Novel regulation of protein kinase C-η

Deepanwita Pal, **Shalini Persaud Outram**[1], and **Alakananda Basu**[*]
Department of Molecular Biology & Immunology, University of North Texas Health Science Center, Fort Worth, Texas, 76107, USA

Abstract

Protein kinase C (PKC) is the receptor for tumor promoting phorbol esters, which are potent activators of conventional and novel PKCs, but persistent treatment with phorbol esters leads to downregulation of these PKCs. However, PKCη, a novel PKC isozyme, resists downregulation by tumor-promoting phorbol esters, but little is known about how PKCη level is regulated. Phosphorylation and dephosphorylation play an important role in regulating activity and stability of PKCs. In the present study, we have investigated the molecular mechanism of PKCη regulation. Several PKC activators, including phorbol 12, 13-dibutyrate, 12-*O*-tetradecanoylphorbol-13-acetate and indolactam V caused upregulation of PKCη whereas the general PKC inhibitor Gö 6983, but not the conventional PKC inhibitor Gö 6976 led to the downregulation of PKCη. Upregulation of PKCη was associated with an increase in phosphorylation of PKCη. Silencing of phosphoinositide-dependent kinase-1, which phosphorylates PKCη at the activation loop, failed to prevent PKC activator-induced upregulation of PKCη. Knockdown of PKCε but not PKCα inhibited PKC activator-induced upregulation of PKCη. Thus, our results suggest that the regulation of PKCη is unique and PKCε is required for the PKC activator-induced upregulation of PKCη.

Keywords

PKCη; PKCε; tumor promoter; upregulation; phosphorylation

1. Introduction

Protein kinase C, a family of phospholipid-dependent serine/threonine kinases, plays a critical role in signal transduction and cell regulation [1,2]. On the basis of their structural features, the PKC family is categorized into three groups, conventional (α, βI, βII, and γ), novel (δ, ε, η, θ) and atypical (ζ, λ/ι). While conventional PKCs require Ca^{2+} and diacylglycerol (DAG) for their activities, novel PKCs are Ca^{2+}-insensitive but DAG-dependent whereas atypical PKCs are insensitive to both Ca^{2+} and DAG [1]. PKC isozymes differ in biochemical properties, tissue-specific distribution and intracellular localization. Most cells express multiple PKC isozymes and they exhibit overlapping as well as distinct functions [3].

[*]Corresponding author. Tel.: +1 817 735 2487; fax: +1 817 735 2118. Alakananda.Basu@unthsc.edu (A. Basu)..
[1]Present Address: Department of Cell Biology and Molecular Medicine, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, NJ 07103, USA

PKC serves as the receptor for tumor-promoting phorbol esters, which are potent activators of conventional and novel PKCs, and can substitute for the physiological activator DAG [3,4]. Sustained stimulation of PKCs by phorbol esters, such as TPA, has implicated the PKC isozymes in tumor promotion [2,5]. Prolonged treatment with tumor-promoting phorbol esters eventually leads to the downregulation of the phorbol ester-sensitive PKCs [6]. Both activation and downregulation of PKCs have been implicated in regulating cellular functions.

PKCs are not only subject to regulation by cofactors, but also via phosphorylation [3]. PKCs are phosphorylated at the conserved residues in the activation loop, turn motif and hydrophobic motif. The phosphorylation of PKCs primes them for activation and regulates their stability and subcellular localization [3,5,7,8]. PKCs are regulated by both autophosphorylation [9] and transphosphorylation [10]. It is generally believed that the priming phosphorylation of PKC occurs at the activation loop by phosphoinositide-dependent kinase-1 (PDK1) and is followed by autophosphorylation at the turn and the hydrophobic motifs [6]. Recent studies, however, suggest that PKCs may also be transphosphorylated by other members of the PKC family [3,6,7,11]. For example, PKCδ has been shown to be transphosphorylated by PKCε and *vice versa* [12]. This cross-regulation of PKCs may be an important way to integrate signals by various PKC isozymes.

PKCη is a member of the novel PKC isozymes that regulates cell proliferation, differentiation, secretion and apoptosis [13,14,15,16,17]. It is primarily expressed in epithelial cells and shares highest homology with PKCε [18]. PKCη is upregulated in breast cancer tissues [19] and overexpression of PKCη has been associated with resistance to chemotherapeutic agents [17,20,21,22,23,24]. Although PKCs have been implicated in tumor promotion, PKCη is the only phorbol ester-sensitive PKC isozyme that resists downregulation upon prolonged treatment with phorbol esters [20,25,26]. Little is known about the unique regulation of PKCη. In the present study, we have investigated the mechanism by which PKCη level is regulated. Our results indicate that in contrast to conventional and novel PKCs, which undergo downregulation following persistent treatment with PKC activators, PKCη is upregulated in response to PKC activators and is downregulated upon treatment with PKC inhibitors. We demonstrate for the first time that the PKC activator-induced upregulation of PKCη is regulated by PKCε, another member of the novel PKC family.

2. Materials and Methods

2.1. Materials

PDBu and TPA were purchased from Alexis Biochemicals (San Diego, CA). ILV was obtained from LC Laboratories (Woburn, MA) and Sigma (St. Louis, MO). Gö 6983 and Gö 6976 were purchased from Calbiochem (San Diego, CA). Polyclonal antibodies to PKCη, PKCδ and PKCε were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal antibody against PDK1 was purchased from Cell Signaling Technology, Inc. (Danvers, MA). Monoclonal antibody to PKCα was obtained from Upstate Biotechnology (Lake Placid, NY) and monoclonal antibody to PKCι was from BD Transduction Laboratories (San Jose, CA). Monoclonal antibody against actin was obtained from Sigma (St. Louis, MO). Horseradish-peroxidase-conjugated donkey anti-rabbit and goat anti-mouse secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). [^{32}P]Orthophosphate was purchased from PerkinElmer, Inc. (Waltham, MA). Poly(vinylidenedifluoride) membrane was obtained from Millipore (Bedford, MA). Enhanced chemiluminescence detection kit was purchased from Amersham (Arlington Heights, IL).

2.2. Cell culture

Breast cancer cells were maintained in RPMI medium supplemented with 10% fetal bovine serum and 2 mM glutamine. Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified minimal essential medium supplemented with 10% fetal bovine serum and 2 mM glutamine. Cells were kept in a humidified incubator at 37°C with 95% air and 5% CO_2.

2.3. Transfection

Control non-targeting siRNA or SMARTpool siRNA against PKC isozymes, and PDK1 were introduced into MCF-7 or T47D cells using Lipofectamine 2000 or Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA) and manufacturer's protocol. 48 h following siRNA transfection, cells were treated as indicated in the text and processed for Western blot analysis.

2.4. Reverse Transcriptase PCR

MCF-7 cells were treated with or without PDBu, ILV or Gö 6983 for 16 h. Total RNA was extracted using TRI Reagent from Molecular Research Center, Inc. (Cincinnati, OH). cDNA was synthesized using random primers and Improm II reverse transcriptase from Promega (Madison, WI). PCR amplification of cDNA was performed using Promega PCR Master Mix (Madison, WI), PKCη and β-actin primers. The sequences of forward and reverse PKCη primers were 5'-ATGCGGTGGAACTTGCCA-3' and 5'-CGTGACCACAGAGCATCTCATAGA-3' respectively. The sequences of the forward and reverse β-actin primers were 5'-ACCCAGCACAATGAAGATCA-3' and 5'-GCGCAAGTTAGGTTTTGTCA-3'. After PCR cycling, a 750 bp product for PKCη and 800-bp product for β-actin was detected by gel electrophoresis.

2.5. Immunoblot Analysis

Cells were lysed in extraction buffer containing 1 mM DTT, protease inhibitors and phosphatase inhibitors. Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred on PVDF membranes. Western blot analysis was performed as described before [17].

2.6. Metabolic labeling

HEK293T cells were transiently transfected with either pcDNA3 or vector containing PKCη construct and radiolabeled with [^{32}P]orthophosphate. Cells were treated with or without PDBu and immunoprecipated with either rabbit IgG or anti-PKCη antibody. Immunocomplexes were processed as described previously [27] and subjected to SDS-PAGE and autoradiography.

3. Results

3.1. Effect of PKC activators and inhibitors on PKCη levels

We have previously demonstrated that persistent treatment with phorbol 12, 13-dibutyrate (PDBu) caused upregulation of PKCη in MCF-7 breast cancer cells [20]. In the present study, we compared the effect of several structurally and functionally distinct PKC activators on PKCη level. While PDBu and TPA belong to the same class of compounds, indolactam V (ILV) is structurally distinct from phorbol esters. All three PKC activators caused substantial upregulation of PKCη (Fig. 1A and 1B). Based on the densitometric quantification of several independent experiments, PKC activators caused a significant increase in PKCη level (Fig. 1B). PKCη appeared as a doublet in the Western blot since it contains two major transcription initiation sites [29]. Prolonged treatment with PDBu and

TPA caused downregulation of conventional PKCα and novel PKCδ although PKCε was less susceptible to PKC activator-induced downregulation (Fig. 1A). The level of phorbol ester-insensitive atypical PKCι remained unaltered, as expected (Fig. 1A). Consistent with our earlier reports, ILV had little effect on the downregulation of PKCα [28]. Thus, the regulation of PKCη is unique in comparison to other conventional and novel PKCs.

Since PKC activators led to PKCη upregulation, we examined whether PKC inhibitors would induce downregulation of PKCη. We compared the effects of the general PKC inhibitor Gö 6983 and conventional PKC inhibitor Gö 6976. Gö 6983 but not Gö 6976 caused substantial downregulation of PKCη (Fig. 1C and 1D). The levels of PKC-α, -δ and -ε were not decreased by Gö 6983 treatment (Fig. 1C). The general PKC inhibitor bisindolylmaleimide also induced selective downregulation of PKCη (data not shown). Since atypical PKCs are phorbol ester-insensitive, these results suggest that the level of PKCη may be regulated by novel PKCs.

To determine if PKC activators and inhibitors alter PKCη expression at the mRNA level, we treated MCF-7 cells with PDBu, ILV or Gö 6983 and examined the mRNA expression by reverse-transcriptase PCR. As shown in Fig. 2A, the treatment of MCF-7 cells with PKC activators and inhibitors did not alter the mRNA expression of PKCη. Taken together, these results suggest that PKCη level is altered at the post-transcriptional level following treatment with PKC activators and inhibitors.

3.2. Effect of PKC activator and inhibitor on PKCη phosphorylation

Since persistent treatment with PKC activators cause activation of PKCs followed by dephosphorylation and downregulation of PKCs, we examined if upregulation of PKCη by PKC activators was associated with an increase in PKCη phosphorylation. We introduced PKCη in HEK293T cells, labeled with [^{32}P]orthophosphate and immunoprecipitated PKCη following treatment with or without PDBu. We did not detect a phosphorylated band corresponding to PKCη in vector-transfected HEK293T cells (Fig. 2B). PKCη was constitutively phosphorylated in HEK293T cells expressing wild-type PKCη and PDBu further increased the level of phospho-PKCη (Fig. 2B). The densitometric scanning from three separate experiments indicated a significant increase in the phosphorylation status of PKCη in response to PDBu (Fig. 2C). These results suggest that upregulation of PKCη is associated with an increase in PKCη phosphorylation.

3.3. Regulation of PKCη level by transphosphorylation

PDK1 is believed to phosphorylate the activation loop of AGC kinases, including PKC isozymes [7,8]. Recent evidence has also implicated PDK1 in phosphorylating PKCη at the activation loop [30]. We therefore examined whether PDK1 was involved in the activator-induced upregulation of PKCη. Fig. 3A shows that the silencing of PDK1 by siRNA decreased the basal level of PKCη but had little effect on the upregulation of PKCη by phorbol esters.

Since cross-regulation of PKC isozymes by other PKC family members has been suggested by several studies [11,12,31], we examined if the knockdown of a particular PKC isozyme affects PKCη level. As shown in Fig. 3B, the depletion of conventional PKCα had little effect on phorbol ester-induced upregulation of PKCη. While knockdown of PKCδ had a modest effect, the knockdown of novel PKCε substantially decreased the ability of PKC activators to enhance PKCη level in both MCF-7 (Fig. 4A) and T47D (Fig. 4B) cells. These results suggest that transphosphorylation of PKCη by novel PKCε may be responsible for PKCη upregulation.

4. Discussion

The results of our present study demonstrate that the regulation of PKCη is unique compared to other PKC isozymes. Although tumor-promoting phorbol esters are potent activators of conventional and novel PKCs [4], persistent treatment with phorbol esters leads to the downregulation of phorbol ester-sensitive PKCs causing termination of PKC signaling [32]. Downregulation of PKCs has important implications in regulating long-term cellular responses, such as cell proliferation, differentiation and tumor promotion [2,13]. We have shown that in contrast to other PKCs, prolonged treatment with PKC activators led to upregulation of PKCη whereas PKC-specific inhibitors triggered downregulation of PKCη. Furthermore, we made a novel observation that novel PKCs are involved in PKC activator-induced upregulation of PKCη.

It is generally believed that treatment with PKC activators leads to membrane translocation of PKCs followed by dephosphorylation [33]. The dephosphorylated PKCs are subject to downregulation by proteases [34]. However, fully phosphorylated PKCα was shown to be downregulated at the plasma membrane via the proteasome-mediated pathway [35]. In addition, the phosphorylated primed form of PKCε was downregulated by phorbol ester treatment independent of its intrinsic kinase activity [36]. It has been reported that active conformation of PKCη is necessary for its downregulation in baby hamster kidney (BHK) cells although TPA failed to downregulate PKCη in these cells [37]. Our results show that prolonged treatment with structurally distinct PKC activators, such as phorbol esters and ILV, caused an upregulation of PKCη in MCF-7 cells (Fig. 1A and 1B). The upregulation of PKCη by PKC activators was not unique to MCF-7 cells, and was observed in several cell types, including T47D, BT-20 and MCF-10CA1d cells (Fig. 4A, 4B and data not shown).

The activity, maturation, stability and localization of PKCs are regulated by phosphorylation and dephosphorylation events [7,8]. Our study suggests that PKCη level is regulated by phosphorylation. First, treatment with several PKC activators, such as TPA, PDBu and ILV induced upregulation of PKCη (Fig. 1A and 1B) but this upregulation was not associated with an increase in PKCη mRNA (Fig. 2A). Second, PKC-specific inhibitors Gö 6983 (Figs. 1C and 1D) and bisindolylmaleimide (data not shown) led to downregulation of PKCη. Third, upregulation of PKCη by PDBu was associated with an increase in PKCη phosphorylation (Fig. 2B and 2C).

Phosphorylation of PKCs is regulated by both autophosphorylation and transphosphorylation [3] and phosphorylation of PKCs at the activation loop is believed to prime them for activation [8]. Phosphoinositide-dependent kinase-1 (PDK1) has been shown to phosphorylate PKCs, at the activation loop and contributes to the stability of cPKCs and PKCε [32,33,38] PDK1 was also shown to phosphorylate PKCη at the activation loop [30]. However, knockdown of PDK1 did not prevent PKC activator-induced upregulation of PKCη (Fig. 3A).

PKCs can also undergo transphosphorylation by other members of the PKC family [3,12]. For example, PKCε rather than PDK1 was shown to phosphorylate PKCδ and PKCε at the activation loop whereas PKCδ induced autophosphorylation as well as transphosphorylation of PKCε at the hydrophobic motif [12]. We recently reported that depletion of PKCε enhanced PDBu-induced downregulation of PKCδ in HeLa cells [11]. Since the general PKC inhibitor Gö 6983 but not the conventional PKC inhibitor Gö 6976 induced PKCη downregulation (Fig. 1C and 1D) and atypical PKCs are phorbol ester insensitive, it is likely that PKCη is also regulated by novel PKC isozyme(s). Consistent with this notion, we found that depletion of cPKCα had little effect on PKC activator-induced upregulation of PKCη whereas knockdown of nPKCε attenuated PKCη upregulation (Fig. 4A and 4B).

The observation that PKCη is the only PKC isozyme upregulated by tumor-promoting phorbol esters suggests that PKCη may play an important role in tumorigenesis. Depending on the cellular context, PKCη may suppress tumorigenesis or promote malignant cell growth. For example, PKCη knockout mice were more susceptible to tumor promotion in two-stage skin carcinogenesis model [39]. In contrast, PKCη has also been implicated in breast cancer [19,20], glioblastoma [21], Hodgkin's lymphoma [40], lung cancer [22,41] and hepatocellular carcinoma [42]. This contrasting function of PKCη in different cell types is not unique to PKCη and has been noted with other novel PKCs, such as PKCδ [5] and PKCε [43]. PKCη is often overexpressed in breast cancer [19] and the level of PKCη is upregulated by estradiol in hormone-sensitive breast cancer cells [44]. Moreover, overexpression of PKCη confers resistance to chemotherapeutic drugs [17,20,21,22,23,24]. Thus, understanding the mechanism of PKCη upregulation has significant implications in cancer therapy.

Acknowledgments

The abbreviations used are

DAG	diacylglycerol
ILV	indolactam V
PDBu	phorbol 12,13-dibutyrate
PDK1	phosphoinositide-dependent protein kinase-1
PKC	protein kinase C
aPKC	atypical PKC
cPKC	conventional PKC
nPKC	novel PKC
siRNA	short interfering RNA
TPA	12-*O*-tetradecanoylphorbol 13-acetate

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Highlights

- The regulation of PKCη is unique

- PKCη is upregulated by PKC activators and downregulated by PKC inhibitors

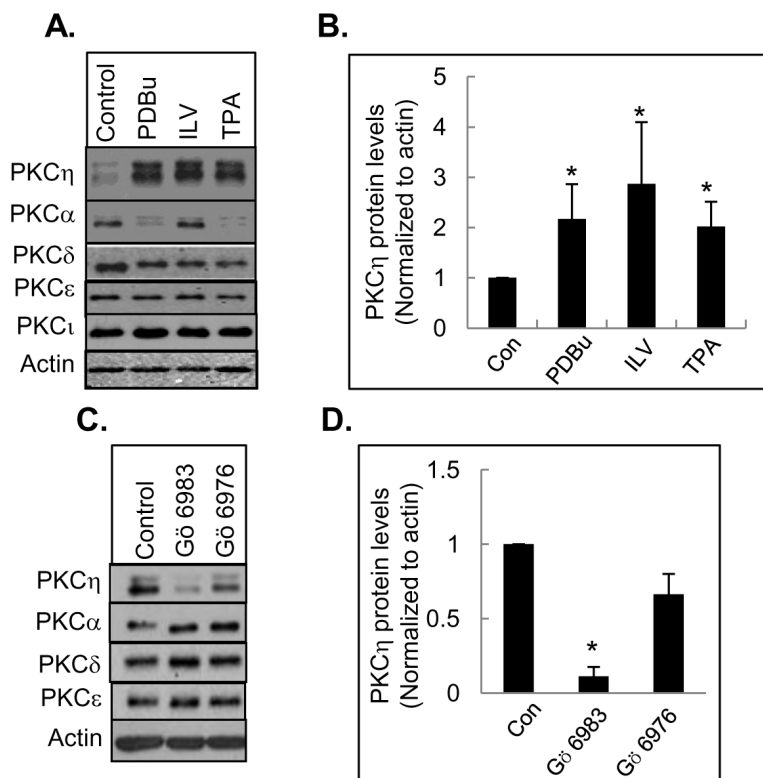- PKC activator-induced upregulation of PKCη involves novel PKCε

Fig. 1.
Effects of PKC activators and inhibitors on PKCη level. MCF-7 cells were treated with 1
μM PDBu, 10 μM ILV and 100 nM TPA for 15 h. (A) Western blot analysis was performed
with total cell extract and probed with the indicated antibodies. Actin was used as a loading
control. (B) Densitometric quantification of PKCη protein level from 3 separate experiments
corrected for loading. Data represents the mean +/− s.e.m. The asterisk (*) indicates
significant difference from control (P<0.05) using paired Student's t-test. (C) MCF-7 cells
were treated with 1 μM Gö 6983 for 15 h. Total cell lysates were subjected to SDS-PAGE
and Western blot analysis was performed using the indicated antibodies. (D) Densitometric
quantification of PKCη protein expression from 3 separate experiments corrected for
loading. Data represents the mean +/− s.e.m. The asterisk (*) indicates significant difference
from the control (P<0.05) using paired Student's t-test.
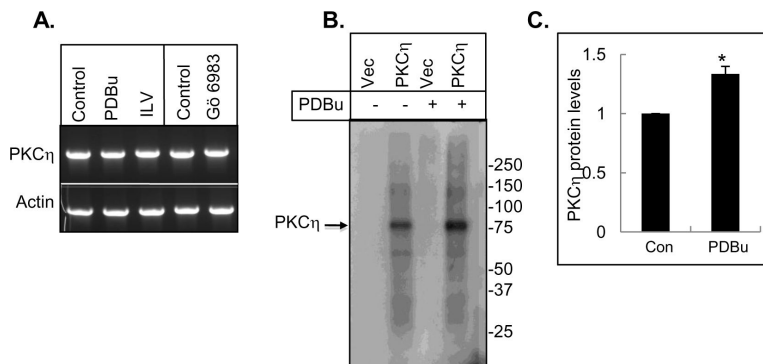
Fig. 2.
Effects of PKC modulators on PKCη mRNA expression and phosphorylation. (A) MCF-7 cells were treated with 1 µM PDBu, 10 µM ILV or 1 µM Gö 6983 for 16 h. Total RNA was extracted and cDNA was synthesized by reverse transcriptase reaction. PKCη and β-actin cDNA were amplified by PCR and electrophoresed. Results are representative of at least 2 independent experiments. (B) HEK293T cells expressing empty vector or vector containing PKCη were radiolabeled with [^{32}P]orthophosphate and immunoprecipitated with PKCη following treatment with or without PDBu. The arrow indicates PKCη. (C) Densitometric quantification of PKCη protein level from 3 separate experiments corrected for loading. Data represents the mean +/− s.e.m. The asterisk (*) indicates significant increase with PDBu treatment (P<0.05) using paired Student's t-test.

A.

siRNA	Con			PDK1		
Treatment	Con	PDBu	TPA	Con	PDBu	TPA
PKCη						
PKCα						
PKCε						
PDK1						
Actin						

B.

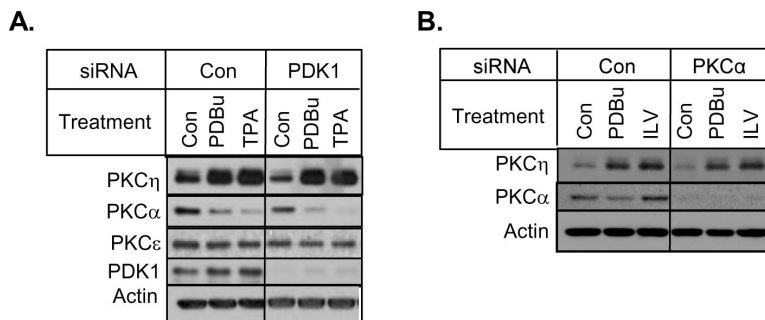| siRNA	Con			PKCα		
Treatment	Con	PDBu	ILV	Con	PDBu	ILV
PKCη						
PKCα						
Actin						

Fig 3.
Effect of PDK1 and PKCα knockdown on PKCη upregulation. (A) and (B) MCF-7 cells were transfected with the indicated siRNAs and then treated with or without 1μM PDBu, 100 nM TPA or 10 μM ILV for 16 h. Western blot analysis was performed with indicated antibodies. Actin was used as a loading control. Results are representative of 3 independent experiments.
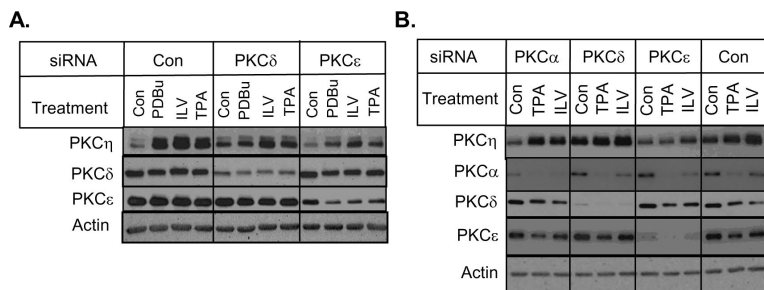
A.

siRNA	Con				PKCδ				PKCε			
Treatment	Con	PDBu	ILV	TPA	Con	PDBu	ILV	TPA	Con	PDBu	ILV	TPA

PKCη
PKCδ
PKCε
Actin

B.

siRNA	PKCα			PKCδ			PKCε			Con		
Treatment	Con	TPA	ILV	Con	TPA	ILV	Con	TPA	ILV	Con	TPA	ILV

PKCη
PKCα
PKCδ
PKCε
Actin

Fig. 4.
Effects of novel PKC isozyme knockdown on PKCη upregulation. MCF-7 (A) and T47D (B) cells were transfected with indicated siRNAs. Cells were treated with or without 1 μM PDBu, 10 μM ILV or 100 nM TPA for 16 h. Western blot analyses were performed using indicated antibodies. Results are representative of 3 independent experiments.