# PKCδ inhibits PKCα-mediated activation of phospholipase D1 in a manner independent of its protein kinase activity

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**Abstract The regulation of phospholipase D1 (PLD1) by protein kinase C (PKC) isoforms was analyzed in human melanoma cell lines. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA)-induced PLD1 activation was suppressed by the introduction of PKCδ as well as its kinase-negative mutant in MeWo cells, which contain PKCα but lack PKCβ. PLD activity was not affected by PKCδ in G361 cells, which have PKCβ but are deficient in PKCα. In MeWo cells introduced by PKCα and PLD1, the association of these proteins was observed, which was enhanced by the TPA treatment. In cells overexpressing PKCδ in addition to PKCα and PLD1, TPA treatment increased the association of PKCδ and PLD1, while it attenuated the association of PKCα and PLD1. These results indicate that PKCδ inhibits TPA-induced PLD1 activation mediated by PKCα through the association with PLD1.**


*Key words:* Phospholipase D; Protein kinase C; 12-*O*-Tetradecanoylphorbol-13-acetate; Protein–protein interaction; Adenovirus vector; Melanoma cell

## 1. Introduction

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine to generate phosphatidic acid and choline, and its catalytic activity is regulated by a variety of agonists through heterotrimeric G protein-coupled and tyrosine kinase-type membrane receptors [1–4]. Phosphatidic acid and its dephosphorylated product, diacylglycerol, are important second messengers, and thus PLD is recognized to play a crucial role in the signal transduction. In mammals, two different PLD genes, PLD1 and PLD2, have been identified, which are expressed in many cell types. The regulation mechanism for PLD1 has been studied extensively, and protein kinase C (PKC) is proposed to mediate the link between receptor stimulation and PLD1 activation. PKC consists of multiple isoforms that are classified into three groups in mammals, cPKC (α, βI, βII, and γ), nPKC (δ, ε, η, and θ), and aPKC (ζ and λ) based on structural characteristics [5–6]. The cPKC and nPKC isoforms are activated by diacylglycerol produced from receptor-mediated hydrolysis of inositol phospholipids, and are the prime targets of tumor-promoting phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA). The α and δ isoforms of the cPKC and nPKC groups, respectively, are expressed universally among the cells and tissues.

The role of PKC in the regulation of PLD1 was implicated by studies that phorbol ester stimulates PLD activity, and that PKC inhibitors suppress the activation of PLD in intact cells [4]. Among the PKC isoforms, PKCα and PKCβ were identified to enhance PLD activity in response to TPA and receptor stimulation in cells [7–10]. On the other hand, activation of PLD1 was revealed to be independent of the protein kinase activity of PKC [11–13]. We have recently reported that PLD1 is dually regulated by the cPKC isoforms α and β through phosphorylation as well as via a protein–protein interaction using the human melanoma cell lines MeWo and G361, the former contains PKCα but lacks PKCβ and the latter has PKCβ but is deficient in PKCα [14].

Furthermore, Hornia et al. [15] have reported that PKCδ of the nPKC group controls PLD activity in intact cells. They show that rottlerin, an inhibitor of PKCδ, as well as the expression of a dominant-negative mutant of PKCδ, increases PLD activity in rat 3Y1 fibroblasts overexpressing the epidermal growth factor (EGF) receptor. Based on these results, they proposed that PLD activity is attenuated by PKCδ through phosphorylation upon EGF treatment. In this study, we investigated the involvement of PKCδ in the regulation of PLD1 using the melanoma cell lines, and revealed that PKCδ suppresses PKCα-mediated activation of PLD1 in a manner independent of its protein kinase activity.

## 2. Materials and methods

### 2.1. Cell culture

Human melanoma MeWo and G361 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan), and cultured in Eagle's minimal essential medium supplemented with 10% fetal calf serum.

### 2.2. Adenovirus vectors

The full-length rat PLD1 cDNA [16] was cloned into the pCMV5

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*Abbreviations:* PtdEtOH, phosphatidylethanol; PFU, plaque-forming unit; PKC, protein kinase C; PLD, phospholipase D; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

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vector with a FLAG-epitope tag to make the amino-terminally FLAG-epitope-tagged PLD1 (FLAG-PLD1). Recombinant adenovirus vectors of FLAG-PLD1, rabbit PKCα [17], mouse PKCδ [18], and a kinase-negative mutant of PKCδ replacing Lys-376 in the ATP-binding site by Ala (KN-PKCδ) [19] were prepared as described previously [14,20]. The cDNA clones of each PKC isoform from different mammalian species encode almost identical deduced amino acid sequences, and thus each PKC isoform is regarded to have properties indistinguishable among the species [5,6]. The adenovirus vectors containing FLAG-PLD1, PKCα, PKCδ, and KN-PKCδ were designated AxPLD1, AxPKCα, AxPKCδ, and AxKN-PKCδ, respectively. The adenovirus designated AxLacZ carrying the β-galactosidase gene (LacZ) from *Escherichia coli* [21] was used as a control virus.

### 2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA (2 μg) was reverse transcribed by using SuperScript II (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The following human PLD1-specific primers [22] were used: 5′-TGGGCTCACCATGAGAA-3′ (sense) and 5′-GTCATGCCAGGGCATCCGGGG-3′ (antisense). PCR was conducted in PCR Thermal Cycler MP (TaKaRa, Tokyo, Japan) in a 50 μl reaction volume using Hot Star Taq (Qiagen, Valencia, CA, USA). Reaction conditions were as follows: 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min for 35 cycles. An aliquot sample of each reaction (10 μl) was analyzed in 2% agarose gel and visualized by ethidium bromide fluorescence staining. Molecular weight markers (Promega, Madison, WI, USA) were used to estimate the size of the amplified fragments.

### 2.4. PLD assay

PLD activity was assayed by monitoring the in vivo transphosphatidylation activity as described previously [14]. Briefly, cells in six-well culture plates were infected with various plaque-forming units (PFU)/cell of adenovirus for 24 h, and then labeled with 1-[1-$^{14}$C]palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine (54.0 mCi/mmol, Amersham Pharmacia Biotech, Buckinghamshire, UK) (0.25 μCi/$10^7$ cells) for 16 h. Then, cells were stimulated with 100 nM TPA (Sigma, St. Louis, MO, USA) in the presence of 1% ethanol for 30 min, and the lipids were extracted and applied to thin layer chromatographic analysis. Radioactivity was quantitated by a Bioimaging analyzer (BAS 2000; Fuji Film, Tokyo, Japan), and PLD activity was expressed as a percentage of the radioactivity in phosphatidylethanol (PtdEtOH) relative to the total lipid radioactivity found in a given lane. Data are expressed as the mean ± S.E.M. ($n = 3$).

### 2.5. Immunoprecipitation and immunoblot analysis

Cells plated out in 10-cm tissue dishes were treated as for the PLD assay described above except for the labeling with the radioactive lipid. Immunoprecipitation and immunoblot analysis were carried out essentially as described previously [14]. Briefly, the cells were lysed in 20 mM Tris–HCl (pH 7.5) containing 1 mM EDTA, 1 mM EGTA, 10 mM 2-mercaptoethanol, 1% Triton X-100, 150 mM NaCl, 10 mM NaF, 1 mM sodium orthovanadate, and 50 μg/ml phenylmethylsulfonyl fluoride. The cell extract was incubated for 3 h at 0–4°C with the antibody against either PKCα (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or PKCδ (Transduction Laboratories, Lexington, KY, USA). Then, protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden) was added to the mixture and incubated for 1 h at 0–4°C with constant mixing. In the case of immunoprecipitation of FLAG-PLD1, anti-FLAG M2 affinity gel (Sigma) was employed instead of protein A-Sepharose. Where indicated, control mouse IgG (Wako Pure Chemical Industries, Osaka, Japan) or protein A-Sepharose incubated with control mouse IgG was used. The immunoprecipitated proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto an Immobilon P membrane (Millipore, Bedford, MA, USA). Immunoblot analysis was carried out using antibodies against PKCα, PKCβ (Transduction Laboratories), PKCδ, and FLAG-epitope tag (M2, Sigma) as the primary antibodies. The alkaline phosphatase-conjugated anti-mouse antibody (Promega) was employed as the secondary antibody using 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium as substrates. Where indicated, the lysates from melanoma cells were directly subjected to immunoblot analysis, and rat brain lysate (Transduction Laboratories) was employed as a marker of PKC isoforms.

## 3. Results and discussion

It has been reported that the melanoma cell lines MeWo and G361 express PKCα and PKCβ of the cPKC, respectively [23]. In contrast, PKCδ was detected in both these melanoma cells (Fig. 1A). The expression of PLD1 in MeWo and G361 cells was investigated by RT-PCR (Fig. 1B). The specific primers to human PLD1 amplified two sequence signals of 533 and 638 bp from both cell lines. These bands derive from the alternatively spliced forms of human PLD1a and PLD1b, respectively [22]. The role of PKCδ in the regulation of PLD activation was examined in these melanoma cell lines (Fig. 2). Without the infection of adenovirus vector for PKCδ, the basal levels of PtdEtOH, the PLD reaction product, were similar in both melanoma cell lines, and the TPA treatment induced PLD activation more efficiently in MeWo cells than in G361 cells (Fig. 2A), as described previously [14]. In MeWo cells, the introduction of PKCδ using the adenovirus vector suppressed the TPA-stimulated PLD activity in a dose-dependent manner, whereas the TPA-stimulated phospholipase activity was not affected by the introduction of PKCδ in G361 cells. In G361 cells infected with adenovirus vector for PKCα, the introduction of PKCδ attenuated the elevation of PLD activity stimulated by TPA (Fig. 2B). These results indicate that PKCδ prevents PLD1 activation mediated by PKCα.

PKCα is revealed to regulate PLD1 through phosphorylation as well as via a protein–protein interaction in vivo [14]. Therefore, the mechanism for suppression of PLD1 activation by PKCδ was analyzed by expressing KN-PKCδ in MeWo cells (Fig. 3). Introduction of PKCα strongly elevated the basal and TPA-stimulated PLD activity as described previously [18], and this potentiation was suppressed by co-introduction with PKCδ as well as KN-PKCδ (Fig. 3A). KN-PKCδ attenuated the elevation of TPA-stimulated PLD activity even more efficiently than the wild type enzyme. In the cells overproducing PLD1, PLD activity was increased and the introduction of PKCδ and KN-PKCδ showed almost identical effects on PLD activity as in the cells without infection of adenovirus vector for PLD1 (Fig. 3B). The protein levels of the recombinant PLD1 were, however, not altered by overexpression of PKCδ and KN-PKCδ. These results support that the attenuation of the elevation of PLD activity in these cells reflects suppression of PKCα-mediated PLD1 activation rather than the reduction of the PLD1 protein levels.

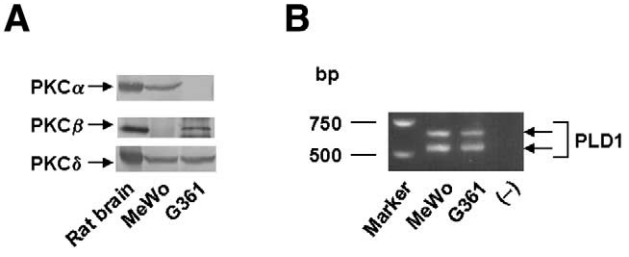


Fig. 1. PKC isoforms and PLD1 in melanoma cell lines. A: Cell lysates of melanoma cells were subjected to immunoblot analysis using antibodies against either PKCα, PKCβ, or PKCδ. Rat brain lysate was used as a positive control. B: Total RNA prepared from melanoma cells was subjected to RT-PCR using specific primers for human PLD1. The control lane without the template is indicated as (−). The sizes of the molecular weight markers are shown in bp, and the positions of the amplified fragments are indicated by arrows.

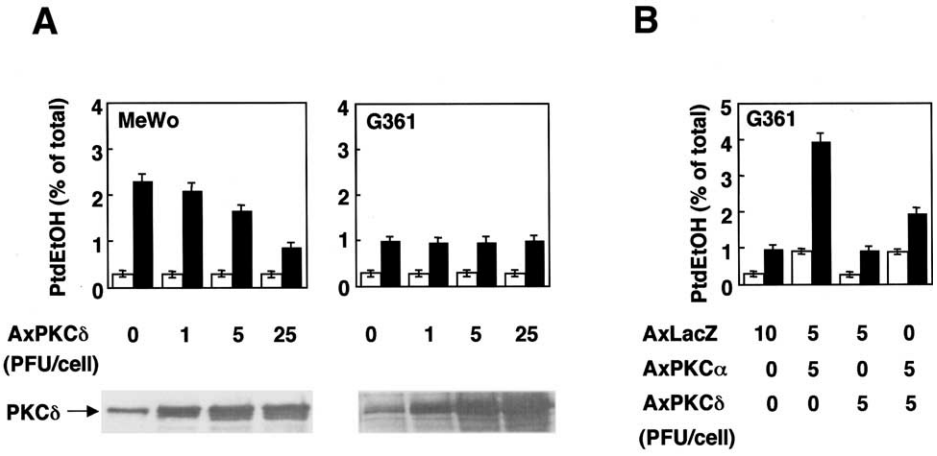


Fig. 2. Effect of overexpression of PKCδ on TPA-induced PLD activation. A: MeWo (left) and G361 (right) cells were infected with different PFU/cell of AxPKCδ. The expression of PKCδ was examined by immunoblot analysis after immunoprecipitation using the antibody against PKCδ. B: G361 cells were infected with AxPKCα and AxPKCδ. PLD activity was assayed after incubation in the presence (solid bar) and absence (open bar) of TPA.

Thus, PKCδ was concluded to repress the PKCα-mediated elevation of PLD activity in a manner independent of its protein kinase activity. Goerke et al. [24] have shown that TPA induces apoptosis in A7r5 vascular smooth muscle cells overexpressing the wild type PKCδ as well as KN-PKCδ. It is therefore plausible that PKCδ can have physiological roles that are independent of its catalytic activity, and thus are likely to be occurring through protein–protein interaction. Hornia et al. [15] have reported that the expression of a dominant-negative mutant of PKCδ elevates PLD activity in the fibroblast cell line. The mutant in their study should have behaved simply as a kinase-negative molecule rather than preventing the role of endogenous PKCδ for the regulation of PLD1. It is known that dominant-negative mutants of PKC isoforms show a broad specificity [25]. For example, the dominant-negative mutant of PKCδ generated by replacing its

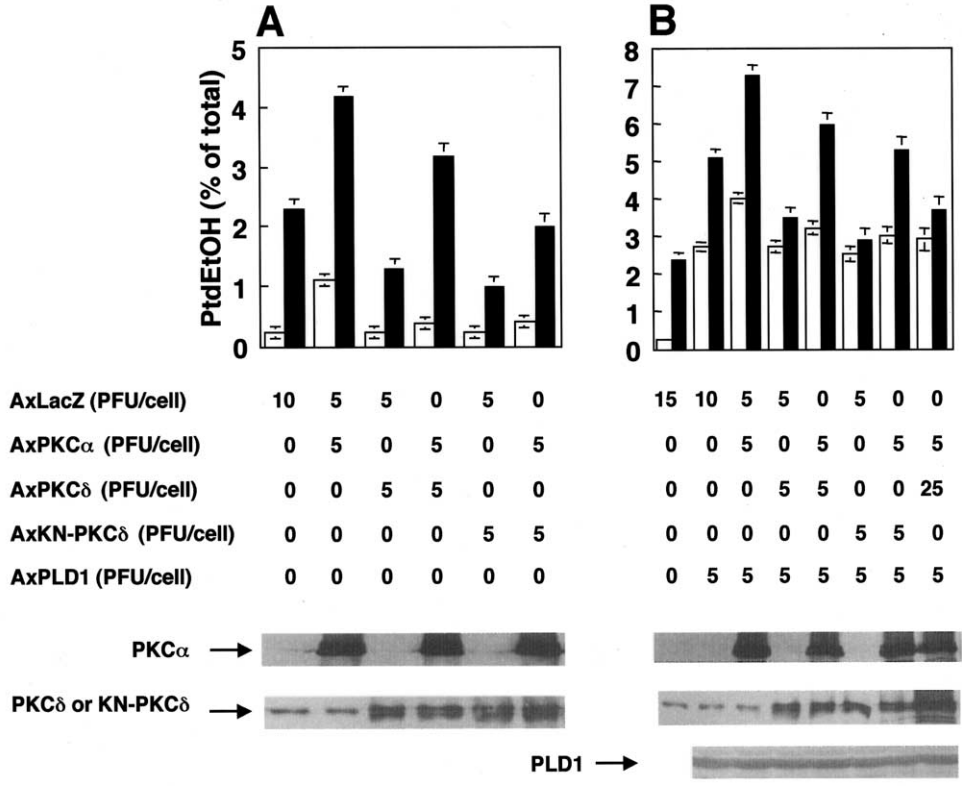


Fig. 3. Suppression of TPA-induced PLD activation by overexpression of PKCδ and KN-PKCδ. MeWo cells were infected with AxPKCα, AxPKCδ, and AxKN-PKCδ in the presence (A) or absence (B) of AxPLD1. The expressions of PKCα, PKCδ and KN-PKCδ, and PLD1 were examined by immunoblot analysis after immunoprecipitation by using the antibodies against PKCα, PKCδ, and FLAG-epitope tag, respectively. PLD activity was assayed after incubation in the presence (solid bar) and absence (open bar) of TPA.

phosphorylation motif sites is capable of inhibiting PKCα activity. In a previous study, however, the kinase-negative PKCα was revealed to enhance PLD activity as the wild type PKCα [14]. Thus, it is less possible that the kinase-negative PKCδ prevents the role of PKCα, because the kinase-negative PKCα itself does not work as a dominant-negative. The kinase-negative PKC isoforms employed in this study replacing the Lys residue in the ATP-binding site may have a property distinct from that of the molecules mutating the phosphorylation motif sites.

To analyze further the mechanism of PLD inhibition by PKCδ, the association of the PKC isoforms with PLD1 was examined in MeWo cells infected with adenovirus vectors (Figs. 4 and 5). In cells co-introduced with PKCα and PLD1, PKCα was detected in the immunoprecipitate of PLD1 and this association was increased upon TPA stimulation (Fig. 4A). The result was confirmed by the immunoprecipitation of PKCα followed by immunoblot analysis using anti-FLAG antibody (Fig. 4B). Effect of PKCδ on the interaction of PKCα and PLD1 was studied by introducing PLD1, PKCα, and PKCδ simultaneously in MeWo cells (Fig. 5). Not only PKCα but also PKCδ was associated with PLD1 in the TPA-treated cells, and a high dose of the PKCδ vector decreased the interaction of PKCα with PLD1, while association of PKCδ with PLD1 further increased in the cells. In the cells overexpressing PKCα, PKCδ, and PLD1, the association of PLD1 and PKC isoforms was observed in cells in the absence

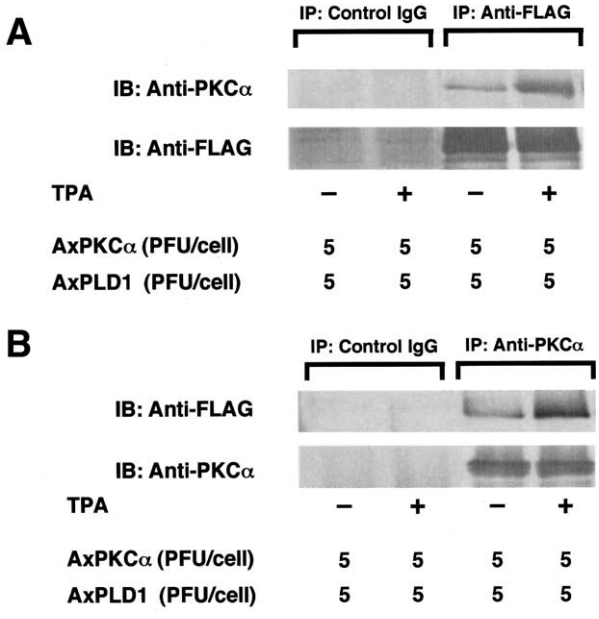


Fig. 4. Association of PKCα to PLD1 in MeWo cells. The cells co-infected with AxPKCα and AxPLD1 were incubated in the presence or absence of TPA. A: The cell lysates were subjected to immunoprecipitation (IP) using the anti-FLAG M2 affinity gel (Anti-FLAG) and immunoblot analysis (IB) was carried out using the antibodies against either PKCα (Anti-PKCα) or FLAG (Anti-FLAG). B: The cell lysates were subjected to immunoprecipitation (IP) using the antibody against PKCα (Anti-PKCα) and immunoblot analysis (IB) was carried out using the antibodies against either FLAG (Anti-FLAG) or PKCα (Anti-PKCα). Control mouse IgG or control mouse IgG coupled with protein A-Sepharose (Control IgG) was employed as a control. The results shown are representative of three independent experiments.

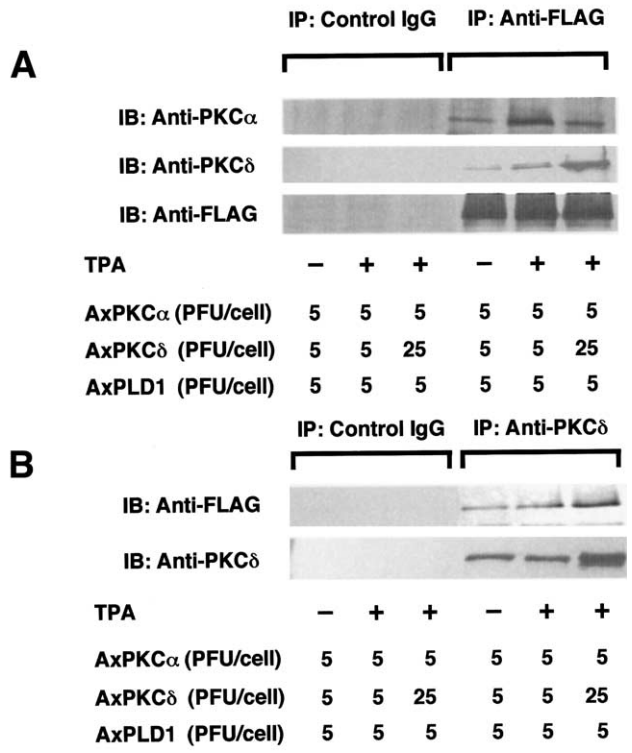


Fig. 5. Association of PKCδ with PLD1 in MeWo cells. The cells co-infected with AxPKCα, AxPLD1 and different doses of AxPKCδ were incubated in the presence or absence of TPA. The association of PKC isoforms with PLD1 was analyzed as in Fig. 4.

of TPA stimulation (Figs. 4 and 5). Complex formation of PKCα and PLD1 has been demonstrated in unstimulated fibroblasts [26]. A part of the PLD1 molecules endogenously expressed in cells may associate with PKC isoforms under resting conditions.

It is established that PKCα is a common and predominant regulator of PLD1, which activates the phospholipase through mechanisms dependent on and independent of phosphorylation. In the present study, we investigated the role of PKCδ in the regulation of PLD1. It was revealed that PKCδ suppresses PKCα-mediated PLD1 activation through protein–protein interaction with PLD1. It is interesting to assume that PLD1 is free from both PKCα and PKCδ in unstimulated cells, and PKCα associates with PLD1 whereas PKCδ prevents the binding in a competitive manner upon treatment of the cells with agonists. Another possibility is that PLD1 is associated with PKCδ even under resting conditions, and cell stimulation induces the binding of PKCα to PLD1. On the other hand, these two PKC isoforms, which are both expressed ubiquitously among various cells [5,6], have been reported to have opposite effects on cell growth and apoptosis [27]. It may be a common way that PKCα and PKCδ contribute distinctly to various signaling processes.

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