Kobe. With this relation, this company offered a 'Nishizuka Memorial Honorary Advisory Room' in its facility where his mementoes, including his stationeries, drafts, his lectures in DVDs and awards such as the Order of Cultural Merit and Albert Lasker Basic Medical Research Award, etc. are exhibited and is open to the public.

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Conflict of interest
None declared.

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