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Happy birthday protein kinase C: Past, present and future of a superfamily

1. Birth and early years

Protein kinase C (PKC) was born in Japan in 1977 in the Department of Biochemistry of the University of Kobe. Inoue, Kishimoto and Takai in Nishizuka's Laboratory, described in two papers in the Journal of Biological Chemistry a cyclic nucleotide-independent, proteolytically modified protein kinase from mammalian brain (named PKM, M for magnesium ions that were indispensable for activation) [1,2]. The full length enzyme [2], subsequently demonstrated to be activated by calcium and phospholipids [3], was named protein kinase C (C, for calcium ions, which fully activated the enzyme at low concentrations, and thus differentiate it from cyclic nucleotide-dependent kinases, protein kinase A and G). The same group observed that unsaturated diacylglycerol (DAG) was an essential activator of PKC [4], linking the previously described receptor-dependent inositol phospholipid hydrolysis [5] to protein phosphorylation, thus thrusting this enzyme into intercellular signal transduction research. PKC inhibitors were characterized in 1980 as phospholipid interfering drugs (such as chlorpromazine, imipramine and dibucaine) [6] and the first direct functional assignment for PKC was made utilizing human platelets in which the thrombin-induced release of serotonin was shown to be mediated by PKC activation [7]. Starting in the early 1980s, the interest in PKC crossed Japan's borders and invaded the rest of the world, making PKC one of the most studied enzymes in biology, with more than 45,000 research papers published up to now.

We thus decided to celebrate the 30th birthday of PKC with a Special Issue of Pharmacological Research, reviewing the more relevant and recent developments in its characterization in physiology and pathology, highlighting the pharmacological implications and in particular the search for isozyme-selective inhibitors and activators.

It is impossible to mention all the discoveries that attracted, and still continue to attract the attention of so many scientists to this enzyme. We will concentrate just on select break-throughts that accompained PKC trentennial research and will divide this continuing story into the developments in each of the three decades (Fig. 1):

- 1977–1986: enzymatic description and modulations of activity;
- 1987–1996: isozyme identifications and their functions;
- 1997–2006: entering the "matured" age (regulation by phosphorylation) and new technological advances.

2. The first 10 years: enzymatic description and modulations of activity

In 1982, the observation by Castagna and colleagues that in human platelets the tumor promoting phorbol derivatives directly activate PKC, mimicking but not generating DAG (i.e. not inducing phospholipid hydrolysis) [8], opened the exciting area of research on PKC involvement in cell growth control. The same year, Kraft and coworkers reported the seminal discovery that activation with phorbol esters [9] leads to translocation (i.e. change in subcellular location) of PKC from the cell soluble to the cell particulate fraction. Radioactive phorbol 12,13 dibutyrate binding [10] was then used to localize and quantify PKC [11]. Subsequently, chronic treatment with phorbol esters was shown to down-regulate PKC phosphorylating activity [12]. The general interest in PKC culminated in a review paper on signal transduction and tumor promotion by Nishizuka, published in 1984, which became the most cited paper of the 1980s [13]. Additional inhibitors, interfering directly with PKC, were described between 1984 and 1986 and utilized extensively: the isoquinolinesulfonamide H7 [14], tamoxifen [15] and the anti-fungal alkaloid staurosporine [16] helped to clarify the implication of PKC in the regulation of several functions, including utilizing long term phorbol esters to "down regulate" the enzyme [12]. Bryostatin 1, a marine bryozoan, is an unconventional PKC activator/inhibitor; it stimulates and subsequently down-regulates PKC (like phorbol esters) but it does not act as a tumor promoter, but rather can inhibit phorbol ester effects under selected conditions [17]. The brain is the highest source for PKC in terms of catalytic activity and levels of expression [18], and one of the more exciting and intriguing aspects of PKC appears to be its involvement in the learning and memory phenomena. After the initial *in vitro* observation that synaptic plasticity is positively influenced by PKC activation [19] and that *in vivo* phorbol esters may antagonize scopolamine-induced amnesia [20], a variety of studies have accumulated providing biochemical, electrophysiological, behavioral, genetic and pharmacological evidence in favor of PKC as one of the

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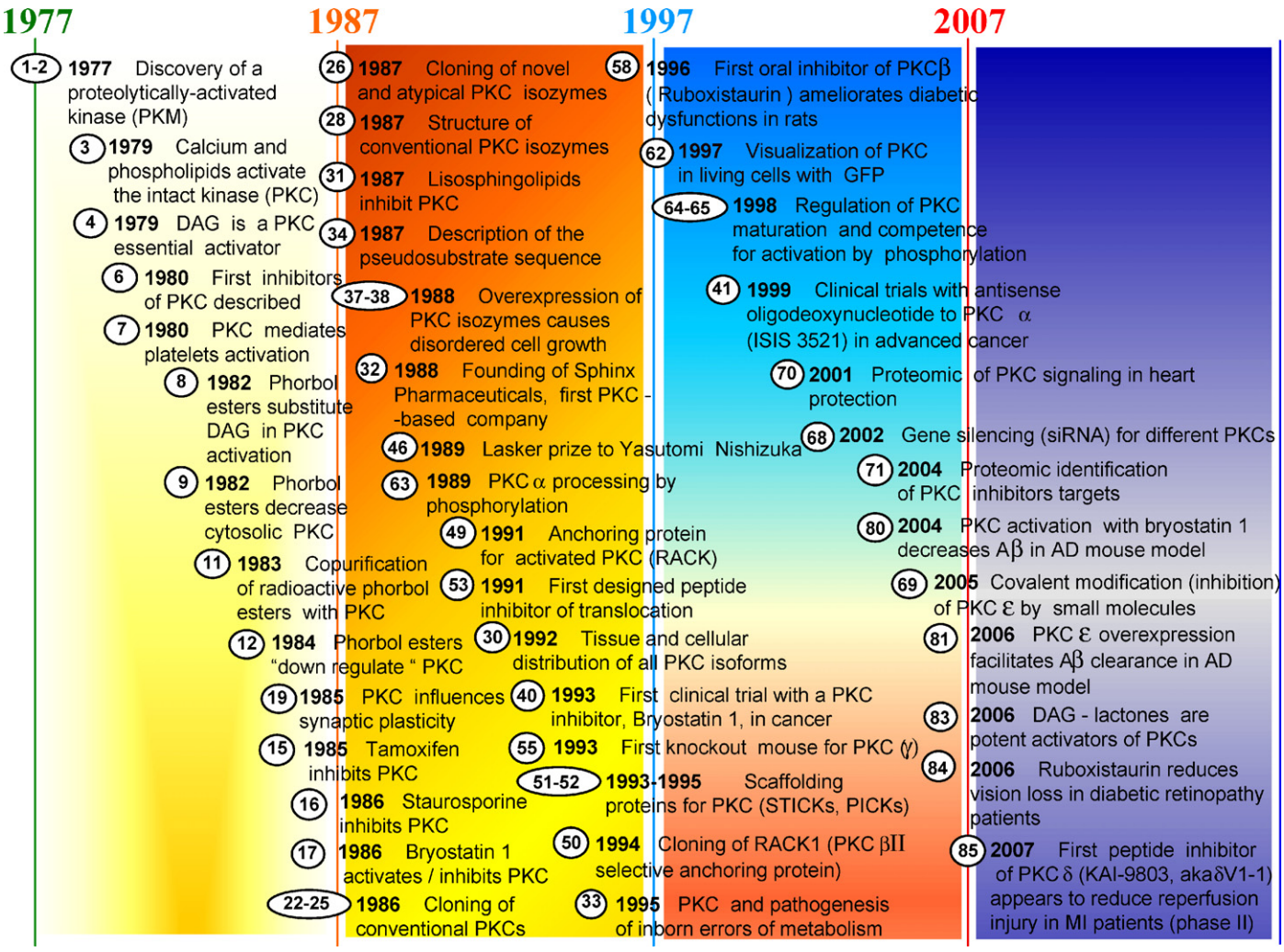

Fig. 1. Chronological studies on protein kinases C. The principal discoveries are reported focusing on pharmacological implications. The numbers in the circles indicate the reference citation. More details are provided in the text. PKM, catalytic fragment of protein kinase C (PKC); DAG, diacylglycerol; RACK, receptor for activated C kinase; STICKs, substrates that interact with C kinases; PICKs, protein that interact with C kinases; GFP, green fluorescent protein; siRNA, small interfering RNA; Aβ, amyloid beta peptide; AD, Alzheimer's disease; MI, myocadial infarction.

relevant players in memory trace formation (for a review see [21]).

The PKC field began to grow in 1986, with the cloning of the calcium-dependent PKCs (or conventional, cPKCs) [22–25], and subsequently the calcium-independent PKCs (or novel, nPKCs) followed by the atypical PKCs (aPKCs) [26]. cPKCs were characterized also by chromatographic techniques, naming them as PKC-I, -II, and -III [27] (corresponding to cPKCγ, β and α, respectively) and consensus followed, identifying the different isozymes with greek letters.

3. The second decade: isozyme identification and their functions

The PKC isozymes contain conserved and variable regions in the catalytic and regulatory subunits [28,29] and isozyme-selective antibodies were thus produced. A detailed study on tissue and cellular distribution of all the isozymes was published in 1992 by Bill Wetsel and co-workers in Y. Hannun's laboratory [30], the same researcher who while in Bob Bell's lab characterized sphingolipids as PKC inhibitors, thus linking PKC to sphingolipidoses [31]. The involvement of different PKC isozymes in the pathogenesis of inborn errors of metabolism (sphingolipidoses, fatty acid oxidation, bile acid and cholesterol) was proposed later, in 1995 [33]. Bob Bell, whose work into diacylglycerol and PKC regulation led to the characterization of how PKC signaling is turned off, founded with Carson Loomis, in 1988 the first PKC-based Company-Sphinx Pharmaceuticals [32]. Spinx (in Research Triangle Park, NC, USA) was acquired in 1994 by Eli Lilly, leading to the discovery of the selective βPKC oral inhibitor, ruboxistaurin (see below).

In 1987, House and Kemp described the pseudosubstrate sequence in the PKC regulatory region that is involved in intramolecular inhibitory interactions [34]. More details and updates on PKC structural composition and intramolecular regulation can be found in the opening review of this issue by

Kheifets and Mochly-Rosen [35]. A peptide corresponding to the pseudosubstrate sequence was found to act as a selective inhibitor of PKC [34], but its use as a pharmacological agent in cells was limited because the peptide does not cross biological membranes. The search for selective pharmacological tools to specifically inhibit PKC was prompted, in part, by the findings that in addition to binding to PKC, phorbol esters interact with other signalling molecules devoid of kinase activity such as the mammalian α– and β–chimaerins, Ras-GRP (Ras guanyl-releasing protein) and the nematode, Unc-13 (with mammalian homologues Munc 13) (more details in [36]).

Overexpression of PKC isozymes in cell cultures opened the area of research aimed at further clarifying the involvement of selected PKC isozymes in abnormal cell growth [37,38]. Further, susceptibility to transformation increased by PKC co-expression with certain oncogenes, i.e. H-*ras*, *myc* and *fos* [39]. These and other data eventually led to the first clinical studies in advanced cancer patients using bryostatin 1 [by CRC in Manchester, UK in 1993 [40]] and with anti-sense oligonucleotides to PKCα [by Isis Inc, in 1999 [41]]. Another line of research focussed on inhibiting PKC as adjuvant to improve conventional chemotherapies [42]. In spite of discouraging results, additional trials are in progress with particular attention to isozyme- and tissue-selective effects (reviewed in [43]). PKC involvement and future in tumor growth control is discussed here in two reviews. *Martiny-Baron and Fabbro* focus on conventional PKCs and the endpoints of all clinical trials with small molecular weight inhibitors and antisense compounds [44]. *Fields and Regala* concentrate on the atypical PKCζ and ι, and how PKCι signalling is targeted to identify a novel drug for human lung cancer [45].

In 1989, in recognition of the contribution to the advancement of the PKC field, the Albert Lasker Basic Medical Research Prize was awarded to the "father" of this enzyme family, Yasutomi Nishizuka [46].

The regulation of PKC activation due to lipid–protein interactions was revisited following the identification of protein–protein (PKC-anchoring protein) interactions for PKC activity and function. Evidence for the presence of anchoring proteins for PKC translocation was first indicated by an *in vitro* study of Gopalkrishna et al. [47], showing that stable interaction of PKC with isolated plasma membranes is lost following pre-treatment of the membranes with proteases. That and the finding that individual isozymes are localized each to a unique subcellular site following activation [48] led to the discovery of Mochly-Rosen and co-workers in 1991 of PKC anchoring proteins, collectively named receptors for activated C kinases (RACKs) [49], and first designed peptide inhibitor of translocation (reviewed in [53]). The first RACK, i.e. the βII-selective RACK1, was cloned in 1994 [50]. A variety of other anchor/scaffolding proteins are known to regulate PKC homeostasis [51,52]. This research led to the development of PKC isozyme-selective peptide activators and inhibitors that induce or inhibit translocation and function of individual isozymes, respectively [53]. *Budas*, *Churchill and Mochly-Rosen* discuss the progress made in establishing cytoprotective mechanisms, which arise as a consequence of εPKC activation and/or δPKC inhibition, and how these may lead to protection in the setting of myocardial ischemia reperfusion [54].

The first PKC knockout mouse was produced and characterized in 1993 and lacked the neuronal-specific cPKCγ [55]. Data on other knockouts and transgenic mice overexpressing various PKCs have since been established and represent an intensive area of research (for a recent review see [56]). Although potential redundant roles of individual PKC isozymes complicate and limit the exact identification of isozyme-selective actions, important new information on the role of each isozyme has been described.

In addition to tumor promotion and growth, other pathologies in which abnormal PKC may be involved were recognized. These include hypertension, diabetes, atherosclerosis, to name a few, suggesting the definition of a potential "PKC syndrome" [57]. The involvement of overfunctional PKCβ in diabetes was established using a staurosporine-derivative inhibitor (i.e. LY333531 or ruboxistaurin). This orally bioavailable compound inhibits PKCβ and ameliorates different vascular dysfunctions in animal models [58]. In this volume, *Das Evcimen and King* discuss the essential role of PKC activation in diabetic cardio- and microvascular complications, the mechanisms by which hyperglycemia causes vascular damage and summarize the clinical trials with PKCβ inhibitors in diabetic complications [59]. The importance of PKCs in respiratory physiology is covered by the contribution of *Dempsey*, *Cool and Littler*, who discuss the relevance of the bidirectional approach (inhibiting and activating different PKC isozymes) as they relate to lung pathology and highlight the differences in lung anatomy between animal models and humans [60]. The importance of PKC theta in T cell functions (activation, proliferation, differentiation and survival), but not in anti-viral responses, is reviewed by *Hayashi and Altman*, who provide their perspective on the potential of this isozyme as a target for controlling allergic and autoimmune diseases [61].

4. The third decade: entering the "matured" age (regulation by phosphorylation) and new technological advances

A diversity of functions are controlled by PKC isozymes present in the same cell. Even upon the same stimulus, individual PKCs move to different subcellular sites (membrane, organelles, cytoskeleton, nucleus) where select substrate phosphorylations leads to diverse and sometimes even opposing functions. The ability to visualize the translocation (activation) of PKC in living cells was made possible in 1997 by tagging PKC with the green fluorescent protein, GFP [62].

Although post-translational processing of PKC by phosphorylation was initially documented in tumor cells in 1989 [63], a new era in PKC regulation started in 1998, when Alexandra Newton's and Peter Parker's groups [64,65] described the

mechanisms for correct maturation and catalytic activity of PKC isozymes, requiring sequential serine/threonine phosphorylation reactions. The initial phosphorylation is common to all the isozymes and is mediated by phosphoinositide-dependent kinase 1 (PDK1), indicating a cross-talk between inositol phospholipid hydrolysis and phosphatidylinositol 3-kinase pathways, whereas the subsequent phosphorylation events are likely isozyme-specific autophosphorylations [66]. *In vivo* tyrosine phosphorylation, initially documented in 1993 for PKCδ [67], also regulates PKC activity in a positive or negative manner by a mechanism specific for each isozyme.

In the search for isozyme-selective PKC inhibitors, new approaches were applied including gene silencing with antisense oligonucleotides [41] and short interfering RNAs [68] as well as covalent modification of PKC isozymes with cysteine-reactive peptide substrate analogs [69]. One of the most frustrating aspects in the PKC field is the difficulty in identifying selective substrates of each isozyme. A recent proteomic approach has been used to study PKC signaling in cardiac protection [70] and to identify PKC targets [71]. In another contribution, *Agnetti et al.* [72] discuss the technologies and applications of proteomics to the study of kinases, in general, and PKC-mediated phosphorylation of cytoskeletal, myofilament and mitochondrial proteins in heart failure, hypertrophy and cardioprotection, in particular.

The involvement of PKC isozymes in the function and pathologies of the central nervous system is another area of intense research and therapeutic prospect. This special issue covers three potential therapeutic indications for PKC regulating drugs. These include: ethanol addiction, pain and aging. *Newton and Ron* summarize data on acute and chronic ethanol effects on PKC isozymes underlining the role of select isozymes as potential therapeutic targets for alcoholism [73]. How PKC may function as a relevant regulator of peripheral and central sensitization that underlies many chronic pain conditions is reviewed by *Velázquez*, *Mohammad and Sweitzer* [74]. Finally, soon after PKC involvement in learning and memory had been recognized, its role in physiological and pathological brain aging has been a focus of reseach (reviewed in [75]). An age- and pathology-related (Alzheimer's disease) deficit in PKC activation and anchoring, rather than changes in isozyme levels, seem to be the most consistent finding, also in non-neuronal tissues [76]. These aspects together with emerging concepts on PKC-dependent mRNA stabilization related to memory substates [77] are sumarized in the review by *Pascale et al.* [78], suggesting that PKC activation could be a useful approach to correct these deficits.

It is possible that the potential of PKC activators as therapeutic compounds may be limited by their tumor promoting actions. However, there are drug candidates, such as bryostatin 1, that activate PKC but are devoid of tumor promoting effects [79]. Furthermore, isozyme-selective activator peptides, targeting PKC isozymes that are not related to uncontrolled growth, have also been identified [53]. Such selective activators may provide benefits for patients with Alzheimer's disease (AD), in which PKC appears to act both upstream and downstream of beta amyloid (Aβ) accumulation in brain tissues. For example, PKC activation with bryostatin 1 decreased Aβ brain deposition in mouse models of AD favoring non-amyloidogenic metabolism of the Aβ precursor [80] and PKCε activation decreased brain Aβ accumulation, favoring its clearance [81]. A general outline of PKC therapeutic potential in AD can be found in a recent publication [82]. In addition to isozyme-selective peptide activators [53], PKC activators belonging to the DAG-lactone chemical structure are also promising compounds for activating specific PKC isozymes [83].

5. The coming years: can PKC go from bench to bedside?

While research on PKC continues to attract interest in the basic research community, applications of PKC regulating drugs has met limited success. PKCα and PKCε inhibitors may be useful to inhibit tumor growth and multi-drug resistance. The cardiovascular field is concentrating on β- and δ-PKC inhibitors and clinical studies have given some hope for approaching diabetic complications such as retinopathy with ruboxistaurin [84] and with a PKCδ inhibitor peptide for acute myocardial infarction. Data from a phase IIa safety and dose escalation clinical trial of KAI Pharmaceuticals indicates that the peptide inhibitor of δPKC appears safe and may provide protection from reperfusion injury when given for patients immediately after acute myocardial infarction [85]. Meanwhile, the concept of "PKC activation" has recently found preclinical support in terms of limiting cardiac and brain degeneration. In this case, PKCε activation could be a good candidate for clinical trials. Finally, promising preclinical work regarding regulation of immune response, alcoholism and possibly other addictions, pain sensation and other clinically relevant conditions suggest that we will see a new surge in drug development that focuses on PKC in the coming years. We hope that this Special Issue will encourage further research in the PKC field that will yield novel drugs for treatment of human diseases.

Disclosure

DM-R is the founder and member of the board of KAI Pharmaceuticals. However, none of the work in her lab is supported by or is in collaboration with the company.

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FB dedicates this issue to the memory of his parents, Giuseppina Rossi and Gaetano Battaini, for their encouragement to pursue the exciting career in science.
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