Protein Kinase Cδ (PKCδ): Activation Mechanisms and Functions

Ushio Kikkawa,[1] Hidenori Matsuzaki, and Toshiyoshi Yamamoto

Biosignal Research Center, Kobe University, Kobe 657-8501

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Protein kinase C (PKC)δ was the first new/novel PKC isoform to be identified by the screening of mammalian cDNA libraries, based on the structural homology of its nucleotide sequences with those of classical/conventional PKC isoforms. PKCδ is expressed ubiquitously among cells and tissues. It is activated by diacylglycerol produced by receptor-mediated hydrolysis of membrane inositol phospholipids as well as by tumor-promoting phorbol ester through the binding of these compounds to the C1 region in its regulatory domain. It is also cleaved by caspase to generate a catalytically active fragment, and it is converted to an active form without proteolysis through the tyrosine phosphorylation reaction. Various lines of evidence indicate that PKCδ activated in distinct ways plays critical roles in cellular functions such as the control of growth, differentiation, and apoptosis. This article briefly summarizes the regulatory mechanisms of PKCδ activity and its functions in cell signaling.

Key words: diacylglycerol, phorbol ester, PKCδ, proteolysis, tyrosine phosphorylation.

Molecular cloning and genomic structure

Protein kinase C (PKC)δ was cloned from a rat brain cDNA library by using fragments encoding classical/conventional PKC (cPKC) isoforms as probes (*1*). It was subsequently obtained from different mammalian species such as mouse (*2, 3*) and human (*4*) and classified as a member of the new/novel PKC (nPKC) subgroup (for reviews: Refs. *5–8*). The open reading frames of rat, mouse, and human clones encode proteins of 673, 674, and 676 amino acid residues, respectively, that are highly homologous and have an almost identical calculated molecular mass of 77.5 kDa. The phylogenic tree of the PKC isoforms (http://www.cellsignal.com/retail/reference/kinase/pkc.asp) shows that the primary structure of PKCδ is most closely related to another nPKC isoform PKCθ. PKCθ is expressed predominantly in muscle and hematopoietic cells as reviewed in this series (*9*), whereas PKCδ is widely distributed among cells and tissues, suggesting that PKCδ has universal rather than cell-type–specific roles in mammals.

The genomic structure of PKCδ is analyzed for human (http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=5580), mouse (http://www.ncbi.nlm.nih.gov/LocusLink/LocRpt.cgi?l=18753), and rat (http://rgd.mcw.edu/tools/genes/genes_view.cgi?id=67383). The PKCδ gene is localized on chromosomes 3 (*10*), 14 (*10*), and 19 (*11*) of human, mouse, and rat, respectively, although the rat gene is assigned to chromosome 16 in the data base above. The 5′-regulatory region of the rat PKCδ gene lacks a TATA box but contains putative binding sites for transcription factors such as AP-1, NFκB, Sp-1, and NGFI-C (nerve growth factor induced-C) (*11*). Functional studies of the promoter regions will clarify the regulatory mechanisms for the expression of PKCδ. In addition, two cDNA clones encoding possible splicing variants of PKCδ have been found: one has an insertion in the middle of the molecule (*12*), and the other is a truncated enzyme lacking its carboxyl-terminal half (*13*), although their physiological roles are yet to be analyzed.

Protein structure

PKCδ has catalytic and regulatory domains in the carboxyl- and amino-terminal halves, respectively (Fig. 1). The catalytic domain contains two conserved regions, C3 and C4, in common with other members of the PKC family, that roughly correspond to the protein kinase subdomains I to XI (*14*). In this review, the amino acid residue numbers of rat PKCδ are employed. The C4 region has a phosphorylation motif site, Thr-505, in the activation loop, and the carboxyl-terminal end of the enzyme has two conserved phosphorylation sites, Ser-643 and Ser-662, which are turn and hydrophobic motif sites, respectively (*15*). The role of phosphorylation in the regulation of catalytic activity will be discussed later. The regulatory domain of the cPKC isoforms has two conserved regions, C1 and C2, whereas PKCδ contains only a C1 region but lacks an authentic C2 region, having instead a C2-like region in the amino-terminal end of the molecule. There is a pseudosubstrate sequence between the C2-like and C1 regions, centered on Ala-147, that is proposed to occupy the substrate recognition site in the catalytic domain of PKCδ to keep this isoform in an inactive conformation.

The three-dimensional structure of intact PKCδ has not been determined, but the precise structures of the two regions in the regulatory domain have been revealed by X-ray crystallographic analysis (*16, 17*). The C1 region of the cPKC and nPKC subgroups binds diacylglycerol and phorbol ester, playing an essential role in its activation, and includes a tandem repeat of Cys-rich motifs named C1A and C1B (*18*). Each Cys-rich motif of cPKC isoforms can bind phorbol ester, whereas the C1A and C1B regions of

[1]To whom correspondence should be addressed. Tel: +81-78-803-5964, Fax: +81-78-803-5972, E-mail: ukikkawa@kobe-u.ac.jp

Abbreviations: PKC, protein kinase C; cPKC, classical/conventional PKC; nPKC, new/novel PKC.

the nPKC subgroup are not equivalent, and the C1B region is the major phorbol ester–binding site of nPKC isoforms (*19–21*). The crystal structure of the C1B region of PKCδ (amino acid resides 231–280) has been determined in complex with phorbol ester, which binds to a pocket between two pulled-apart β sheets at the tip of the region (*16*). The C2 region is found in various proteins involved in membrane trafficking and signal transduction (*22*), and the domain in cPKC isoforms binds membrane phospholipids in a Ca^{2+}-dependent manner. PKCδ does not require Ca^{2+} for its activity, and crystallographic analysis indicated consistently that the C2-like region of the PKC isoform (amino acid resides 1–123) is a variant of the C2 fold that lacks sequences necessary for Ca^{2+} coordination (*17*). Recently, two-dimensional crystal structures of intact PKCδ and its regulatory domain were analyzed (*23*). Intact PKCδ and the regulatory domain on the lipid monolayer show a donut-shaped structure, to which the crystal structure of the C1B region is accommodated by overlaying the images. Precise structural information on intact PKCδ is essential to elucidate the regulatory mechanism of the enzyme.

Enzymatic properties

The expression product of the rat PKCδ clone recovered from transfected COS-7 cells showed a protein kinase activity dependent on diacylglycerol in the presence of phospholipid (*1*). Later, the enzymes isolated from transfected mammalian cells (*2, 24, 25*) and insect cells infected by baculovirus vector (*4, 26*) were revealed to act independently of Ca^{2+} when activated by diacylglycerol or phorbol ester. PKCδ was subsequently confirmed to be activated in intact cells by phorbol ester treatment as well as by physiological stimuli inducing inositol phospholipid hydrolysis, such as bombesin, serum, platelet-derived growth factor, and epidermal growth factor, as judged by its membrane translocation, down-regulation, and phosphorylation (*27, 28*). As a native enzyme sample, a phorbol ester-activated, Ca^{2+}-unresponsive protein kinase was purified from the Triton X-100 extract of porcine spleen and identified as PKCδ by immunoblot analysis using a specific antibody (*29*). PKCδ was also purified from the detergent-soluble fraction of ABPL-3 mouse myeloid cells (*3*) and rat brain (*25*). Its biochemical properties have been analyzed using these native and recombinant enzyme preparations. For example, some PKC isoforms are activated efficiently by fatty acids *in vitro* and are proposed to be regulated in the phospholipase A_2 pathway, but the PKCδ activity is not significantly enhanced by fatty acids (*25, 30*).

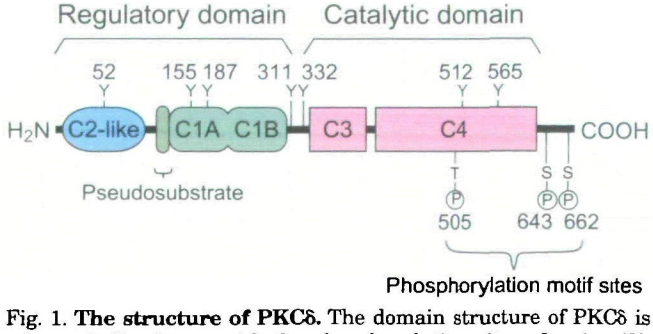

Fig. 1. **The structure of PKCδ.** The domain structure of PKCδ is schematically shown, with the phosphorylation sites of serine (S), threonine (T), and tyrosine (Y) residues.

Activation mechanisms

PKCδ is regulated not only by the binding of diacylglycerol or phorbol ester but also by molecular mechnisms such as phosphorylation and proteolytic reactions (Fig. 2).

Phosphorylation at motif sites. A number of protein kinases are regulated by phosphorylation (*31*), and the PKC family members have phosphorylation motif sites of conserved serine or threonine residues (*8, 15*). One is a threonine residue in the activation loop that is common among members of the PKC family. In addition, cPKC and nPKC isoforms are phosphorylated at turn and hydrophobic motif sites in the carboxyl-terminal end region, whereas atypical PKC isoforms have the turn motif site but not the hydrophobic motif site, which is replaced by a phosphate mimic, Glu. Studies of cPKC isoforms have shown that the sequential phosphorylation of these threonine and serine residues render them catalytically competent. cPKC isoforms are first phosphorylated in the activation loop by an upstream kinase, PDK-1 or a related enzyme, which is essential for its catalytic activity. Then, turn and hydrophobic motif sites are autophosphorylated, and cPKC isoforms adopt a mature and stable conformation ready to be activated by diacylglycerol or phorbol ester.

PKCδ has activation loop, turn, and hydrophobic motif sites at Thr-505, Ser-643, and Ser-662, respectively, and these sites are substantially phosphorylated *in vivo* (*32*). PKCδ differs from cPKC isoforms, however, in the regulatory mechanism by phosphorylation. Namely, PKCδ expressed in bacteria, which is unphosphorylated at Thr-505, shows a modest catalytic activity. Furthermore, a point-mutant molecule with Ala replacing Thr-505 is active, although the mutation of the corresponding threonine residue in cPKC isoforms makes them kinase-dead (*33*). The acidic residue Glu-500 in the activation loop sequence, which is unique in PKCδ, is proposed to partially fulfill the role of phosphorylation for catalytic competence of the enzyme (*34*). In embryonic stem cells lacking PDK-1, the in-

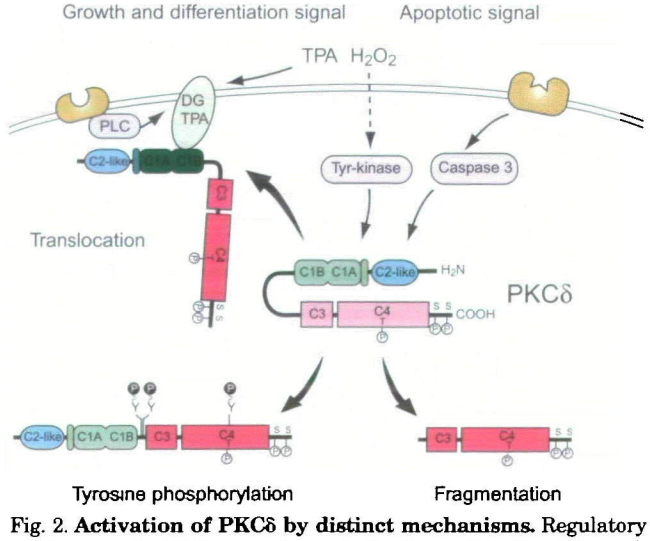

Fig. 2. **Activation of PKCδ by distinct mechanisms.** Regulatory mechanisms for PKCδ are schematically shown. DG, diacylglycerol; PLC, phospholipase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; Tyr-kinase, tyrosine kinase.

tracellular level of endogenously expressed PKCδ is greatly reduced, suggesting that phosphorylation at Thr-505 by PDK-1 is required for the stability of the enzyme in mammalian cells (*35*). Ser-643 is autophosphorylated (*34, 36*), but Ser-662 is recognized by an upstream kinase (*37, 38*). PKCζ, an atypical PKC isoform, has been identified as a component of the upstream kinase responsible for phosphorylation of Ser-662, although it is not clear whether PKCζ directly recognizes this site. In addition, rapamycin, an immunosuppressant, blocks the *in vivo* phosphorylation reaction of Ser-662, and thus phosphorylation of this hydrophobic site is regulated by a pathway involving the mammalian target of rapamycin (mTOR) (*39*). In fact, mTOR has been shown to interact with PKCδ (*40*). Phosphorylation at Ser-662 as well as at Thr-505 is regulated in intact cells (*32, 41*), and thus it is necessary to evaluate the role of phosphorylation at these motif sites in comparison with other PKC isoforms.

Tyrosine phosphorylation. PKC isoforms such as α, βI, γ, δ, ε, ζ, θ, and ι/λ are further phosphorylated on tyrosine upon stimulation of the cells (*32, 42–48*). In contrast to phosphorylation at serine and threonine motif sites, the phosphorylated tyrosine residues thus far identified such as Tyr-52 (*48*), Tyr-155 (*48*), Tyr-187 (*49*), Tyr-311 (*32, 50*), Tyr-332 (*32*), and Tyr-565 (*48*) of PKCδ, Tyr-90 of PKCθ (*46*), and Tyr-256, Tyr-271, and Tyr-325 of PKCι (*47*), are not conserved among members of the PKC family, although Tyr-512 of PKCδ is an exception (*32, 45*). Tyrosine phosphorylation appears to be an isoform-specific modification rather than one common to the whole family. In PKCδ, which is most efficiently tyrosine-phosphorylated among the PKC family, different tyrosine residues appear to be phosphorylated depending on cell stimuli. In fact, PKCδ is phosphorylated by various tyrosine kinases, and in some cases, it is associated with tyrosine kinases, such as Src (*50–55*), Fyn (*43, 51, 52*), Lyn (*48, 55*), Abl (*56, 57*), PYK2 (*58*), Lck (*32*), and growth factor receptors (*43, 52*). Therefore, it is interesting to know the effects of tyrosine phosphorylation on the catalytic activity of PKC. The catalytic activity of PKCδ was shown to be reduced by tyrosine phosphorylation in *v-ras*–transformed keratinocytes (*42*) and in epidermal cells treated with epidermal growth factor (*52*) or phosphorylated by Src family kinases (*59*). On the other hand, tyrosine phosphoryation enhances the enzymatic activity in various cells stimulated with substances such as phorbol ester, growth factors, and hormones (*43, 48, 60–66*). In some cases, the enzymatic specificity is altered by this modification (*67–69*). We have found that PKCδ is tyrosine-phosphorylated at Tyr-311, Tyr-332, and Tyr-512 in the H_2O_2-treated cells, and that the enzyme recovered is constitutively active and is independent of diacylglycerol (*32, 45*). Consistent with this, PKCδ does not translocate to membranes but apparently stays in the cytosol after H_2O_2 stimulation, whereas it associates with membranes in cells stimulated by a receptor agonist (*70*). Therefore, PKCδ is activated by tyrosine phosphorylation in the H_2O_2-treated cells through a mechanism unrelated to receptor-coupled hydrolysis of inositol phospholipids. Also, PKCδ phosphorylated at Tyr-311 *in vitro*, the major tyrosine phosphorylation site in the H_2O_2-treated cells, shows an enhanced catalytic activity (*32*). The PKC isoform phosphorylated at Tyr-311 is, however, further activated by diacylglycerol, indicating that modification of this single residue is insufficient to generate the fully active enzyme. The active form of PKCδ may be generated *in vivo* by phosphorylation at more than one residue. It is noteworthy that the targets of the tyrosine-phosphorylated PKCδ have been analyzed (*71–73*). In addition, a receptor-type tyrosine phosphatase, CD45, is shown to be involved in dephosphorylation of PKCδ (74), although the treatment of the cells with protein–tyrosine phosphatase inhibitors does not induce tyrosine phosphorylation or generation of the active PKCδ (*75*). It seems, therefore, that the H_2O_2 treatment facilitates the tyrosine phosphorylation reaction of PKCδ rather than preventing dephosphorylation of the enzyme. On the other hand, PKC is suggested to be regulated by redox modification (*76*). It is interesting to assume that the catalytic activity of PKCδ is regulated by the combination of phosphorylation on tyrosine, serine, and threonine residues, as well as by the oxidative modification.

Active fragment. A catalytically active fragment of PKCδ is generated by proteolysis in cells induced to undergo apoptosis in response to ionizing radiation, DNA-damaging drugs, and anti-Fas antibody (*77–80*). The catalytic fragment of PKCδ, presumably cleaved by caspase 3 or a related enzyme between Asp-327 and Asn-328, inhibits the function of DNA-dependent protein kinase and contributes to DNA damage–induced apoptosis (*81*).

In the CHO cell line overproducing PKCδ, H_2O_2-induced apoptosis is enhanced compared with that in wild-type cells (*82*). Under such conditions, PKCδ is recovered as the active form by tyrosine phosphorylation as described above, but the catalytic fragment is not detected in the cell line. Similarly, apoptosis is potentiated by overexpressing PKCδ in LNCaP prostate cancer cells without proteolytic activation of PKCδ (*83*). This cleavage site of PKCδ by caspase 3 is located between two phosphorylation sites of Tyr-311 and Tyr-332. Phosphorylation at Tyr-311 is shown to promote degradation of PKCδ (*50*), presumably after ubiquitination (*84*). PKCδ phosphorylated at Tyr-311 and probably at Tyr-332 may be insensitive to proteolysis by caspase. The catalytic fragment and the tyrosine-phosphorylated active form of PKCδ seem to contribute to promotion of cell death independently. The splicing variant of PKCδ having an insertion in the middle of the molecule (*12*) appears to be insusceptible to the protease because it lacks the caspase 3-recognition site. It is interesting to speculate that the splicing variant may be activated not by the cleavage but through the tyrosine phosphorylation.

On the other hand, ultraviolet radiation activates PKCδ by different mechanisms. Low doses of ultraviolet radiation, which generate reactive oxygen species, activate PKCδ by tyrosine phosphorylation without proteolysis in a keratinocyte cell line HaCaT (*85*), whereas PKCδ is cleaved after high doses of ultraviolet radiation in normal human keratinocytes (*86*). PKCδ seems to be regulated by reversible and irreversible mechanisms depending on cell stimuli.

Analysis of the roles

PKCδ shares properties with other PKC isoforms and is activated by diacylglycerol and phorbol ester. The functions of PKCδ *in vivo* have been analyzed by many techniques.

Activators and inhibitors. Phorbol esters and related tumor promoters are widely employed as PKC activators. The *in vivo* effects of these compounds should be evaluated carefully, because, for example, phorbol esters not only acti-

vate the PKC family but bind to other proteins such as chimaerin (*87, 88*) and Ras exchange factor RasGRP3 (*89*). Furthermore, these compounds exhibit different activities toward the activation, intracellular translocation, and down-regulation of PKC isoforms (*6, 90*). In particular, bryostatin, a macrocyclic lactone that activates PKC, protects PKCδ selectively from phorbol ester–induced down-regulation of the PKC isoforms (*91, 92*). Thus, bryostatin has been employed in combination with phorbol esters to elucidate specific roles of PKCδ such as tumor suppresser function (*93*), inhibition of the expression of glutamine synthetase (*94*), and contact inhibition of growth (*95*). Recently, some bryostatin analogues were synthesized that bind selectively to the C1 region peptides of PKCδ (*96*), and thus it will become possible to design compounds that specifically regulate PKCδ.

On the other hand, antisense oligonucleotides have been introduced into cultured cells to suppress the expression of the PKC isoform. This technique has revealed that PKCδ is implicated in several physiological functions including differentiation of murine erythroleukemia cells (*97*), the regulation of cation-chloride cotransporter (*98, 99*), activation of mitogen-activated protein kinases (*100*), expression of nitric oxide synthase (*101*), and stimulation of pyruvate dehydrogenase (*102*).

Among the PKC inhibitors, rottlerin was found to show a narrow spectrum and has been employed to distinguish the roles of PKCδ from those of other PKC isoforms, although it also attenuates calmodulin-dependent protein kinase III at low concentrations (*103*). *In vitro* analysis showed later that rottlerin does not suppress PKCδ, whereas it inhibits some other enzymes such as p38-regulated/activated kinase and mitogen-activated protein kinase-activated protein kinase 2 (*104*). The effects of rottlerin may depend on the assay conditions or the preparation of the compound (the home page of LC Laboratories, http://www.lclabs.com/PRODFILE/P-R/R-9630.php3). More recently, rottlerin was revealed to be a mitochondria uncoupler and suggested to block the PKCδ activity indirectly *in vivo* (*105*). It also inhibited the pervanadate-induced tyrosine phosphorylation in PKCδ-null mast cells (*106*). Results obtained by using rottlerin need to be evaluated cautiously.

As a novel approach to investigate the role of PKCδ based on structural modeling of the C2-like region, PKCδ-selective activator and inhibitor peptides were synthesized that correspond to a potential sequence resembling its isoform-specific anchoring protein and a possible binding site for the anchoring protein, respectively (*107*). The activator and inhibitor peptides regulate the intracellular translocation of PKCδ, and increased and suppressed ischemic damage of heart cells, respectively, when introduced by cross-linking to *Drosophila antennapedia* homeodomain-derived carrier peptide. It will be interesting to see if this method can be applied to other cells.

Overexpression. By using isolated cDNA, cell lines stably overproducing the wild type PKCδ have been established to elucidate the role of the PKC isoform. Phorbol ester-induced growth inhibition is generally observed in such transformants constructed by using CHO cells (*108*), NIH 3T3 cells (*109*), 32D myeloid progenitor cells (*110*), A7r5 vascular smooth muscle cells (*111*), and RFPEC endothelial cells (*112*). In particular, growth arrest at G_2/M (*108*) and G_0/G_1 (*111*) phases and cell differentiation (*109, 110*) are observed. Differentiation is also observed in normal keratinocytes carrying PKCδ introduced with an adenovirus vector (*113*). Furthermore, H_2O_2-inducded apoptosis is enhanced in the CHO cell line overproducing this PKC isoform as described above (*82*). On the other hand, PKCδ is increased in highly metastatic mammary tumor cell lines, and the expression of its regulatory domain inhibits anchorage-independent growth in the tumor cell lines, suggesting that PKCδ contributes to cell growth and that the regulatory domain works as a dominant negative fragment (*114*). Kinase-negative mutants in which Lys-376 in the ATP-binding site was replaced by Ala (*115*) and by Arg (*116, 117*) have also been used as dominant negatives. In contrast, a mutant molecule in which both Arg-144 and Arg-145 in the pseudosubstrate sequence were replaced by Ala is a constitutively active molecule (*115*). Chimeric molecules have also been constructed by swapping the regulatory and catalytic domains between PKC isoforms to determine the role of each domain in isoform-specific function, and the catalytic domain of PKCδ was shown to be responsible for phorbol ester-induced cell differentiation (*118–120*).

Transgenic mice have been developed that overexpress PKCδ in basal epidermal cells under the control of the keratin 14 promoter, and that are resistant to skin tumor formation by phorbol ester (*121*). Furthermore, phorbol ester induced a several-fold increase of ornithine decarboxylase, the rate-limiting step enzyme for polyamine synthesis, and the administration of an irreversible inhibitor of ornithine decarboxylase, α-difluoromethylornithine, did not affect the skin tumor multiplicity in the transgenic mice (*122*). Therefore, PKCδ is involved both in tumor suppression and polyamine synthesis in epidermal cells, but these two signaling pathways appear to be independent.

Knockout mice. Recently, mice deficient in PKCδ were generated independently by two groups (*106, 123–126*). The knockout mice developed normally and were fertile (*123*) and viable up to twelve months (*126*). PKCδ is proposed to act as tumor suppresser (*93, 121, 127, 128*), although no obvious increase of cancer-induced death was observed in PKCδ-deficient mice (*126*). The PKCδ-null mice, however, showed increased proliferation of B lymphocytes and were prone to autoimmune disease (*126*). Also, the deficiency of PKCδ prevented B cell tolerance and allowed maturation and terminal differentiation of self-reactive B cells (*124*). These results suggest that PKCδ is involved in negative regulation of proliferation, especially the induction of tolerance in B cells. In PKCδ-deficient bone marrow-derived mast cells, a sustained Ca^{2+} mobilization and a high level of degranulation were observed, indicating that PKCδ reduces antigen-induced degranulation (*125*). In addition, severe arteriosclerotic lesions were found in the vein grafts of PKCδ-deficient animals, in which veins were isografted to carotid arteries (*123*). The increase of vascular smooth muscle cells, namely, decreased cell death, observed in the arteriosclerotic lesions suggested that PKCδ maintains homeostasis of smooth muscle cells, in particular by inducing apoptosis. It is rather unexpected that PKCδ-deficient mice show a clear phenotype only in certain cells, even though PKCδ is expressed ubiquitously.

PKCδ has a proapoptotic role in various cells (*73, 129–132*), and it is worth noting that PKCδ translocates to mitochondria to alter its function (*102, 133, 134*). PKCδ may

have a role in the regulation of apoptosis that is common to all cell types. In contrast, PKCδ is involved in growth regulation such as neuritogenesis (*135, 136*), shedding of the ectodomain of the heparin-binding epidermal growth factor-like growth factor (*137*), and the interleukin-induced transcription (*138*). In particular, PKCδ regulates the mitogen-activated protein kinase cascade (*136, 139–142*) and interacts with a novel protein kinase, DIK (*143*). Furthermore, the role of PKCδ in cell cycle regulation has been demonstrated (*144–147*). More detailed studies of the PKCδ-deficient mice will give important clues to elucidate the roles of PKCδ.

Conclusion

PKCδ is regulated by distinct molecular mechanisms: activation by diacylglycerol after serine and threonine phosphorylation at the motif sites, the formation of the active enzyme by tyrosine phosphorylation, and the generation of the catalytic fragment. This enzyme is, in other words, activated by the receptor-coupled mechanisms as well as in manners independent of membrane receptors. On the other hand, PKCδ contributes to both general and cell type-specific functions. It is interesting to assume that PKCδ activated by distinct mechanisms plays different roles, and thus further studies are required to investigate the functions of PKCδ in each signaling pathway.

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