Pal, Deepanwita, Protein kinase C-eta signaling in breast cancer. Doctor of Philosophy (Biochemistry and Molecular Biology), November, 2013, 117 pp, 14 illustrations, 260 references

Protein kinase C-eta (PKCη) is a novel member of the PKC family that is important for several cellular processes. PKCη is overexpressed in breast cancer and has been associated with chemotherapeutic resistance. PKCη is the only phorbol ester-sensitive PKC isozyme that resists downregulation upon prolonged treatment with tumor-promoting phorbol esters suggesting its unique regulation. The purpose of this dissertation is to elucidate the mechanism of PKCη regulation and its functional relevance in breast cancer. We have shown that PKCη is upregulated by several structurally and functionally distinct PKC activators in contrast to other PKC isozymes. Activator-induced upregulation of PKCη was associated with its phosphorylation. Our results indicate that novel PKCs are involved in the upregulation of PKCη by PKC activators. We also made a novel observation that PKCη is downregulated via two distinct mechanisms. While inhibition of PKC caused the downregulation of PKCη via proteasome-independent pathway, inhibition of PDK1 led to PKCη downregulation via proteasome-dependent pathway. We further demonstrated that PKCη is important for the growth and survival of breast cancer cells. The unique regulation of PKCη and its implications on breast cancer growth and survival suggests that this pathway could be selectively exploited for targeted therapies for breast cancer.

PROTEIN KINASE C-ETA SIGNALING IN BREAST CANCER

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Deepanwita Pal, M.S.

Fort Worth, Texas

November, 2013

ACKNOWLEDGEMENTS

Groucho Marx once said “Life is a whim of several billion cells to be you for a while.” For the past few years, my several billion cells were on an expedition to study other sets of several billion cells and it has culminated in a profound journey, for which I am thankful to a lot of people. I would like to express my deepest gratitude to my advisor, Dr. Alakananda Basu for having provided me the opportunity to work in her laboratory. It has been an immensely fruitful experience for which I am indebted to her. I greatly value the training and the lessons that I have learned during my stay here. I would like to acknowledge all my committee members for their advice and encouragement – Drs. Laszlo Prokai, Michael Forster, Raghu Krishnamoorthy and Yogesh Awasthi for guiding me throughout my graduate studies and Dr. Meharvan Singh for his willingness to serve on my committee and his enthusiasm to understand my circuitous project at the last moment.

I have been fortunate to have had the company of wonderful labmates. I am grateful to Drs. Qiang Zeng, Shalini Persaud Outram and Eswar Shankar for training me. In particular, I am thankful to Shalini for her instrumental help with my project. Working in the lab has been all the more worthwhile thanks to the helpful discussions and the entertaining chats with Ms. Kirti Jain, Dr. Soumya Krishnamurthy and specially Ms. Savitha Sridharan. I greatly appreciate their unrelenting support. I would like to thank the graduate school and the former department of Molecular Biology and Immunology for having supported my education here and for providing a nurturing environment.

I have been privileged to have amazing friends here and back home. While many of them go unmentioned here, their contribution has not gone unnoticed. I am particularly grateful to Ms. Smrithi Rajendiran and Mrs. Amruta Oak Sathe for their overwhelming encouragement and confidence in me.

I dedicate my work to my family, especially my most avid supporters and strongest critics, Dr. Dilipkumar Pal, Mrs. Banani Pal and Mr. Nachiket Rajderkar. Baba and Ma, I would not be here today if not for your love, inspiration and your resolute belief in my abilities. I am greatly indebted to Nachiket for the unflinching support, the laughter, the much-needed chides and his endless patience through my crazy times. I am grateful to my partner-in-crime, Mrs. Deedhiti Pal Mudliar for having stuck with me through thick and thin and having borne with my "grad-school moods." I am thankful to my uncle, Mr. Dhrubajyoti Biswas for his unwavering faith in me. Finally, I am thankful to the Lord Almighty for making me understand the subtle value of earning a degree in philosophy despite having worked in biomedical science.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF ABBREVIATIONS

aPKC	Atypical protein kinase C
ATP	Adenosine triphosphate
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
Bid	BH3-interacting domain death agonist
BIM	Bisindolylmaleimide
Cdk	Cyclin-dependent kinase
cPKC	Conventional protein kinase C
DAG	Diacylglycerol
DGK	Diacylglycerol kinase
ER	Endoplasmic reticulum
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
GFP	Green fluorescent protein
HEK	Human embryonic kidney

Hsp	Heat shock protein
IFN	Interferon
ILV	Indolactam V
IP3	Inositol triphosphate
JNK	c-Jun N-terminal kinase
MAPK	Mitogen activated protein kinase
Mcl-1	Myeloid cell leukemia sequence 1
MDR1	Multidrug resistance protein 1
mTOR	Mammalian target of Rapamycin
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor kappa B
nPKC	Novel protein kinase C
PB1	Phox and Bem 1
PDBu	Phorbol 12, 13-dibutyrate
PDK1	Phosphoinositide-dependent kinase 1
PDZ	Post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1) and Zonula occudens-1 protein (zo-1)
PHLPP	PH domain and leucine rich repeat protein phosphatase

PI3K	Phosphatiylinositol 3-kinase
Pin1	Peptidyl prolyl isomerase
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
Pro	Proline
RACK	Receptors for activated C kinase
Rb	Retinoblastoma
Ser	Serine
siRNA	Short interfering RNA
Thr	Threonine
TLR2	Toll-like receptor 2
TNF	Tumor necrosis factor
TPA	12-O-tetradecanoylphorbol 13-acetate.
uORF	Upstream open reading frame
UTR	Untranslated region

VEGF	Vascular endothelial growth factor
XIAP	X-linked inhibitor of apoptosis

CHAPTER I

INTRODUCTION

Breast Cancer

Breast cancer is the second leading cause of death in American women (1). In the United States, about 1 in 8 women develop invasive breast cancer during the course of their lifetime claiming around 40,000 lives each year (1). While surgery, radiation, hormonal treatment, chemo- and targeted therapies are the conventional modes of treatment, they are associated with several problems like toxicity, lack of specificity and acquisition of resistance (2). Thus, understanding the regulation of signaling molecules that promote cancer and chemotherapeutic resistance will facilitate the development of effective therapeutics.

Protein Kinase C (PKC)

Protein kinase C was originally identified by Nishizuka and colleagues as a cyclic nucleotide-independent, Ca^{2+}- and lipid-dependent kinase from bovine and rat cerebellum (3, 4). Results from southern hybridization experiments later revealed a multi-gene family of related isoforms (5). The PKC family has since been established as a family of serine/threonine kinases that play diverse roles in fundamental cellular processes including cell proliferation, cell death and

differentiation (6, 7). PKCs respond to extracellular signals that promote phospholipid hydrolysis and facilitate the generation of diacylglycerol (DAG) and release of Ca^{2+} from intracellular stores. Ca^{2+} and DAG act as cofactors for PKCs and subsequently initiate membrane translocation and activation of PKC (7). Based on the structural features and cofactor requirements, the PKC family consists of 10 isozymes categorized as the conventional or the classical (c) PKCs (α, βI, βII, γ), the novel (n) PKCs (δ, ε, η, θ) and the atypical (a) PKCs (ζ, λ/ι) (6).

PKC isoform structure

Protein kinase C is a member of a larger superfamily of protein kinases, the AGC family that shares basic structural features (8). All PKC isozymes contain a common structural backbone comprised of a highly conserved catalytic domain at the C-terminal and a regulatory domain at the N-terminal (Figure 1). The catalytic domain consists of motifs that are required for ATP/substrate binding and catalysis. PKCs possess 4 conserved modules (C1–4): C1 and C2 are the membrane targeting modules that along with the pseudosubstrate region form the regulatory domain; C3 and C4 comprise the catalytic domain (9). A proteolytically labile hinge region connects the regulatory domain to the catalytic domain (3).

The N-terminal contains the autoinhibitory pseudosubstrate sequence that contains an alanine in place of the serine/threonine phosphoacceptor site, but otherwise resembles a PKC substrate. The pseudosubstrate thus holds the enzyme in an inactive conformation by occupying the catalytic site (10). The membrane targeting modules regulate the spatial distribution of the PKC isozymes. The C1 domain is a globular structure that contains cysteine-rich motifs. The conventional and the novel PKCs have a tandem C1 domain (C1A and C1B) (11). The physiological stimulator

diacylglycerol and the pharmacological activators, such as tumor-promoting phorbol esters bind to the C1 domain. In addition, C1 domain confers selectivity for phosphatidylserine that acts as the activator for PKCs (12). Atypical PKCs have only one cysteine-rich motif in their C1 domain which renders them incapable of binding to DAG or phorbol esters (11). C2 domain can also serve as the recognition site for anionic lipids such as phosphatidylserine and in some enzymes, the calcium binding site. The C2 domain of novel PKCs lacks the key aspartic residues to bind calcium. Consequently, cPKCs are Ca^{2+}- and DAG/phorbol ester-sensitive, nPKCs are Ca^{2+}-insensitive, but they are activated by DAG/phorbol esters and aPKCs are insensitive to both Ca^{2+} and DAG/phorbol esters. Their function is driven by protein–protein interactions mediated via the N-terminal Phox and Bem 1 (PB1) domain and the carboxyl-terminal PDZ ligand motif (9). The distinct structural and biochemical features of the PKC isozymes pave the way for the differential cellular responses attributed to the PKC family.

Modes of regulation

The PKC isozymes are under tight structural and spatial regulation that underlies their biochemical functions, intracellular localization and tissue distribution (9). PKCs can be regulated by phosphorylation, cofactor binding and membrane targeting through interaction with scaffold proteins (13).

Regulation by phosphorylation

PKCs have to be first processed by a series of ordered phosphorylation events before it can become catalytically competent (13). PKCs have three conserved phosphorylation sites: *activation loop* (which is located in a cleft in the kinase domain at the entrance of the catalytic site), *turn motif* (which is at a turn in the structure) and the *hydrophobic motif* (which is

surrounded by hydrophobic residues). It is generally believed that first of the phosphorylation events occurs at the activation loop site followed by subsequent phosphorylations at the turn motif and the hydrophobic motif on the C-terminal (14). The priming phosphorylation is mediated by phosphoinositide-dependent kinase 1 (PDK1) (15, 16). When PKC is newly synthesized, it remains associated with the membrane in an open conformation that allows PDK1 access to the activation loop site and the substrate binding pocket. Phosphorylation of PKC at the activation loop by PDK1 triggers additional phosphorylations at the C-terminus (14). There are, however, controversies regarding the phosphorylation of novel PKCs at the activation loop (17-19). For example, PKCε rather than PDK1 has been reported to phosphorylate PKCδ and PKCε at the activation loop (18, 19). Although activation loop phosphorylation is considered an early event in the priming of PKCs, recent studies have identified an earlier step in PKC maturation which involves chaperone proteins (20). Heat shock protein-90 (Hsp90) and co-chaperone Cdc37 bind to a molecular clamp in the kinase domain of PKC, an event that is essential for the subsequent priming phosphorylations (20). Activation loop phosphorylation serves two purposes- it allows for the proper positioning of the residues for catalysis and it unmasks the substrate binding site (21). Although activation loop phosphorylation is crucial for the maturation of PKC, once PKC is phosphorylated at the C-terminal sites, phosphorylation at the activation loop is dispensable (22). Unlike the activation loop phosphorylation, phosphorylation at the turn motif is absolutely essential for the function of PKC. Negative charge at the turn motif is pre-requisite and sufficient for the function of the mature PKC (22). It is generally accepted that this site is modified by autophosphorylation (23). It also serves as the docking site for protein-protein interactions with other effectors such as 14-3-3 class of proteins (24). The phosphorylation at the turn motif is followed by phosphorylation at the hydrophobic motif. The

mTORC2 complex has been implicated in the regulation of the hydrophobic motif (25, 26). The hydrophobic site phosphorylation determines subcellular localization and stability but is not necessary for its catalytic function (22, 27, 28). This series of phosphorylation events lead to a conformation of PKC that is primed for activation by cofactors (14).

Regulation of PKC by cofactors and membrane targeting

Once PKC is phosphorylated, it localizes to the cytosol in an inactive form with the pseudosubstrate bound to the catalytic site (13). Engagement of growth factor or cytokine to their receptors activates phospholipase C that cleaves phosphatidylinositol 4, 5-bisphosphate to generate the second messengers: DAG and inositol trisphosphate (IP3). IP3 facilitates the release of calcium from intracellular stores. The production of DAG recruits PKCs to the plasma membrane through the action of receptors for activated C kinases (RACKs), where they become activated by acidic phospholipids, such as phosphatidylserine (29). RACKs bind to the PKC isozymes and serve as anchoring proteins thus facilitating proper spatial localization of PKCs (30). Binding of DAG and Ca^{2+} to the C1 and C2 domains respectively, changes the conformation of PKC releasing the pseudosubstrate from the substrate binding pocket and allows for the downstream signaling of PKCs (12, 31) (Figure 2).

Pharmacological modulators of PKC

PKCs are the receptors for tumor-promoting phorbol esters which are potent activators of PKCs and mimic the action of DAG (32, 33). While membrane recruitment and activation of PKCs by DAG is transient, phorbol esters induce sustained activation of PKCs. However, chronic activation of PKCs by phorbol esters ultimately leads to desensitization and downregulation of the PKCs (13). Diterpene esters like mezerein have also been shown to activate PKC by

mimicking the action of DAG (34). PKCs can also be activated by synthetic analogs (indolactams) related to the tumor promoter teleocidin (35, 36). Besides tumor-promoting phorbol esters, other non-tumor-promoting agents can also activate PKCs like bryostatins, ingenol mebutate and the relatively newer class of synthetic analogs of DAG known as DAG-lactones (37-39). Bryostatins, belonging to the family of macrocyclic lactones however, act as partial agonists of PKCs and can counteract the action of phorbol esters and have been widely investigated in clinical trials for cancer therapy (40, 41).

The function of PKCs can also be modulated by PKC inhibitors that can be either categorized as ATP-competitive inhibitors or regulatory site inhibitors. Bisindolylmaleimide and its analogs and staurosporine derivatives such as Gö 6983 compete for the ATP-binding site and serve as potent inhibitors of PKC activity (42-45). Calphostin C acts as regulatory site inhibitor by interaction with the DAG/phorbol ester binding domains (46). Peptide inhibitors or antisense oligonucleotides such as aprinocarsen can also inhibit PKC function by interfering in the interaction of PKCs with anchoring proteins (40, 47). Recent studies have also described bisubstrate-based inhibitors, which are capable of binding both the ATP binding site as well as the substrate binding groove by use of a pseudosubstrate analog ensuring better selectivity (48, 49).

Signal termination and downregulation of PKC isozymes

Termination of PKC signaling can occur via different mechanisms such as release of PKC isozymes from the membrane, metabolism of DAG by DAG kinases (DGKs) (50, 51), agonist-induced degradation or the removal of priming phosphorylation which leads to downregulation and rapid degradation (29, 50, 52). Desensitization of G-protein coupled receptors by

autophosphorylation facilitates reverse translocation and leads to the release of PKCs from the membrane and subsequent downregulation (51). Since co-factors such as DAG are important for PKC regulation, the removal of these second messengers leads to inactivation of PKC signaling. DGKs phosphorylate DAG to generate phosphatidic acid, thereby effectively depleting the levels of DAG in the cell, thus inducing downregulation of PKC (51). While the interaction of PKC with DAG is transient (53), high-affinity binding of phorbol esters leads to sustained activation of PKC (54). This renders PKC in an open conformation and predisposes them to the action of proteases (55). In the open conformation, the sensitivity of PKC to dephosphorylation is also increased (13). Dephosphorylation of PKCs is believed to precede their downregulation (56) although it was reported that phosphorylation of PKCδ is required for activation-induced downregulation (57). In addition, fully phosphorylated PKCα was shown to undergo downregulation (52). PKCs are also susceptible to phosphatases such as PHLPP which can target PKCs for ubiquitin-mediated degradation (58). However, dephosphorylated PKCs can be rescued by the action of heat-shock proteins such as Hsp70 (59). PKCs can be stabilized by the binding of Hsp70 to the dephosphorylated turn motif which allows rephosphorylation of PKC, thereby prolonging its lifetime (59). Recently, the peptidyl-prolyl isomerase (Pin1) was implicated in the downregulation of conventional protein kinase C isozymes (60). Binding of Pin1 to the conventional PKCβII led to conformational changes in the Thr-Pro peptidyl bond in the C-terminal tail facilitating downregulation. In novel PKCs, the absence of the Pro residue in the turn motif prevents this conformation-dependent degradation switch. However, PKCη is the only novel isozyme which has the Thr-Pro peptidyl bond in its turn motif (60).

Several mechanisms of degradation have been proposed for the PKC isozymes. Conventional PKCs are believed to be downregulated by calcium-activated proteases, such as calpains (61, 62)

whereas PKCα, -δ and –ε were shown to be degraded via proteasome-mediated pathway (63-65). PKCs are also subject to caspase-mediated cleavage and can be subsequently degraded by other proteases (66-68). Studies have reported that PKCs are trafficked to the endosomal compartments upon activation with phorbol esters (56, 69, 70) and subsequently accumulate in the perinuclear region by a caveolae-dependent process where it is dephosphorylated and degraded (56, 70).

Role of PKC isozymes in breast cancer

Since their discovery as receptors for tumor promoters, PKC isozymes have been attractive targets for cancer therapy (33, 40). Individual PKC isozymes exhibit distinct biochemical properties, tissue distribution and subcellular localization which lead to divergent responses in distinct cancers and sometimes even within the same cancer type (71, 72). While PKCα was shown to be overexpressed in human breast tumor samples (73), downregulation of PKCα in breast cancer has also been reported (74). However, several lines of evidence have implicated PKCα in promoting migration and epithelial-mesenchymal transition of breast cancer cells thereby implicating PKCα in invasion and metastasis of breast cancer cells (75, 76). Numerous reports have established PKCβ as a potential therapeutic target in breast cancer (77). While both PKCβI and PKCβII are important for cell cycle progression and cell proliferation (78), PKCβII has been reported to be a key mediator of vascular endothelial growth factor (VEGF)-induced proliferation thereby suggesting a role in angiogenesis (79, 80). Similar to PKCα, PKCδ also has conflicting roles in breast cancer; it can facilitate both tumorigenesis and tumor suppression (81, 82). It is well documented that PKCδ mediates survival of breast cancer cells (83) through the NF-κB and the MEK pathways (84, 85) and promotes metastasis (86). In contrast, PKCδ promotes an anti-proliferative response via suppression of Akt and the ERK pathways as well as

by regulating hypophosphorylation of Rb (87). While the proteolytic cleavage of PKCδ is generally believed to be important for apoptosis, depending on the apoptotic stimulus, the catalytic fragment can elicit both pro- and anti-apoptotic functions (88-90). On the other hand, PKCε promotes oncogenic signaling in several cancers including breast cancer (91). Overexpression of PKCε correlates with high tumor-grade, ER negative status and poor survival in breast cancer patients, thus serving as a predictive biomarker for breast cancer aggressiveness (92). Similarly, PKCι is believed to be an oncogenic kinase with crucial roles in transformation and tumorigenicity (93, 94). Immunohistochemical analysis has revealed increased expression and abnormal cellular localization of PKCι in breast cancer tissue samples (95). Overexpression of PKCζ has been reported in breast carcinomas (96). In mouse mammary cells, PKCζ was shown to activate the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, leading to cell proliferation and migration (97). Studies have also implicated PKCζ in breast cancer cell chemotaxis and cell motility (98, 99). Thus, the PKC isozymes exhibit pleiotropic functions in cancer.

Regulation of Bcl-2 family members by PKC isozymes

The PKC isozymes are known to exert their effects in cancer signaling via several mechanisms including the regulation of the Bcl-2 family members which are central regulators of cell survival and apoptosis and are frequently deregulated in cancer (7, 81, 100). It is well documented that PKCα can promote cell survival by the suppression of apoptosis (75). Overexpression of PKCα has been shown to inhibit apoptosis via the phosphorylation of Bcl-2 at Ser-70 which leads to stabilization of Bcl-2 (101). In SKBR3 breast cancer cells, inhibition of PKCα augmented apoptosis by downregulation of Bcl-2 (102). On the other hand, PKCδ acts downstream of Syk kinase and stabilizes anti-apoptotic Mcl-1 through inhibition of GSK3β thereby inhibiting

apoptosis (103). PKCδ can also exert its anti-apoptotic effect by promoting the proteasomal degradation of pro-apoptotic Bim (104). In contrast, the catalytic fragment of PKCδ was shown to phosphorylate Mcl-1 and target it for degradation, thus facilitating cell death (88). PKCδ can also inhibit cell survival through the activation of Bax and Bak proteins through interaction with the p38/MAPK pathway (105). PKCε is believed to protect against apoptosis and has been implicated in chemotherapeutic resistance in tumor cells (106). In lung cancer, PKCε was shown to upregulate the pro-survival factors X-linked inhibitor of apoptosis (XIAP) and Bcl-xL (107). We have previously reported that PKCε overexpression in breast cancer cells increased the Bcl-2 expression at the mRNA and protein levels with concomitant decrease in the levels of pro-apoptotic Bid (106). Recent evidence has also showed that downregulation of PKCε by miR-31 sensitized breast cancer cells to apoptosis which was accompanied by decrease in NF-κB activity and Bcl-2 expression (108). Sequestration of PKCι and PKCζ by the pro-apoptotic Par-4 protein has been demonstrated to induce apoptosis (109). PKCζ has been shown to phosphorylate and inhibit the pro-apoptotic Bax in lung cancer (110) while PKCι promotes cell survival through inhibition of Bad in glioblastoma (111). Although limited evidence is available for the role of PKCθ in the regulation of the Bcl-2 family, PKCθ-deficient mice displayed reduced expression of Bcl-2 and Bcl-xL (112).

Protein kinase C-eta

Protein kinase C-eta (PKCη) is a novel member of the PKC family. It is classified as a calcium-independent but DAG/phorbol ester-dependent PKC (113). It was first isolated from a cDNA library of mouse epidermis (113). PKCη is assigned to human chromosome 14 (14q22-23) and mouse chromosome 12 (12C3-D2) (114, 115) and contains an open reading frame encoding 683 amino acid residues (116). Contrary to other PKCs which are primarily enriched in the brain

tissue, PKCη is mainly expressed in lung, skin and heart tissues (117). PKCη participates in diverse cellular processes including proliferation, differentiation, secretion and apoptosis (118-124). Recent reports have revealed the role of PKCη in immune function (125, 126). PKCη was shown to be important for T-cell proliferation and homeostasis (127). PKCη was also implicated in the regulation of toll-like receptor-2 (TLR-2) responses in macrophages (128).

Structure, regulation and localization of PKCη

The structure of PKCη comprises of the catalytic domain and the regulatory domain similar to other PKCs (9). A characteristic cysteine-rich region is present in the C1 domain of PKCη along with a protein kinase domain, both of which are conserved features in the PKC family (129). PKCη shares greatest homology with PKCε, another member of the novel PKC family (117). Similar to other PKC isozymes, PKCη has three conserved phosphorylation sites- activation loop (Thr-513), turn motif (Thr-655) and hydrophobic domain (Ser-674) (9). Although the order of priming phosphorylations of PKCη is not well established, PDK1 is believed to phosphorylate PKCη at the activation loop *in vitro* (130). In mouse A9 fibroblasts infected with parovirus, Lachmann et.al demonstrated that PKCλ phosphorylates PKCη at the hydrophobic site thus allowing PDK1 access to the activation loop (131). The C2 domain structure of PKCη was found to be similar to PKCε with significant differences at the putative lipid binding site. Mass spectrometric analysis of the C2 domain of PKCη revealed two autophosphorylation sites at Ser-28 and Ser-32 (132). The autophosphorylation site at Ser-28 but not Ser-32 is conserved in PKCε (132). It has been speculated that autophosphorylation at these sites could affect the lipid-binding of PKCη (132).

Anionic phospholipids like phosphatidylserine and DAG/phorbol esters regulate PKCη (113, 117). However, contrary to other phorbol-ester sensitive PKC isozymes, PKCη resists downregulation by prolonged treatment with phorbol esters (119, 133, 134). PKCη is specifically activated by cholesterol sulfate and sulfatide (135). It was reported that cholesterol sulfate-mediated activation of PKCη involved casein kinase I (136). In addition, PKCη was shown to be activated by treatment with type I interferons (IFNs) like IFNα or IFNβ in chronic myeloid leukemia cells (137). Interestingly, other novel PKC isozymes like PKCδ, -ε and -θ are also activated by Type I and Type II IFNs and participate in Type I and/or Type II IFN-induced responses (138-141). PKCη is also elevated in response to estradiol treatment in estrogen-sensitive breast cancer cells in a time- and concentration-dependent manner (142). Differential expression analysis in the neoplastic cell line 8701-BC demonstrated that PKCη downregulation can be induced by type V collagen (143).

Furthermore, PKCη is subject to translational regulation under both normal and stressed conditions caused by amino acid starvation (144). Raveh-Amit and colleagues reported that the 5'-UTR of PKCη is unusually long (659 nucleotides) and rich in GC content and identified two upstream open reading frames (uORF) in the 5'-UTR which function as repressive elements under normal growth conditions. However, under amino acid starvation, the repression is removed by leaky scanning leading to the translational upregulation of PKCη (144). PKCε is the only other PKC isozyme for which the presence of a regulatory uORF has been reported (145).

PKCη is localized in the Golgi, endoplasmic reticulum (ER) and the nuclear envelope (146). Although the C1A domain of PKCη lacks a Golgi localization signal similar to the other members of the novel PKC family, the C1B domain of PKCη facilitates its translocation to the Golgi complex (146). In response to serum starvation and PMA, PKCη translocates to the

nuclear envelope. While C1B domain is sufficient to drive Golgi translocation of PKCη, both the C1 and the pseudosubstrate region are required for the localization at the nuclear envelope and ER (146). Furthermore, a recent study reported that in hepatocellular carcinoma cells, PKCη is targeted to lipid droplets where it limited the formation of larger lipid droplets (147).

Role of PKCη in breast cancer

The role of PKCη in cancer is controversial owing to its diverse responses in different cancers. Although, PKCη-deficient mice were more susceptible to tumor promotion in two-stage skin carcinogenesis model (148), PKCη mediates chemotherapeutic resistance in breast cancer (118, 149), glioblastoma (150), lung cancer (151) and several other cancers (152, 153). It has been reported that PKCη is downregulated in hepatocellular carcinoma (154), however, it is associated with the progression of renal cell carcinoma (155). Thus, PKCη may promote or inhibit malignant growth depending on the cellular context.

PKCη is a regulator of mammary gland development (156). It is upregulated in the rat mammary gland during the transition from the resting to the pregnant state (156). Furthermore, a marked decrease in PKCη levels was observed during gland regression which is typically characterized by the onset of apoptotic processes leading to involution (156). Qualitative and quantitative alterations in PKCη have been reported in human breast cancer tissues (157). PKCη expression was increased in locally invasive breast tumor tissues and high levels of PKCη were detected in invasive tumors associated with significant lymph node metastases which suggests a role of PKCη in cancer progression (157). This is consistent with a report which demonstrated the importance of PKCη in maintaining tight junction integrity via interaction and subsequent phosphorylation of occludin on its C-terminal domain (158). Since key changes in the barrier

function of tight junctions have been shown to be critical in cancer progression (159), it is likely that PKCη may have potential roles in survival and progression of cancer cells.

PKCη mRNA is elevated in multidrug-resistant breast tumors (160). Overexpression of PKCη has been shown to protect against apoptosis (118, 119, 123). We have previously reported that overexpression of PKCη attenuated caspase activation and TNF-induced cell death in breast cancer cells (118). PKCη also protects against camptothecin-induced DNA damage by activating NF-κB and promoting nuclear localization of RelA/p65 in breast cancer (123). Upon etoposide-induced stress, PKCη is tethered to the nuclear membrane and confers protection against cell death (161). Moreover, PKCη was effective in blocking apoptosis via the suppression of c-Jun N-terminal kinase (JNK) activity upon UV irradiation (149). PKCη is also critical for cell cycle control. Although PKCη induced growth arrest in NIH3T3 fibroblasts and keratinocytes (162, 163), it enhanced cell cycle progression in breast cancer cells (120). Induced expression of PKCη led to an increase in the levels of cyclin E and cyclin D (120). While the levels of the cell cycle inhibitor p27 (kip1) were unaltered by PKCη overexpression, it however, facilitated the removal of the cell cycle inhibitor p27 (kip1) from the cyclin E/cdk2 complex thereby activating the cyclin E/cdk2 complex (120). While these studies implicate PKCη in breast cancer, the mechanism by which PKCη contributes to breast cancer development is largely unknown.

Objectives of the present study

Deregulation of cellular processes is the hallmark of cancer. The signaling pathways that are altered in cancer can greatly influence clinical outcome. The protein kinase C (PKC) family is critical for the regulation of cellular processes like cell proliferation, cell survival and tumor promotion (6, 7). Thus, they serve as potent targets for cancer therapy. Protein kinase C (PKC) serves as the receptor for tumor promoting phorbol esters, which are activators of conventional and novel PKCs and can substitute for the physiological activator DAG (33). However, persistent treatment with phorbol esters leads to downregulation of these PKCs (13). PKCη, a novel PKC isozyme, resists downregulation by tumor-promoting phorbol esters, suggesting that the regulation of PKCη is unique (119, 133, 134). PKCη levels were found to be altered in human breast tumors and correlated with positive lymph node metastases in invasive tumors (157). Moreover, significant positive correlations were established between PKCη expression and MDR1 (multidrug resistance-associated protein 1) genes in breast cancer (160). However, little is known about how PKCη level is regulated. The objective of this study is to examine the regulation of PKCη and to elucidate its role in breast cancer.

Hypothesis and specific aims

Our preliminary studies demonstrate that PKCη is the only isozyme that resists downregulation in response to persistent treatment with tumor-promoting phorbol esters in contrast to the other PKCs which are either downregulated or remain unaltered. The unique upregulation of PKCη in response to tumor-promoters suggests its role in tumor development. Furthermore, we have observed that the protein levels of PKCη increased with the aggressiveness of breast cancer in the progressive MCF-10A series, suggesting that PKCη may contribute to the survival of breast

cancer cells leading to breast cancer progression. Overexpression of PKCη has been observed in human breast tumors and it contributes to chemoresistance in several cancers including breast cancer (118, 119, 123). Hence, we hypothesize that ***the upregulation of PKCη contributes to breast cancer cell survival***. We will pursue the following specific aims in order to test our hypothesis.

Aim 1. ***To determine the mechanism of upregulation of PKCη in breast cancer***

Aim 2. ***To elucidate how inhibition of PKCη phosphorylation leads to its downregulation***

Aim 3. ***To determine the consequence of PKCη downregulation on breast cancer cell survival***

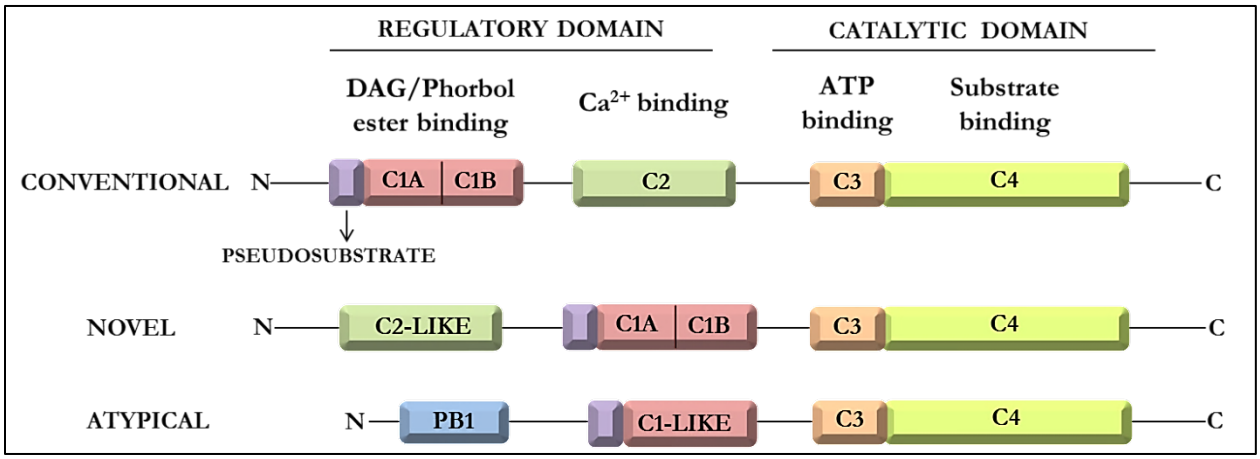


Figure 1. Domain structure of PKC isozymes. *The PKC family comprises of three classes- conventional, novel and atypical. Each isozyme has a regulatory domain (C1, C2) and a catalytic domain (C3, C4). The C1 domain binds phosphatidylserine for all PKCs and consists of motifs that form the DAG/phorbol ester binding site for the conventional and novel PKCs while C2 domain binds anionic lipids and* Ca^{2+} *for conventional PKCs. Atypical PKCs possess a PB1 module for protein-protein interactions. C3 and C4 form the ATP and the substrate binding domains of PKCs respectively.*

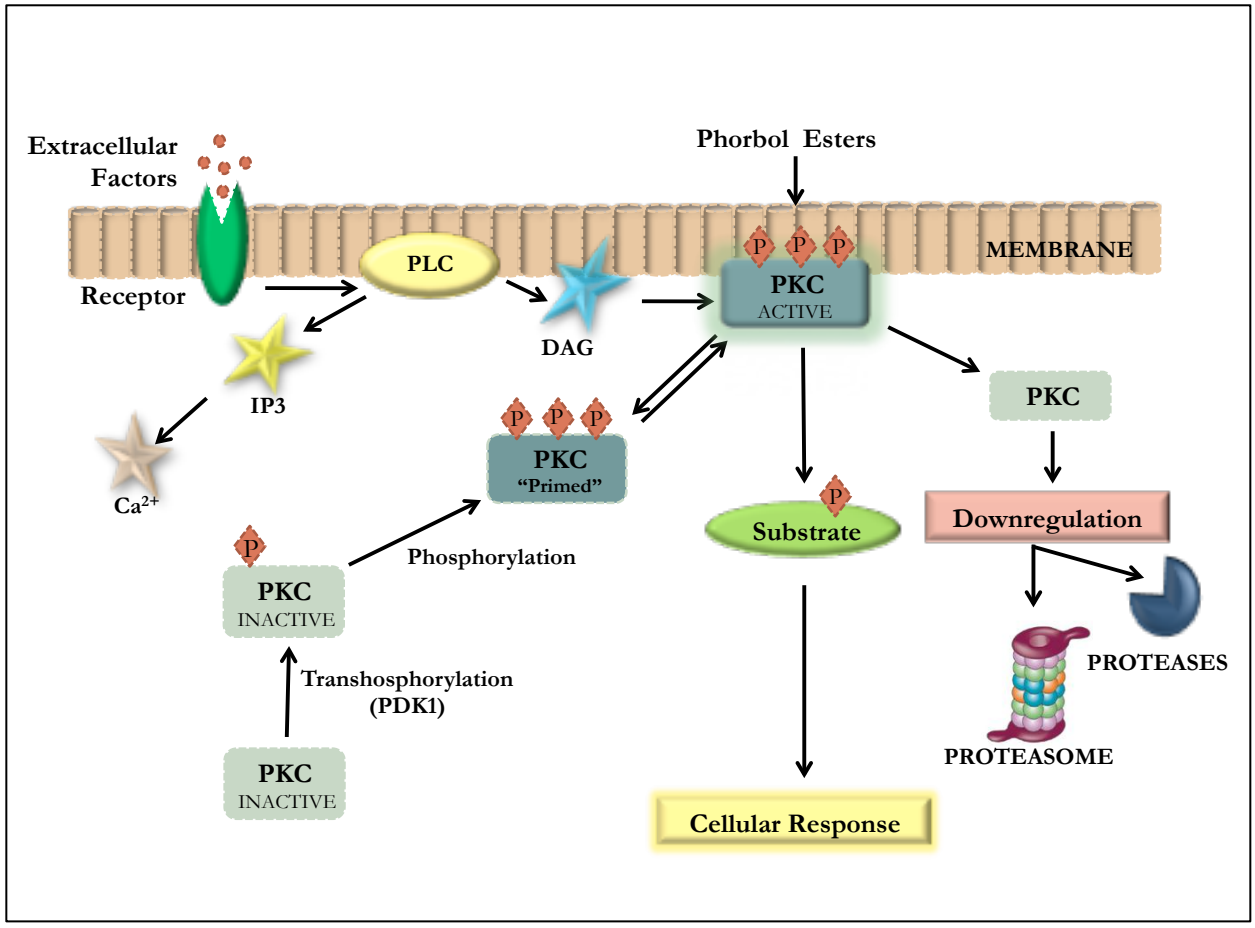


Figure 2. Regulation of PKC. *Inactive PKCs are localized in the cytosol where they are processed by ordered phosphorylations which prime them for activation. Signals that promote phospholipid hydrolysis facilitate the generation of DAG and* Ca^{2+} *which induce the translocation of the PKCs from the cytosol to the membrane leading to activation. Upon activation, the pseudosubstrate is released which allows substrate binding thus eliciting a cellular response. The open, active conformation of PKC is susceptible to dephosphorylation and degradation. Persistent activation by phorbol esters can also lead to downregulation of PKCs via protease- or proteasome-dependent pathways causing downregulation of PKCs.*

References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin. 2013;63:11-30.

2. Lonning PE. Molecular basis for therapy resistance. Mol Oncol. 2010;4:284-300.

3. Inoue M, Kishimoto A, Takai Y, Nishizuka Y. Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent protease from rat brain. J Biol Chem. 1977;252:7610-6.

4. Takai Y, Kishimoto A, Inoue M, Nishizuka Y. Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. I. Purification and characterization of an active enzyme from bovine cerebellum. J Biol Chem. 1977;252:7603-9.

5. Ono Y, Fujii T, Ogita K, Kikkawa U, Igarashi K, Nishizuka Y. The structure, expression, and properties of additional members of the protein kinase C family. J Biol Chem. 1988;263:6927-32.

6. Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science. 1992;258:607-14.

7. Griner EM, Kazanietz MG. Protein kinase C and other diacylglycerol effectors in cancer. Nat Rev Cancer. 2007;7:281-94.

8. Pearce LR, Komander D, Alessi DR. The nuts and bolts of AGC protein kinases. Nat Rev Mol Cell Biol. 2010;11:9-22.

9. Steinberg SF. Structural basis of protein kinase C isoform function. Physiol Rev. 2008;88:1341-78.

10. House C, Kemp BE. Protein kinase C contains a pseudosubstrate prototope in its regulatory domain. Science. 1987;238:1726-8.

11. Hurley JH, Newton AC, Parker PJ, Blumberg PM, Nishizuka Y. Taxonomy and function of C1 protein kinase C homology domains. Protein Sci. 1997;6:477-80.

12. Johnson JE, Giorgione J, Newton AC. The C1 and C2 domains of protein kinase C are independent membrane targeting modules, with specificity for phosphatidylserine conferred by the C1 domain. Biochemistry. 2000;39:11360-9.

13. Gould CM, Newton AC. The life and death of protein kinase C. Curr Drug Targets. 2008;9:614-25.

14. Parekh DB, Ziegler W, Parker PJ. Multiple pathways control protein kinase C phosphorylation. Embo J. 2000;19:496-503.

15. Chou MM, Hou W, Johnson J, Graham LK, Lee MH, Chen CS, et al. Regulation of protein kinase C zeta by PI 3-kinase and PDK-1. Curr Biol. 1998;8:1069-77.

16. Dutil EM, Toker A, Newton AC. Regulation of conventional protein kinase C isozymes by phosphoinositide-dependent kinase 1 (PDK-1). Curr Biol. 1998;8:1366-75.

17. Rybin VO, Guo J, Gertsberg Z, Elouardighi H, Steinberg SF. Protein kinase Cepsilon (PKCepsilon) and Src control PKCdelta activation loop phosphorylation in cardiomyocytes. J Biol Chem. 2007;282:23631-8.

18. Rybin VO, Sabri A, Short J, Braz JC, Molkentin JD, Steinberg SF. Cross-regulation of novel protein kinase C (PKC) isoform function in cardiomyocytes. Role of PKC epsilon in activation loop phosphorylations and PKC delta in hydrophobic motif phosphorylations. J Biol Chem. 2003;278:14555-64.

19. Basu A, Sridharan S, Persaud S. Regulation of protein kinase C delta downregulation by protein kinase C epsilon and mammalian target of rapamycin complex 2. Cell Signal. 2009;21:1680-5.

20. Gould CM, Kannan N, Taylor SS, Newton AC. The chaperones Hsp90 and Cdc37 mediate the maturation and stabilization of protein kinase C through a conserved PXXP motif in the C-terminal tail. J Biol Chem. 2009;284:4921-35.

21. Johnson LN, O'Reilly M. Control by phosphorylation. Curr Opin Struct Biol. 1996;6:762-9.

22. Bornancin F, Parker PJ. Phosphorylation of protein kinase C-alpha on serine 657 controls the accumulation of active enzyme and contributes to its phosphatase-resistant state. J Biol Chem. 1997;272:3544-9.

23. Flint AJ, Paladini RD, Koshland DE, Jr. Autophosphorylation of protein kinase C at three separated regions of its primary sequence. Science. 1990;249:408-11.

24. Yaffe MB, Rittinger K, Volinia S, Caron PR, Aitken A, Leffers H, et al. The structural basis for 14-3-3:phosphopeptide binding specificity. Cell. 1997;91:961-71.

25. Parekh D, Ziegler W, Yonezawa K, Hara K, Parker PJ. Mammalian TOR controls one of two kinase pathways acting upon nPKCdelta and nPKCepsilon. J Biol Chem. 1999;274:34758-64.

26. Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, et al. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. Dev Cell. 2006;11:859-71.

27. Edwards AS, Newton AC. Phosphorylation at conserved carboxyl-terminal hydrophobic motif regulates the catalytic and regulatory domains of protein kinase C. J Biol Chem. 1997;272:18382-90.

28. Gysin S, Imber R. Phorbol-ester-activated protein kinase C-alpha lacking phosphorylation at Ser657 is down-regulated by a mechanism involving dephosphorylation. Eur J Biochem. 1997;249:156-60.

29. Newton AC. Protein kinase C: poised to signal. Am J Physiol Endocrinol Metab. 2010;298:E395-402.

30. Kheifets V, Mochly-Rosen D. Insight into intra- and inter-molecular interactions of PKC: design of specific modulators of kinase function. Pharmacol Res. 2007;55:467-76.

31. Cho W. Membrane targeting by C1 and C2 domains. J Biol Chem. 2001;276:32407-10.

32. Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U, Nishizuka Y. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J Biol Chem. 1982;257:7847-51.

33. Blumberg PM. Protein kinase C as the receptor for the phorbol ester tumor promoters: sixth Rhoads memorial award lecture. Cancer Res. 1988;48:1-8.

34. Sharkey NA, Hennings H, Yuspa SH, Blumberg PM. Comparison of octahydromezerein and mezerein as protein kinase C activators and as mouse skin tumor promoters. Carcinogenesis. 1989;10:1937-41.

35. Irie K, Nakagawa Y, Ohigashi H. Indolactam and benzolactam compounds as new medicinal leads with binding selectivity for C1 domains of protein kinase C isozymes. Curr Pharm Des. 2004;10:1371-85.

36. Fujiki H, Sugimura T. New classes of tumor promoters: teleocidin, aplysiatoxin, and palytoxin. Adv Cancer Res. 1987;49:223-64.

37. Garcia-Bermejo ML, Leskow FC, Fujii T, Wang Q, Blumberg PM, Ohba M, et al. Diacylglycerol (DAG)-lactones, a new class of protein kinase C (PKC) agonists, induce

apoptosis in LNCaP prostate cancer cells by selective activation of PKCalpha. J Biol Chem. 2002;277:645-55.

38. Serova M, Ghoul A, Benhadji KA, Faivre S, Le Tourneau C, Cvitkovic E, et al. Effects of protein kinase C modulation by PEP005, a novel ingenol angelate, on mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling in cancer cells. Mol Cancer Ther. 2008;7:915-22.

39. Szallasi Z, Smith CB, Pettit GR, Blumberg PM. Differential regulation of protein kinase C isozymes by bryostatin 1 and phorbol 12-myristate 13-acetate in NIH 3T3 fibroblasts. J Biol Chem. 1994;269:2118-24.

40. Mackay HJ, Twelves CJ. Targeting the protein kinase C family: are we there yet? Nat Rev Cancer. 2007;7:554-62.

41. Hennings H, Blumberg PM, Pettit GR, Herald CL, Shores R, Yuspa SH. Bryostatin 1, an activator of protein kinase C, inhibits tumor promotion by phorbol esters in SENCAR mouse skin. Carcinogenesis. 1987;8:1343-6.

42. Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, et al. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J Biol Chem. 1991;266:15771-81.

43. Way KJ, Chou E, King GL. Identification of PKC-isoform-specific biological actions using pharmacological approaches. Trends Pharmacol Sci. 2000;21:181-7.

44. Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, et al. Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. J Biol Chem. 1993;268:9194-7.

45. Wu-Zhang AX, Newton AC. Protein kinase C pharmacology: refining the toolbox. Biochem J. 2013;452:195-209.

46. Kobayashi E, Nakano H, Morimoto M, Tamaoki T. Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. Biochem Biophys Res Commun. 1989;159:548-53.

47. Dean NM, McKay R, Condon TP, Bennett CF. Inhibition of protein kinase C-alpha expression in human A549 cells by antisense oligonucleotides inhibits induction of intercellular adhesion molecule 1 (ICAM-1) mRNA by phorbol esters. J Biol Chem. 1994;269:16416-24.

48. Parang K, Cole PA. Designing bisubstrate analog inhibitors for protein kinases. Pharmacol Ther. 2002;93:145-57.

49. van Wandelen LT, van Ameijde J, Mady AS, Wammes AE, Bode A, Poot AJ, et al. Directed modulation of protein kinase C isozyme selectivity with bisubstrate-based inhibitors. ChemMedChem. 2012;7:2113-21.

50. Feng X, Zhang J, Barak LS, Meyer T, Caron MG, Hannun YA. Visualization of dynamic trafficking of a protein kinase C betaII/green fluorescent protein conjugate reveals differences in G protein-coupled receptor activation and desensitization. J Biol Chem. 1998;273:10755-62.

51. Crotty T, Cai J, Sakane F, Taketomi A, Prescott SM, Topham MK. Diacylglycerol kinase delta regulates protein kinase C and epidermal growth factor receptor signaling. Proc Natl Acad Sci U S A. 2006;103:15485-90.

52. Leontieva OV, Black JD. Identification of two distinct pathways of protein kinase Calpha down-regulation in intestinal epithelial cells. J Biol Chem. 2004;279:5788-801.

53. Carrasco S, Merida I. Diacylglycerol, when simplicity becomes complex. Trends Biochem Sci. 2007;32:27-36.

54. Junco M, Webster C, Crawford C, Bosca L, Parker PJ. Protein kinase C V3 domain mutants with differential sensitivities to m-calpain are not resistant to phorbol-ester-induced down-regulation. Eur J Biochem. 1994;223:259-63.

55. Parker PJ, Bosca L, Dekker L, Goode NT, Hajibagheri N, Hansra G. Protein kinase C (PKC)-induced PKC degradation: a model for down-regulation. Biochem Soc Trans. 1995;23:153-5.

56. Prevostel C, Alice V, Joubert D, Parker PJ. Protein kinase C(alpha) actively downregulates through caveolae-dependent traffic to an endosomal compartment. J Cell Sci. 2000;113 (Pt 14):2575-84.

57. Srivastava J, Procyk KJ, Iturrioz X, Parker PJ. Phosphorylation is required for PMA- and cell-cycle-induced degradation of protein kinase Cdelta. Biochem J. 2002;368:349-55.

58. Gao T, Brognard J, Newton AC. The phosphatase PHLPP controls the cellular levels of protein kinase C. J Biol Chem. 2008;283:6300-11.

59. Gao T, Newton AC. The turn motif is a phosphorylation switch that regulates the binding of Hsp70 to protein kinase C. J Biol Chem. 2002;277:31585-92.

60. Abrahamsen H, O'Neill AK, Kannan N, Kruse N, Taylor SS, Jennings PA, et al. Peptidyl-prolyl isomerase Pin1 controls down-regulation of conventional protein kinase C isozymes. J Biol Chem. 2012;287:13262-78.

61. Pontremoli S, Melloni E, Damiani G, Salamino F, Sparatore B, Michetti M, et al. Effects of a monoclonal anti-calpain antibody on responses of stimulated human neutrophils. Evidence for a role for proteolytically modified protein kinase C. J Biol Chem. 1988;263:1915-9.

62. Savart M, Letard P, Bultel S, Ducastaing A. Induction of protein kinase C down-regulation by the phorbol ester TPA in a calpain/protein kinase C complex. Int J Cancer. 1992;52:399-403.

63. Lee HW, Smith L, Pettit GR, Smith JB. Bryostatin 1 and phorbol ester down-modulate protein kinase C-alpha and -epsilon via the ubiquitin/proteasome pathway in human fibroblasts. Mol Pharmacol. 1997;51:439-47.

64. Lee HW, Smith L, Pettit GR, Vinitsky A, Smith JB. Ubiquitination of protein kinase C-alpha and degradation by the proteasome. J Biol Chem. 1996;271:20973-6.

65. Lu Z, Liu D, Hornia A, Devonish W, Pagano M, Foster DA. Activation of protein kinase C triggers its ubiquitination and degradation. Mol Cell Biol. 1998;18:839-45.

66. Emoto Y, Manome Y, Meinhardt G, Kisaki H, Kharbanda S, Robertson M, et al. Proteolytic activation of protein kinase C delta by an ICE-like protease in apoptotic cells. Embo J. 1995;14:6148-56.

67. Smith L, Wang Z, Smith JB. Caspase processing activates atypical protein kinase C zeta by relieving autoinhibition and destabilizes the protein. Biochem J. 2003;375:663-71.

68. Basu A, Lu D, Sun B, Moor AN, Akkaraju GR, Huang J. Proteolytic activation of protein kinase C-epsilon by caspase-mediated processing and transduction of antiapoptotic signals. J Biol Chem. 2002;277:41850-6.

69. Becker KP, Hannun YA. Isoenzyme-specific translocation of protein kinase C (PKC)betaII and not PKCbetaI to a juxtanuclear subset of recycling endosomes: involvement of phospholipase D. J Biol Chem. 2004;279:28251-6.

70. Hansra G, Garcia-Paramio P, Prevostel C, Whelan RD, Bornancin F, Parker PJ. Multisite dephosphorylation and desensitization of conventional protein kinase C isotypes. Biochem J. 1999;342 (Pt 2):337-44.

71. Koivunen J, Aaltonen V, Peltonen J. Protein kinase C (PKC) family in cancer progression. Cancer Lett. 2006;235:1-10.

72. Urtreger AJ, Kazanietz MG, Bal de Kier Joffe ED. Contribution of individual PKC isoforms to breast cancer progression. IUBMB Life. 2012;64:18-26.

73. Nakashima S. Protein kinase C alpha (PKC alpha): regulation and biological function. J Biochem. 2002;132:669-75.

74. Kerfoot C, Huang W, Rotenberg SA. Immunohistochemical analysis of advanced human breast carcinomas reveals downregulation of protein kinase C alpha. J Histochem Cytochem. 2004;52:419-22.

75. Lonne GK, Cornmark L, Zahirovic IO, Landberg G, Jirstrom K, Larsson C. PKCalpha expression is a marker for breast cancer aggressiveness. Mol Cancer. 2010;9:76.

76. Parsons M, Keppler MD, Kline A, Messent A, Humphries MJ, Gilchrist R, et al. Site-directed perturbation of protein kinase C- integrin interaction blocks carcinoma cell chemotaxis. Mol Cell Biol. 2002;22:5897-911.

77. Sledge GW, Jr., Gokmen-Polar Y. Protein kinase C-beta as a therapeutic target in breast cancer. Semin Oncol. 2006;33:S15-8.

78. Li H, Weinstein IB. Protein kinase C beta enhances growth and expression of cyclin D1 in human breast cancer cells. Cancer Res. 2006;66:11399-408.

79. Teicher BA, Alvarez E, Menon K, Esterman MA, Considine E, Shih C, et al. Antiangiogenic effects of a protein kinase Cbeta-selective small molecule. Cancer Chemother Pharmacol. 2002;49:69-77.

80. Xia P, Aiello LP, Ishii H, Jiang ZY, Park DJ, Robinson GS, et al. Characterization of vascular endothelial growth factor's effect on the activation of protein kinase C, its isoforms, and endothelial cell growth. J Clin Invest. 1996;98:2018-26.

81. Reyland ME. Protein kinase C isoforms: Multi-functional regulators of cell life and death. Front Biosci (Landmark Ed). 2009;14:2386-99.

82. Basu A, Pal D. Two faces of protein kinase Cdelta: the contrasting roles of PKCdelta in cell survival and cell death. ScientificWorldJournal. 2010;10:2272-84.

83. McCracken MA, Miraglia LJ, McKay RA, Strobl JS. Protein kinase C delta is a prosurvival factor in human breast tumor cell lines. Mol Cancer Ther. 2003;2:273-81.

84. Lonne GK, Masoumi KC, Lennartsson J, Larsson C. Protein kinase Cdelta supports survival of MDA-MB-231 breast cancer cells by suppressing the ERK1/2 pathway. J Biol Chem. 2009;284:33456-65.

85. Lu ZG, Liu H, Yamaguchi T, Miki Y, Yoshida K. Protein kinase Cdelta activates RelA/p65 and nuclear factor-kappaB signaling in response to tumor necrosis factor-alpha. Cancer Res. 2009;69:5927-35.

86. Kiley SC, Clark KJ, Duddy SK, Welch DR, Jaken S. Increased protein kinase C delta in mammary tumor cells: relationship to transformtion and metastatic progression. Oncogene. 1999;18:6748-57.

87. Vucenik I, Ramakrishna G, Tantivejkul K, Anderson LM, Ramljak D. Inositol hexaphosphate (IP6) blocks proliferation of human breast cancer cells through a PKCdelta-

dependent increase in p27Kip1 and decrease in retinoblastoma protein (pRb) phosphorylation. Breast Cancer Res Treat. 2005;91:35-45.

88. Sitailo LA, Tibudan SS, Denning MF. The protein kinase C delta catalytic fragment targets Mcl-1 for degradation to trigger apoptosis. J Biol Chem. 2006;281:29703-10.

89. Mohanty S, Huang J, Basu A. Enhancement of cisplatin sensitivity of cisplatin-resistant human cervical carcinoma cells by bryostatin 1. Clin Cancer Res. 2005;11:6730-7.

90. Huang J, Mohanty S, Basu A. Cisplatin resistance is associated with deregulation in protein kinase C-delta. Biochem Biophys Res Commun. 2004;316:1002-8.

91. Toton E, Ignatowicz E, Skrzeczkowska K, Rybczynska M. Protein kinase Cepsilon as a cancer marker and target for anticancer therapy. Pharmacol Rep. 2011;63:19-29.

92. Pan Q, Bao LW, Kleer CG, Sabel MS, Griffith KA, Teknos TN, et al. Protein kinase C epsilon is a predictive biomarker of aggressive breast cancer and a validated target for RNA interference anticancer therapy. Cancer Res. 2005;65:8366-71.

93. Kampfer S, Windegger M, Hochholdinger F, Schwaiger W, Pestell RG, Baier G, et al. Protein kinase C isoforms involved in the transcriptional activation of cyclin D1 by transforming Ha-Ras. J Biol Chem. 2001;276:42834-42.

94. Fields AP, Regala RP. Protein kinase C iota: human oncogene, prognostic marker and therapeutic target. Pharmacol Res. 2007;55:487-97.

95. Kojima Y, Akimoto K, Nagashima Y, Ishiguro H, Shirai S, Chishima T, et al. The overexpression and altered localization of the atypical protein kinase C lambda/iota in breast cancer correlates with the pathologic type of these tumors. Hum Pathol. 2008;39:824-31.

96. Schondorf T, Kurbacher CM, Becker M, Warm M, Kolhagen H, Gohring UJ. Heterogeneity of proteinkinase C activity and PKC-zeta expression in clinical breast carcinomas. Clin Exp Med. 2001;1:1-8.

97. Urtreger AJ, Grossoni VC, Falbo KB, Kazanietz MG, Bal de Kier Joffe ED. Atypical protein kinase C-zeta modulates clonogenicity, motility, and secretion of proteolytic enzymes in murine mammary cells. Mol Carcinog. 2005;42:29-39.

98. Sun R, Gao P, Chen L, Ma D, Wang J, Oppenheim JJ, et al. Protein kinase C zeta is required for epidermal growth factor-induced chemotaxis of human breast cancer cells. Cancer Res. 2005;65:1433-41.

99. Liu Y, Wang J, Wu M, Wan W, Sun R, Yang D, et al. Down-regulation of 3-phosphoinositide-dependent protein kinase-1 levels inhibits migration and experimental metastasis of human breast cancer cells. Mol Cancer Res. 2009;7:944-54.

100. Tait SW, Green DR. Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol. 2010;11:621-32.

101. Ruvolo PP, Deng X, Carr BK, May WS. A functional role for mitochondrial protein kinase Calpha in Bcl2 phosphorylation and suppression of apoptosis. J Biol Chem. 1998;273:25436-42.

102. Le XF, Marcelli M, McWatters A, Nan B, Mills GB, O'Brian CA, et al. Heregulin-induced apoptosis is mediated by down-regulation of Bcl-2 and activation of caspase-7 and is potentiated by impairment of protein kinase C alpha activity. Oncogene. 2001;20:8258-69.

103. Baudot AD, Jeandel PY, Mouska X, Maurer U, Tartare-Deckert S, Raynaud SD, et al. The tyrosine kinase Syk regulates the survival of chronic lymphocytic leukemia B cells through

PKCdelta and proteasome-dependent regulation of Mcl-1 expression. Oncogene. 2009;28:3261-73.

104. Quadros MR, Connelly S, Kari C, Abrams MT, Wickstrom E, Rodeck U. EGFR-dependent downregulation of Bim in epithelial cells requires MAPK and PKC-delta activities. Cancer Biol Ther. 2006;5:498-504.

105. Choi SY, Kim MJ, Kang CM, Bae S, Cho CK, Soh JW, et al. Activation of Bak and Bax through c-abl-protein kinase Cdelta-p38 MAPK signaling in response to ionizing radiation in human non-small cell lung cancer cells. J Biol Chem. 2006;281:7049-59.

106. Basu A, Sivaprasad U. Protein kinase Cepsilon makes the life and death decision. Cell Signal. 2007;19:1633-42.

107. Pardo OE, Wellbrock C, Khanzada UK, Aubert M, Arozarena I, Davidson S, et al. FGF-2 protects small cell lung cancer cells from apoptosis through a complex involving PKCepsilon, B-Raf and S6K2. Embo J. 2006;25:3078-88.

108. Korner C, Keklikoglou I, Bender C, Worner A, Munstermann E, Wiemann S. MicroRNA-31 sensitizes human breast cells to apoptosis by direct targeting of protein kinase C epsilon (PKCepsilon). J Biol Chem. 2013;288:8750-61.

109. Diaz-Meco MT, Municio MM, Frutos S, Sanchez P, Lozano J, Sanz L, et al. The product of par-4, a gene induced during apoptosis, interacts selectively with the atypical isoforms of protein kinase C. Cell. 1996;86:777-86.

110. Xin M, Gao F, May WS, Flagg T, Deng X. Protein kinase Czeta abrogates the proapoptotic function of Bax through phosphorylation. J Biol Chem. 2007;282:21268-77.

111. Desai S, Pillai P, Win-Piazza H, Acevedo-Duncan M. PKC-iota promotes glioblastoma cell survival by phosphorylating and inhibiting BAD through a phosphatidylinositol 3-kinase pathway. Biochim Biophys Acta. 2011;1813:1190-7.

112. Barouch-Bentov R, Lemmens EE, Hu J, Janssen EM, Droin NM, Song J, et al. Protein kinase C-theta is an early survival factor required for differentiation of effector CD8+ T cells. J Immunol. 2005;175:5126-34.

113. Osada S, Mizuno K, Saido TC, Akita Y, Suzuki K, Kuroki T, et al. A phorbol ester receptor/protein kinase, nPKC eta, a new member of the protein kinase C family predominantly expressed in lung and skin. J Biol Chem. 1990;265:22434-40.

114. Quan T, Fisher GJ. Cloning and characterization of the human protein kinase C-eta promoter. J Biol Chem. 1999;274:28566-74.

115. Chida K, Nakada T, Otsuka H, Kuroki T, Satoh H. Assignment of protein kinase C eta (Pkch) to mouse chromosome band 12C3-D2 by in situ hybridization. Cytogenet Cell Genet. 1998;82:30-1.

116. Kashiwagi M, Ohba M, Chida K, Kuroki T. Protein kinase C eta (PKC eta): its involvement in keratinocyte differentiation. J Biochem. 2002;132:853-7.

117. Bacher N, Zisman Y, Berent E, Livneh E. Isolation and characterization of PKC-L, a new member of the protein kinase C-related gene family specifically expressed in lung, skin, and heart. Mol Cell Biol. 1991;11:126-33.

118. Akkaraju GR, Basu A. Overexpression of protein kinase C-eta attenuates caspase activation and tumor necrosis factor-alpha-induced cell death. Biochem Biophys Res Commun. 2000;279:103-7.

119. Basu A. The involvement of novel protein kinase C isozymes in influencing sensitivity of breast cancer MCF-7 cells to tumor necrosis factor-alpha. Mol Pharmacol. 1998;53:105-11.

120. Fima E, Shtutman M, Libros P, Missel A, Shahaf G, Kahana G, et al. PKCeta enhances cell cycle progression, the expression of G1 cyclins and p21 in MCF-7 cells. Oncogene. 2001;20:6794-804.

121. Fima E, Shahaf G, Hershko T, Apte RN, Livneh E. Expression of PKCeta in NIH-3T3 cells promotes production of the pro-inflammatory cytokine interleukin-6. Eur Cytokine Netw. 1999;10:491-500.

122. Hussaini IM, Karns LR, Vinton G, Carpenter JE, Redpath GT, Sando JJ, et al. Phorbol 12-myristate 13-acetate induces protein kinase ceta-specific proliferative response in astrocytic tumor cells. J Biol Chem. 2000;275:22348-54.

123. Raveh-Amit H, Hai N, Rotem-Dai N, Shahaf G, Gopas J, Livneh E. Protein kinase Ceta activates NF-kappaB in response to camptothecin-induced DNA damage. Biochem Biophys Res Commun. 2011;412:313-7.

124. Ohba M, Ishino K, Kashiwagi M, Kawabe S, Chida K, Huh NH, et al. Induction of differentiation in normal human keratinocytes by adenovirus-mediated introduction of the eta and delta isoforms of protein kinase C. Mol Cell Biol. 1998;18:5199-207.

125. Fu G, Gascoigne NR. Protein kinase Ceta, an emerging player in T-cell biology. Cell Cycle. 2012;11:837-8.

126. Quann EJ, Liu X, Altan-Bonnet G, Huse M. A cascade of protein kinase C isozymes promotes cytoskeletal polarization in T cells. Nat Immunol. 2011;12:647-54.

127. Fu G, Hu J, Niederberger-Magnenat N, Rybakin V, Casas J, Yachi PP, et al. Protein kinase C eta is required for T cell activation and homeostatic proliferation. Sci Signal. 2011;4:ra84.

128. Park DW, Lee HK, Lyu JH, Chin H, Kang SW, Kim YJ, et al. TLR2 stimulates ABCA1 expression via PKC-eta and PLD2 pathway. Biochem Biophys Res Commun. 2013;430:933-7.

129. Hashimoto Y, Osada S, Ohno S, Kuroki T. A Ca(2+)-independent protein kinase C, nPKC eta: its structure, distribution and possible function. Tohoku J Exp Med. 1992;168:275-8.

130. Balendran A, Hare GR, Kieloch A, Williams MR, Alessi DR. Further evidence that 3-phosphoinositide-dependent protein kinase-1 (PDK1) is required for the stability and phosphorylation of protein kinase C (PKC) isoforms. FEBS Lett. 2000;484:217-23.

131. Lachmann S, Bar S, Rommelaere J, Nuesch JP. Parvovirus interference with intracellular signalling: mechanism of PKCeta activation in MVM-infected A9 fibroblasts. Cell Microbiol. 2008;10:755-69.

132. Littler DR, Walker JR, She YM, Finerty PJ, Jr., Newman EM, Dhe-Paganon S. Structure of human protein kinase C eta (PKCeta) C2 domain and identification of phosphorylation sites. Biochem Biophys Res Commun. 2006;349:1182-9.

133. Chen CC, Wang JK, Chen WC. TPA induces translocation but not down-regulation of new PKC isoform eta in macrophages, MDCK cells and astrocytes. FEBS Lett. 1997;412:30-4.

134. Resnick MS, Luo X, Vinton EG, Sando JJ. Selective up-regulation of protein kinase C eta in phorbol ester-sensitive versus -resistant EL4 mouse thymoma cells. Cancer Res. 1997;57:2209-15.

135. Chida K, Murakami A, Tagawa T, Ikuta T, Kuroki T. Cholesterol sulfate, a second messenger for the eta isoform of protein kinase C, inhibits promotional phase in mouse skin carcinogenesis. Cancer Res. 1995;55:4865-9.

136. Okano M, Yokoyama T, Miyanaga T, Ohtsuki K. Activation of C-kinase eta through its cholesterol-3-sulfate-dependent phosphorylation by casein kinase I in vitro. Biol Pharm Bull. 2004;27:109-12.

137. Redig AJ, Sassano A, Majchrzak-Kita B, Katsoulidis E, Liu H, Altman JK, et al. Activation of protein kinase C{eta} by type I interferons. J Biol Chem. 2009;284:10301-14.

138. Uddin S, Sassano A, Deb DK, Verma A, Majchrzak B, Rahman A, et al. Protein kinase C-delta (PKC-delta) is activated by type I interferons and mediates phosphorylation of Stat1 on serine 727. J Biol Chem. 2002;277:14408-16.

139. Srivastava KK, Batra S, Sassano A, Li Y, Majchrzak B, Kiyokawa H, et al. Engagement of protein kinase C-theta in interferon signaling in T-cells. J Biol Chem. 2004;279:29911-20.

140. Ivaska J, Bosca L, Parker PJ. PKCepsilon is a permissive link in integrin-dependent IFN-gamma signalling that facilitates JAK phosphorylation of STAT1. Nat Cell Biol. 2003;5:363-9.

141. Venkatesan BA, Mahimainathan L, Ghosh-Choudhury N, Gorin Y, Bhandari B, Valente AJ, et al. PI 3 kinase-dependent Akt kinase and PKCepsilon independently regulate interferon-gamma-induced STAT1alpha serine phosphorylation to induce monocyte chemotactic protein-1 expression. Cell Signal. 2006;18:508-18.

142. Karp G, Maissel A, Livneh E. Hormonal regulation of PKC: estrogen up-regulates PKCeta expression in estrogen-responsive breast cancer cells. Cancer Lett. 2007;246:173-81.

143. Luparello C, Sirchia R, Longo A. Type V collagen and protein kinase C eta down-regulation in 8701-BC breast cancer cells. Mol Carcinog. 2013;52:348-58.

144. Raveh-Amit H, Maissel A, Poller J, Marom L, Elroy-Stein O, Shapira M, et al. Translational control of protein kinase Ceta by two upstream open reading frames. Mol Cell Biol. 2009;29:6140-8.

145. Morrish BC, Rumsby MG. The 5' UTR of protein kinase C epsilon confers translational regulation in vitro and in vivo. Biochem Biophys Res Commun. 2001;283:1091-8.

146. Maissel A, Marom M, Shtutman M, Shahaf G, Livneh E. PKCeta is localized in the Golgi, ER and nuclear envelope and translocates to the nuclear envelope upon PMA activation and serum-starvation: C1b domain and the pseudosubstrate containing fragment target PKCeta to the Golgi and the nuclear envelope. Cell Signal. 2006;18:1127-39.

147. Suzuki M, Iio Y, Saito N, Fujimoto T. Protein kinase Ceta is targeted to lipid droplets. Histochem Cell Biol. 2013;139:505-11.

148. Chida K, Hara T, Hirai T, Konishi C, Nakamura K, Nakao K, et al. Disruption of protein kinase Ceta results in impairment of wound healing and enhancement of tumor formation in mouse skin carcinogenesis. Cancer Res. 2003;63:2404-8.

149. Rotem-Dai N, Oberkovitz G, Abu-Ghanem S, Livneh E. PKCeta confers protection against apoptosis by inhibiting the pro-apoptotic JNK activity in MCF-7 cells. Exp Cell Res. 2009;315:2616-23.

150. Hussaini IM, Carpenter JE, Redpath GT, Sando JJ, Shaffrey ME, Vandenberg SR. Protein kinase C-eta regulates resistance to UV- and gamma-irradiation-induced apoptosis in glioblastoma cells by preventing caspase-9 activation. Neuro Oncol. 2002;4:9-21.

151. Sonnemann J, Gekeler V, Ahlbrecht K, Brischwein K, Liu C, Bader P, et al. Down-regulation of protein kinase Ceta by antisense oligonucleotides sensitises A549 lung cancer cells to vincristine and paclitaxel. Cancer Lett. 2004;209:177-85.

152. Sonnemann J, Gekeler V, Sagrauske A, Muller C, Hofmann HP, Beck JF. Down-regulation of protein kinase Ceta potentiates the cytotoxic effects of exogenous tumor necrosis factor-related apoptosis-inducing ligand in PC-3 prostate cancer cells. Mol Cancer Ther. 2004;3:773-81.

153. Abu-Ghanem S, Oberkovitz G, Benharroch D, Gopas J, Livneh E. PKCeta expression contributes to the resistance of Hodgkin's lymphoma cell lines to apoptosis. Cancer Biol Ther. 2007;6:1375-80.

154. Lu HC, Chou FP, Yeh KT, Chang YS, Hsu NC, Chang JG. Analysing the expression of protein kinase C eta in human hepatocellular carcinoma. Pathology. 2009;41:626-9.

155. Brenner W, Farber G, Herget T, Wiesner C, Hengstler JG, Thuroff JW. Protein kinase C eta is associated with progression of renal cell carcinoma (RCC). Anticancer Res. 2003;23:4001-6.

156. Masso-Welch PA, Verstovsek G, Darcy K, Tagliarino C, Ip MM. Protein kinase C eta upregulation and secretion during postnatal rat mammary gland differentiation. Eur J Cell Biol. 1998;77:48-59.

157. Masso-Welch PA, Winston JS, Edge S, Darcy KM, Asch H, Vaughan MM, et al. Altered expression and localization of PKC eta in human breast tumors. Breast Cancer Res Treat. 2001;68:211-23.

158. Suzuki T, Elias BC, Seth A, Shen L, Turner JR, Giorgianni F, et al. PKC eta regulates occludin phosphorylation and epithelial tight junction integrity. Proc Natl Acad Sci U S A. 2009;106:61-6.

159. Martin TA, Mason MD, Jiang WG. Tight junctions in cancer metastasis. Front Biosci (Landmark Ed). 2011;16:898-936.

160. Beck J, Bohnet B, Brugger D, Bader P, Dietl J, Scheper RJ, et al. Multiple gene expression analysis reveals distinct differences between G2 and G3 stage breast cancers, and correlations of PKC eta with MDR1, MRP and LRP gene expression. Br J Cancer. 1998;77:87-91.

161. Tamarkin A, Zurgil U, Braiman A, Hai N, Krasnitsky E, Maissel A, et al. DNA damage targets PKCeta to the nuclear membrane via its C1b domain. Exp Cell Res. 2011;317:1465-75.

162. Cabodi S, Calautti E, Talora C, Kuroki T, Stein PL, Dotto GP. A PKC-eta/Fyn-dependent pathway leading to keratinocyte growth arrest and differentiation. Mol Cell. 2000;6:1121-9.

163. Livneh E, Shimon T, Bechor E, Doki Y, Schieren I, Weinstein IB. Linking protein kinase C to the cell cycle: ectopic expression of PKC eta in NIH3T3 cells alters the expression of cyclins and Cdk inhibitors and induces adipogenesis. Oncogene. 1996;12:1545-55.

CHAPTER II

NOVEL REGULATION OF PROTEIN KINASE C-η

Deepanwita Pal, Shalini Persaud Outram and Alakananda Basu

Biochem Biophys Res Commun. 2012 Sep 7;425(4):836-41

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.

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).

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 (13-17). 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 (20-24). 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 (28). 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 (29). 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 (20-24). Thus, understanding the mechanism of PKCη upregulation has significant implications in cancer therapy.

Figure 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.

Figure 1.

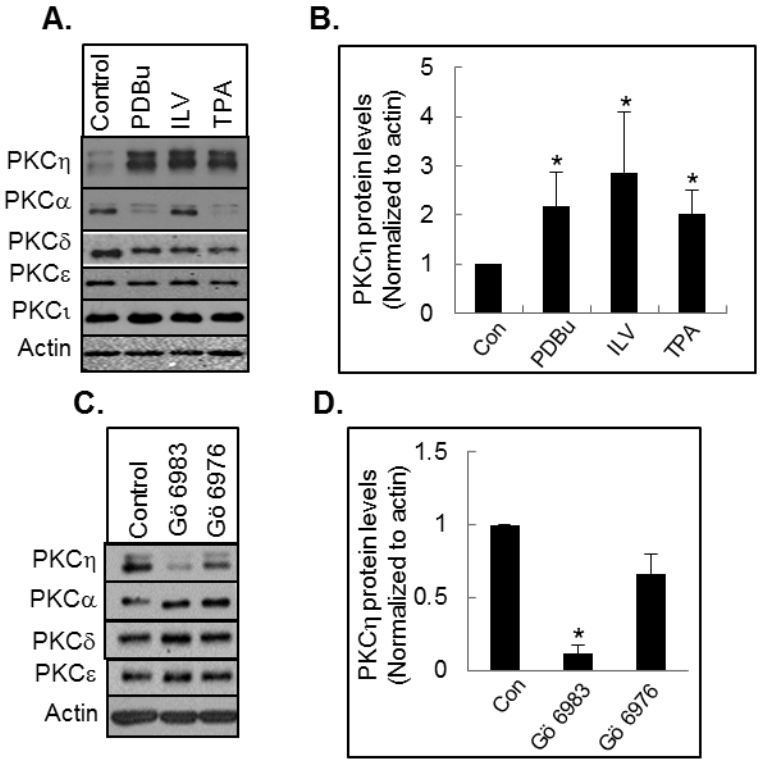

A.
Control
PDBu
ILV
TPA
PKCη
PKCα
PKCδ
PKCε
PKCι
Actin
B.
PKCη protein levels
(Normalized to actin)
5
4
3
2
1
0
*
*
*
Con
PDBu
ILV
TPA
C.
Control
Gö 6983
Gö 6976
PKCη
PKCα
PKCδ
PKCε
Actin
D.
PKCη protein levels
(Normalized to actin)
1.5
1
0.5
0
*
Con
Gö 6983
Gö 6976

Figure 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.

Figure 2.

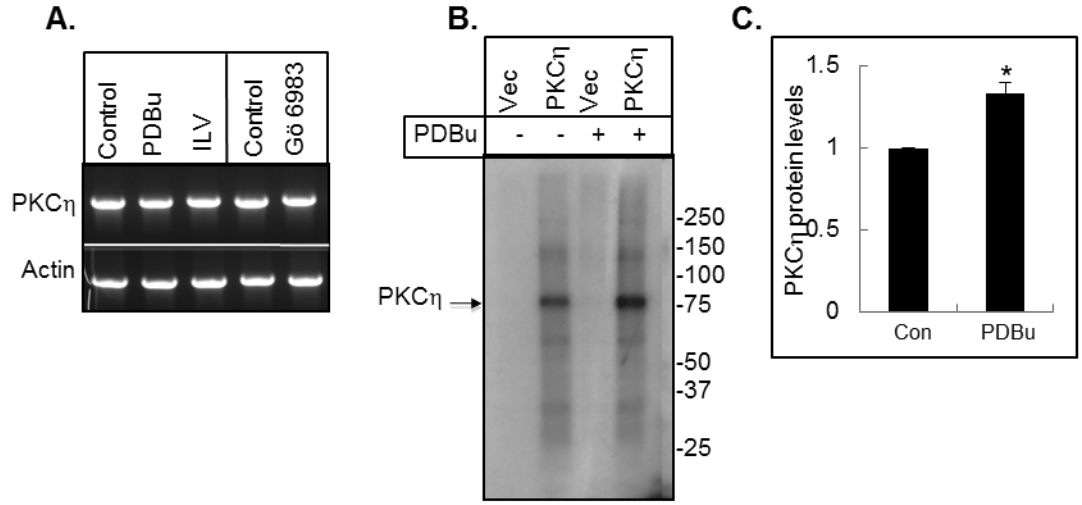

A.
Control
PDBu
ILV
Control
Gö 6983
PKCη
Actin
B.
Vec
PKCη
Vec
PKCη
PDBu
-
-
+
+
PKCη
-250
-150
-100
-75
-50
-37
-25
C.
PKCη protein levels
1.5
1
0.5
0
*
Con
PDBu

Figure 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.

Figure 3.

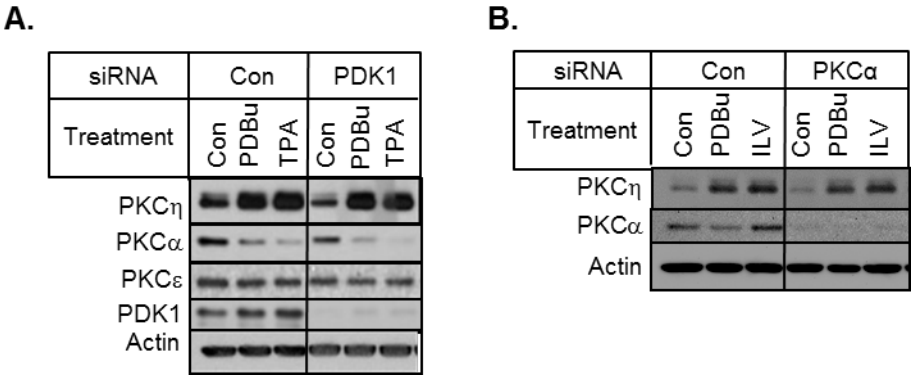
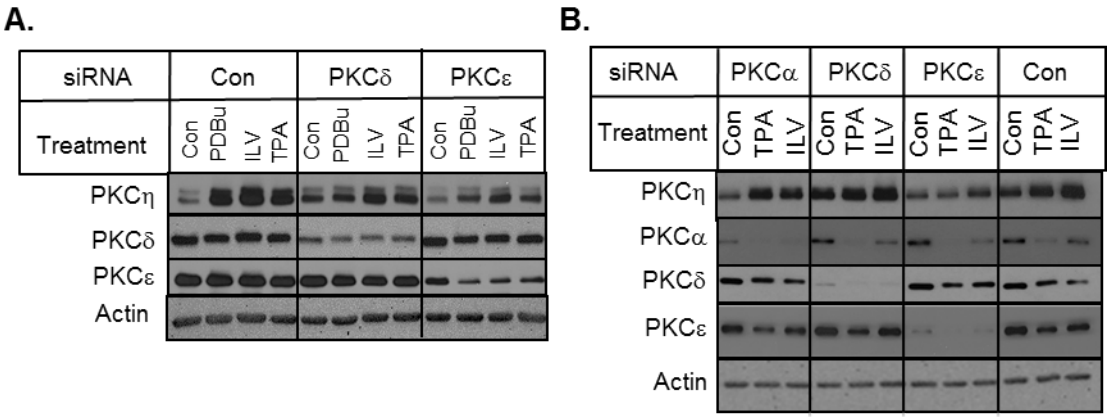

A.
siRNA
Con
PDK1
Treatment
Con
PDBu
TPA
Con
PDBu
TPA
PKCη
PKCα
PKCε
PDK1
Actin
B.
siRNA
Con
PKCα
Treatment
Con
PDBu
ILV
Con
PDBu
ILV
PKCη
PKCα
Actin

Figure 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.

Figure 4.


A.
siRNA
Con
PKCδ
PKCε
Treatment
Con
PDBu
ILV
TPA
PKCη
PKCδ
PKCε
Actin
B.
siRNA
PKCα
PKCδ
PKCε
Con
Treatment
Con
TPA
ILV
PKCη
PKCα
PKCδ
PKCε
Actin

References

1. Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science. 1992;258:607-14.

2. Griner EM, Kazanietz MG. Protein kinase C and other diacylglycerol effectors in cancer. Nat Rev Cancer. 2007;7:281-94.

3. Steinberg SF. Structural basis of protein kinase C isoform function. Physiol Rev. 2008;88:1341-78.

4. Blumberg PM. Protein kinase C as the receptor for the phorbol ester tumor promoters: sixth Rhoads memorial award lecture. Cancer Res. 1988;48:1-8.

5. Basu A, Pal D. Two faces of protein kinase Cdelta: the contrasting roles of PKCdelta in cell survival and cell death. ScientificWorldJournal. 2010;10:2272-84.

6. Gould CM, Newton AC. The life and death of protein kinase C. Curr Drug Targets. 2008;9:614-25.

7. Parekh DB, Ziegler W, Parker PJ. Multiple pathways control protein kinase C phosphorylation. EMBO J. 2000;19:496-503.

8. Newton AC. Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. Biochem J. 2003;370:361-71.

9. Flint AJ, Paladini RD, Koshland DE, Jr. Autophosphorylation of protein kinase C at three separated regions of its primary sequence. Science. 1990;249:408-11.

10. Dutil EM, Keranen LM, DePaoli-Roach AA, Newton AC. In vivo regulation of protein kinase C by trans-phosphorylation followed by autophosphorylation. J Biol Chem. 1994;269:29359-62.

11. Basu A, Sridharan S, Persaud S. Regulation of protein kinase C delta downregulation by protein kinase C epsilon and mammalian target of rapamycin complex 2. Cell Signal. 2009;21:1680-5.

12. Rybin VO, Sabri A, Short J, Braz JC, Molkentin JD, Steinberg SF. Cross-regulation of novel protein kinase C (PKC) isoform function in cardiomyocytes. Role of PKC epsilon in activation loop phosphorylations and PKC delta in hydrophobic motif phosphorylations. J Biol Chem. 2003;278:14555-64.

13. Basu A. The potential of protein kinase C as a target for anticancer treatment. Pharmacol Ther. 1993;59:257-80.

14. Kashiwagi M, Ohba M, Chida K, Kuroki T. Protein kinase C eta (PKC eta): its involvement in keratinocyte differentiation. J Biochem. 2002;132:853-7.

15. Fima E, Shtutman M, Libros P, Missel A, Shahaf G, Kahana G, et al. PKCeta enhances cell cycle progression, the expression of G1 cyclins and p21 in MCF-7 cells. Oncogene. 2001;20:6794-804.

16. Hussaini IM, Karns LR, Vinton G, Carpenter JE, Redpath GT, Sando JJ, et al. Phorbol 12-myristate 13-acetate induces protein kinase ceta-specific proliferative response in astrocytic tumor cells. J Biol Chem. 2000;275:22348-54.

17. Akkaraju GR, Basu A. Overexpression of protein kinase C-eta attenuates caspase activation and tumor necrosis factor-alpha-induced cell death. Biochem Biophys Res Commun. 2000;279:103-7.

18. Hashimoto Y, Osada S, Ohno S, Kuroki T. A Ca(2+)-independent protein kinase C, nPKC eta: its structure, distribution and possible function. Tohoku J Exp Med. 1992;168:275-8.

19. Masso-Welch PA, Winston JS, Edge S, Darcy KM, Asch H, Vaughan MM, et al. Altered expression and localization of PKC eta in human breast tumors. Breast Cancer Res Treat. 2001;68:211-23.

20. Basu A. The involvement of novel protein kinase C isozymes in influencing sensitivity of breast cancer MCF-7 cells to tumor necrosis factor-alpha. Mol Pharmacol. 1998;53:105-11.

21. Hussaini IM, Carpenter JE, Redpath GT, Sando JJ, Shaffrey ME, Vandenberg SR. Protein kinase C-eta regulates resistance to UV- and gamma-irradiation-induced apoptosis in glioblastoma cells by preventing caspase-9 activation. Neuro Oncol. 2002;4:9-21.

22. Sonnemann J, Gekeler V, Ahlbrecht K, Brischwein K, Liu C, Bader P, et al. Down-regulation of protein kinase Ceta by antisense oligonucleotides sensitises A549 lung cancer cells to vincristine and paclitaxel. Cancer Lett. 2004;209:177-85.

23. Raveh-Amit H, Hai N, Rotem-Dai N, Shahaf G, Gopas J, Livneh E. Protein kinase Ceta activates NF-kappaB in response to camptothecin-induced DNA damage. Biochemical and biophysical research communications. 2011;412:313-7.

24. Sonnemann J, Gekeler V, Sagrauske A, Muller C, Hofmann HP, Beck JF. Down-regulation of protein kinase Ceta potentiates the cytotoxic effects of exogenous tumor necrosis factor-related apoptosis-inducing ligand in PC-3 prostate cancer cells. Mol Cancer Ther. 2004;3:773-81.

25. Chen CC, Wang JK, Chen WC. TPA induces translocation but not down-regulation of new PKC isoform eta in macrophages, MDCK cells and astrocytes. FEBS Lett. 1997;412:30-4.

26. Resnick MS, Luo X, Vinton EG, Sando JJ. Selective up-regulation of protein kinase C eta in phorbol ester-sensitive versus -resistant EL4 mouse thymoma cells. Cancer Res. 1997;57:2209-15.

27. Lu D, Huang J, Basu A. Protein kinase Cepsilon activates protein kinase B/Akt via DNA-PK to protect against tumor necrosis factor-alpha-induced cell death. J Biol Chem. 2006;281:22799-807.

28. Bacher N, Zisman Y, Berent E, Livneh E. Isolation and characterization of PKC-L, a new member of the protein kinase C-related gene family specifically expressed in lung, skin, and heart. Molecular and cellular biology. 1991;11:126-33.

29. Basu A, Kozikowski AP, Lazo JS. Structural requirements of lyngbyatoxin A for activation and downregulation of protein kinase C. Biochemistry. 1992;31:3824-30.

30. Lachmann S, Bar S, Rommelaere J, Nuesch JP. Parvovirus interference with intracellular signalling: mechanism of PKCeta activation in MVM-infected A9 fibroblasts. Cell Microbiol. 2008;10:755-69.

31. Ziegler WH, Parekh DB, Le Good JA, Whelan RD, Kelly JJ, Frech M, et al. Rapamycin-sensitive phosphorylation of PKC on a carboxy-terminal site by an atypical PKC complex. Curr Biol. 1999;9:522-9.

32. Hansra G, Garcia-Paramio P, Prevostel C, Whelan RD, Bornancin F, Parker PJ. Multisite dephosphorylation and desensitization of conventional protein kinase C isotypes. Biochem J. 1999;342 (Pt 2):337-44.

33. Lee HW, Smith L, Pettit GR, Smith JB. Bryostatin 1 and phorbol ester down-modulate protein kinase C-alpha and -epsilon via the ubiquitin/proteasome pathway in human fibroblasts. Mol Pharmacol. 1997;51:439-47.

34. Prevostel C, Alice V, Joubert D, Parker PJ. Protein kinase C(alpha) actively downregulates through caveolae-dependent traffic to an endosomal compartment. J Cell Sci. 2000;113 (Pt 14):2575-84.

35. Leontieva OV, Black JD. Identification of two distinct pathways of protein kinase Calpha down-regulation in intestinal epithelial cells. J Biol Chem. 2004;279:5788-801.

36. Cameron AJ, Escribano C, Saurin AT, Kostelecky B, Parker PJ. PKC maturation is promoted by nucleotide pocket occupation independently of intrinsic kinase activity. Nat Struct Mol Biol. 2009;16:624-30.

37. Kang BS, French OG, Sando JJ, Hahn CS. Activation-dependent degradation of protein kinase C eta. Oncogene. 2000;19:4263-72.

38. Balendran A, Hare GR, Kieloch A, Williams MR, Alessi DR. Further evidence that 3-phosphoinositide-dependent protein kinase-1 (PDK1) is required for the stability and phosphorylation of protein kinase C (PKC) isoforms. FEBS Lett. 2000;484:217-23.

39. Chida K, Hara T, Hirai T, Konishi C, Nakamura K, Nakao K, et al. Disruption of protein kinase Ceta results in impairment of wound healing and enhancement of tumor formation in mouse skin carcinogenesis. Cancer Res. 2003;63:2404-8.

40. Abu-Ghanem S, Oberkovitz G, Benharroch D, Gopas J, Livneh E. PKCeta expression contributes to the resistance of Hodgkin's lymphoma cell lines to apoptosis. Cancer Biol Ther. 2007;6:1375-80.

41. Krasnitsky E, Baumfeld Y, Freedman J, Sion-Vardy N, Ariad S, Novack V, et al. PKCeta is a novel prognostic marker in non-small cell lung cancer. Anticancer research. 2012;32:1507-13.

42. Lu HC, Chou FP, Yeh KT, Chang YS, Hsu NC, Chang JG. Analysing the expression of protein kinase C eta in human hepatocellular carcinoma. Pathology. 2009;41:626-9.

43. Basu A, Sivaprasad U. Protein kinase Cepsilon makes the life and death decision. Cell Signal. 2007;19:1633-42.

44. Karp G, Maissel A, Livneh E. Hormonal regulation of PKC: estrogen up-regulates PKCeta expression in estrogen-responsive breast cancer cells. Cancer Lett. 2007;246:173-81.

CHAPTER III

UPREGULATION OF PKCη BY PKCε AND PDK1 INVOLVES TWO DISTINCT MECHANISMS AND PROMOTES BREAST CANCER CELL SURVIVAL

Deepanwita Pal*, Shalini Persaud Outram* and Alakananda Basu

Biochim Biophys Acta. 2013 Aug; 1830(8):4040-5.

*Both authors contributed equally.

ABSTRACT

***Background*:** Protein kinase C (PKC) serves as the receptor for tumor-promoting phorbol esters, which are potent activators of conventional (c) and novel (n) PKCs. We recently showed that these activators induced selective upregulation of PKCη in breast cancer cells. The objective of this study is to understand unique regulation of PKCη and its importance in breast cancer.

***Methods*:** The levels of PKC isozymes were monitored in breast cancer cells following treatment with inhibitors of kinases, proteasome and proteases by Western blotting. PKCε was introduced by adenoviral delivery. PKCη and PDK1 were depleted by siRNA silencing. Cell growth was determined by the MTT or clonal assay.

***Results*:** The general PKC inhibitors Gö 6983 and bisindolylmaleimide but not cPKC inhibitor Gö 6976 led to substantial PKCη downregulation, which was partly rescued by the introduction of nPKCε. Inhibition of phosphoinositide-dependent kinase-1 (PDK1) by Ly294002 or knockdown of PDK1 also led to downregulation of basal PKCη but had no effect on PKC activator-induced upregulation of PKCη. Proteasome inhibitors blocked PKCη downregulation triggered by PDK1 inhibition/depletion but not by Gö 6983. PKCη level increased in malignant but not in non-tumorigenic or pre-malignant cells in the progressive MCF-10A series associated with activated PDK1, and knockdown of PKCη inhibited breast cancer cell growth and clonogenic survival.

***Conclusion*:** Upregulation of PKCη contributes to breast cancer cell growth and targeting either PKCε or PDK1 triggers PKCη downregulation but involves two distinct mechanisms.

General significance: The status of PKCη may serve as a potential biomarker for breast cancer malignancy.

1. Introduction

Protein kinase C, a family of phospholipid-dependent serine/threonine kinases, plays a critical role in growth factor signal transduction pathways (1, 2). The PKC family is classified into conventional (α, βI, βII, γ), novel (δ, ε, η, θ) and atypical PKCs (ζ, λ/ι) based on their structural features and cofactor sensitivity (1, 2). Diacylglycerol (DAG) and Ca^{2+} are required for the activity of conventional PKCs whereas novel PKCs are insensitive to Ca^{2+} but dependent on DAG; atypical PKCs are insensitive to both Ca^{2+} and DAG (1, 2).

Tumor-promoting phorbol esters are potent activators of PKCs and can substitute for DAG. However, sustained activation by phorbol esters leads to downregulation of PKCs causing termination of the PKC signaling. Calcium-activated proteases, such as calpains and ubiquitin proteasome-mediated pathways have been implicated in PKC activator-induced downregulation of PKCs. Calpains are believed to downregulate cPKCs (3, 4) whereas PKCα, -δ and -ε were shown to be degraded via proteasome-mediated pathway (5-7). Downregulation of PKCs has important implications in regulating long-term cellular responses such as cell proliferation, differentiation and tumor promotion (1, 8, 9).

PKCη is a member of the novel PKC family and shares highest homology with PKCε (10). However, the regulation of PKCη appears to be unique. We have recently shown that several different PKC activators, including tumor-promoting phorbol esters, induce upregulation rather than downregulation of PKCη (11). Moreover, phosphorylation of PKCη by novel PKCε appears to be responsible for PKC activator-induced upregulation of PKCη (11). Depending on the cellular context, inhibition of PKCη could either promote or suppress tumor promotion (12-19). Since PKCη is upregulated by tumor promoters, we examined the mechanism of PKCη

downregulation. Our results indicate that inhibition of either PKC or PDK1 induces PKCη downregulation but involves two distinct mechanisms. While inhibition of PKC resulted in PKCη downregulation via proteasome-independent pathway, inhibition of PDK1 led to PKCη downregulation via proteasome-dependent pathway. We also showed that PKCη level is increased in malignant but not in non-tumorigenic or pre-malignant cells in the progressive MCF-10A series and knockdown of PKCη inhibited breast cancer cell growth.

2. Materials and Methods

2.1. Materials

PDBu was purchased from Alexis Biochemicals (San Diego, CA). Ly294002 was purchased from Calbiochem (San Diego, CA) and Cell Signaling Technology, Inc (Danvers, MA). Gö 6983, Gö 6976, KT5720, Rottlerin, PD98059, BIM, U0126, MG-132, lactacystin, calpeptin and cathepsin inhibitor I were obtained from Calbiochem (San Diego, CA). Polyclonal antibodies to PKCη, PKCδ, PKCε and monoclonal antibody to GAPDH were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibody to PKCα was obtained from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibody against PDK1 was purchased from Cell Signaling Technology, Inc. (Danvers, MA). 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). Control non-targeting siRNA and siRNA specific for PDK1 and PKCη were obtained from Dharmacon (Lafayette, CO). Poly(vinylidenedifluoride) membrane was from Millipore (Bedford, MA) and enhanced chemiluminescence detection kit was from Amersham (Arlington Heights, IL).

2.2. Cell culture

MCF-7 and T47D cells were maintained in RPMI 1640 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. The MCF-10A series developed by Dr. Fred Miller and colleagues (20), was obtained from the Barbara Ann Karmanos Cancer Institute (Detroit, MI). They were cultured as described previously (21).

2.3. Transfection

Control non-targeting siRNA or SMARTpool siRNA against PDK1 or PKCη were introduced into cells using Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA) and manufacturer's protocol. Cells were treated as indicated in the text and processed for Western blot analysis.

2.4. 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 previously (22).

2.5. Clonogenic assay

Cells transfected with or without control non-targeting or PKCη siRNA were cultured at 37°C in a humidified incubator with 5% CO_2 until there were at least 50 cells per colony. At the end of the incubation, the cells were washed with PBS and incubated with 0.025% crystal violet solution for 15 minutes. Colonies were counted using ImageJ software (http://rsbweb.nih.gov/ij/), and the plate was photographed using the BioChemi System (BioImaging System, UVP, Upland, CA).

2.6. MTT assay

Cells transfected with or without control non-targeting or PKCη siRNA were plated in microtiter plates and incubated at 37°C and 5% CO_2. After 4 days, the number of viable cells was determined using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetra-zolium bromide (MTT) as described previously (23).

2.7. Statistical analysis

Statistical significance was determined by Student's *t* test by using PASW Statistics (SPSS, Inc.). p values < 0.05 were considered statistically significant.

3. Results

3.1. Inhibitors of PKC and PI3K induce downregulation of PKCη

We have previously shown that the regulation of PKCη is unique since it is upregulated rather than downregulated by PKC activators in breast cancer cells (11). In the present study, we examined if inhibition of PKC induces its downregulation. Figure 1A shows that the general PKC inhibitor bisindolylmaleimide caused substantial downregulation of PKCη whereas rottlerin, which inhibits novel PKCδ, had no effect; the conventional PKC inhibitor Gö 6976 caused only a modest decrease in PKCη level in MCF-7 cells. The PI3K inhibitor Ly294002 also induced PKCη downregulation whereas rapamycin, PD98059 and KT5720, which inhibit mTOR, MAPK and PKA, respectively had little or no effect (Fig. 1A). To determine if the ability of PKC or PI3K inhibitor to downregulate PKCη is a general phenomenon, we tested several different breast cancer cell lines. Figure 1B shows that similar to MCF-7 cells, bisindolylmaleimide and Ly294002 induced substantial downregulation of PKCη in T47D cells whereas mTOR inhibitor rapamycin had a modest effect and MEK inhibitor U0126 had no effect. These results suggest that both PKC and PI3K pathways are involved in regulating PKCη level.

3.2. PKC and PI3K inhibitors induce PKCη downregulation via two distinct pathways.

Since PKC activators induce upregulation of PKCη, we examined if the general PKC inhibitor Gö 6983 or the PI3K inhibitor Ly294002 could block PKC activator-induced upregulation of PKCη. Figure 2 shows that while the PKC activator PDBu induced downregulation of PKCα, -δ and -ε, it caused upregulation of PKCη. Consistent with the notion that PKC activity is required for PKC activator-induced downregulation of PKCs (5, 7, 24), the

general PKC inhibitor Gö 6983 prevented PDBu-induced downregulation of PKCα and PKCδ. However, Gö 6983 was able to induce PKCη downregulation in PDBu-treated cells. The PI3K inhibitor Ly294002 caused a substantial decrease in basal PKCη level but the constitutive levels of PKCα, -δ and -ε were not altered by the Ly294002 treatment. In contrast to Gö 6983, Ly294002 was unable to induce PKCη downregulation in PDBu-treated cells. These results suggest that Gö 6983 and Ly294002 induced PKCη downregulation via two distinct pathways.

3.3. Ly294002 induces PKCη downregulation via ubiquitin proteasome-mediated pathway

Both calcium-activated proteases, such as calpains as well as ubiquitin proteasome-mediated pathway have been implicated in the activation-induced downregulation of PKCs (4, 5, 7). Therefore, we examined the ability of calpain inhibitor calpeptin, cathepsin inhibitor I and proteasome inhibitor MG-132 or lactacystin in preventing downregulation of PKCη by Gö 6983 or Ly294002. Figure 3 shows that neither calpain inhibitor nor cathepsin inhibitor blocked PKCη downregulation induced by either Gö 6983 or Ly294002. Although the proteasome inhibitor MG-132 prevented PKCη downregulation by Ly294002, it had only a modest effect on Gö 6983-mediated downregulation of PKCη (Figs. 4A & 4B). Similar results were observed in T47D cells where two different proteasome inhibitors MG-132 and lactacystin blocked Ly294002-mediated but not Gö 6983-mediated downregulation of PKCη (Fig. 4C). These results demonstrate that Ly294002 but not Gö 6983 induces PKCη downregulation via the proteasome-mediated pathway.

3.4. Inhibition of PDK1 and PKCε triggers PKCη downregulation

Since PDK1 is believed to phosphorylate PKCη at the activation loop (25) and Ly294002 inhibits PDK1 which acts downstream of PI3K, we examined if knockdown of PDK1 induces

downregulation of PKCη. Figure 5A shows that similar to Ly294002, depletion of PDK1 by siRNA decreased basal PKCη level. The proteasome inhibitors MG-132 and lactacystin caused a modest increase in PKCη level in cells transfected with control non-targeting siRNA or in untransfected cells, and blocked PKCη downregulation by both Ly294002 and PDK1 knockdown (Fig. 5A). These results suggest that inhibition of PDK1 by Ly294002 targets PKCη to proteasome-mediated downregulation.

Since we have previously shown that novel PKCε was responsible for PKC activator-induced upregulation of PKCη (11), we speculated that the general PKC inhibitor Gö 6983 triggers PKCη downregulation by inhibiting PKCε. Since T47D cells were more dependent on PKCε-mediated upregulation of PKCη (11), we infected T47D cells with adenoviral vector expressing either GFP or PKCε. Figure 5B shows that adenoviral delivery of PKCε but not GFP increased basal PKCε level and partially blocked Gö 6983-induced downregulation of PKCη. Taken together, these results suggest that inhibition of PDK1 and novel PKCε by Ly294002 and Gö 6983, respectively triggers PKCη downregulation via two distinct pathways.

3.5. PKCη is progressively increased in MCF-10A series and promotes breast cancer cell survival

Since PKCη level is regulated by PKC and PI3K/PDK1 pathways that have been implicated in breast cancer (26-28), we compared PKCη level in non-tumorigenic MCF-10A cells and breast cancer cells. The level and activation status of PDK1 was increased in the progressive MCF-10A series (21) that includes spontaneously immortalized non-tumorigenic mammary epithelial MCF-10A cells, premalignant MCF-10AT cells, ductal carcinoma in situ (DCIS) and highly malignant MCF-10CA1d cells (20) . Figure 6A shows that PKCη is expressed only in

malignant breast cancer cells but not in non-tumorigenic MCF-10A or premalignant MCF-10AT cells. The levels of other PKCs, such as PKCα, PKCδ, PKCε or PKCζ were not altered with the malignancy of the MCF-10A series. Similar to MCF-7 and T47D cells, both Gö 6983 and Ly294002 led to PKCη downregulation in CA1d cells (Fig. 6B).

To determine the functional significance of PKCη upregulation in breast cancer cells, we determined the consequence of PKCη depletion on the viability of breast cancer cells. Depletion of PKCη by siRNA (Fig. 7A) decreased MCF-7 cell survival in a long-term clonogenic assay (Figs. 7B & 7C). Knockdown of PKCη also inhibited the growth of both T47D (Fig. 7D) and DCIS (Fig. 7E) cells. These results suggest that PKCη contributes to the viability of breast cancer cells.

4. Discussion

Because of the pivotal role of the PKC family members in signal transduction and cell regulation, there have been significant efforts in understanding their function and regulation. Among the novel PKCs, most of the studies thus far have been focused on PKCδ and -ε but little is known about the regulation of novel PKCη. Our results suggest that the regulation of PKCη is unique. We recently reported that persistent treatment with PKC activators induce upregulation of PKCη rather than downregulation which is seen with other phorbol ester-sensitive conventional and novel PKCs. In the present study, we show that downregulation of PKCη can be achieved via two distinct pathways. While inhibition of PKC induces PKCη downregulation via a proteasome-independent pathway, inhibition of PDK1 results in PKCη downregulation via a proteasome-dependent pathway. We also made a novel observation that PKCη is the only PKC isozyme which is upregulated in breast cancer cells compared to non-malignant cells and downregulation of PKCη inhibits breast cancer cell growth.

PKCη has been implicated in both tumor promotion and tumor suppression. For example, PKCη expression was decreased in hepatocellular carcinoma (16), and PKCη deficiency enhanced susceptibility to tumor formation in a two-stage skin carcinogenesis model (12, 13). Moreover, overexpression of PKCη induced differentiation in keratinocytes (17, 19). In contrast, PKCη has been implicated in breast cancer (29), glioblastoma (15), lung cancer (14) and renal cell carcinoma (18). In addition, overexpression of PKCη induced anchorage-independent growth in NIH3T3 cells (30) and provided proliferative advantage in astrocytic tumor cells (31). The contrasting function of PKCη is not unique and several other PKC isoforms have been shown to exert opposite effects depending on the cell type (1, 9, 27, 32). Thus, it is important to

understand how PKCη is regulated in a particular cellular context in order to understand its function.

We recently reported that the regulation of PKCη in breast cancer cells is distinct from other PKC isozymes. While brief exposure to PKC activators, such as tumor-promoting phorbol esters leads to activation of conventional and novel PKCs, persistent treatment with these activators leads to their downregulation (1, 2). We showed that prolonged treatment with several structurally and functionally distinct PKC activators caused upregulation rather than downregulation of PKCη and a general PKC inhibitor led to PKCη downregulation (11). When we compared the effects of pharmacological inhibitors of several different kinases, we found that not only PKC inhibitors but also the PI3K inhibitor Ly294002 induced PKCη downregulation.

PKCs are phosphorylated by autophosphorylation and transphosphorylation by other members of the PKC family as well as PDK1 (33-35). We recently reported that transphosphorylation of PKCη by novel PKCε is responsible for its upregulation (11). Since two different general PKC inhibitors bisindolylmaleimide and Gö 6983 induced PKCη downregulation whereas the conventional PKC inhibitor Gö 6976 had only a modest effect, it is likely that inhibition of PKCη phosphorylation by novel PKCε leads to its downregulation. Our observation that adenoviral delivery of PKCε increased PKCη level both in control and Gö 6983-treated cells supports this notion. We, however, found that overexpression of PKCε was unable to completely prevent Gö 6983-mediated downregulation of PKCη; presumably because Gö 6983 could inhibit PKCε in PKCε-overexpressing cells albeit to a lesser extent compared to endogenous PKCε.

It has been reported that PDK1 directly phosphorylates PKCη resulting in its activation (25) but knockdown of PDK1 had little effect on PKC activator-induced upregulation of PKCη (11). Although PDK1 was not required for PKC activator-induced upregulation of PKCη, depletion of PDK1 by siRNA or its inhibition by the PI3K inhibitor Ly294002 decreased basal PKCη level, suggesting that phosphorylation of PKCη by PDK1 also protects it from downregulation. However, the mechanism by which Gö 6983 and Ly294002 induced PKCη downregulation was distinct. While both Gö 6983 and Ly294002 decreased basal PKCη level, only Gö 6983 but not Ly294002 could induce PKCη downregulation in the presence of PDBu. Moreover, two distinct proteasome inhibitors MG-132 and lactacystin could prevent PKCη downregulation caused by Ly294002 or PDK1 knockdown but not by Gö 6983. These results suggest that while inhibition of PDK1 triggers PKCη downregulation via proteasome-dependent pathway, inhibition of PKC triggers PKCη downregulation via proteasome-independent pathway.

Both PKC and PI3K/PDK1/Akt signaling pathways play important roles in the development and progression of breast cancer (1, 26-28). It has been reported that the level and activation status of PDK1 is progressively increased in the MCF-10A series (21) which includes spontaneously immortalized non-tumorigenic mammary epithelial MCF-10A, premalignant MCF-10AT and MCF10AT3G, ductal carcinoma in situ DCIS and fully malignant MCF10CA1a and MCF10CA1d cells (20, 21). The MCF-10A series provides a unique cell culture model system to study the alterations in signaling molecules leading to breast cancer since these cells share common genetic background. We made a novel observation that PKCη is the only PKC isozyme that is progressively increased in the MCF-10A series but there was no correlation between the levels of conventional PKCα, novel PKCδ and -ε and atypical PKCζ and the

malignant status of MCF10A-derived cells. Our results suggest that while PKCη level correlates with the level/activation status of PDK1 in the MCF10 cell series, there is no correlation between PKCη level and hormone receptor status. For example, it is difficult to detect PKCη in triple-negative MDA-MB-231 cells (data not shown). However, both PKC inhibitor Gö 6983 and PI3K/PDK1 inhibitor Ly294002 induced downregulation of PKCη in basal type MCF-10CA1d cells (Fig. 6B) and MCF-10CA1a cells (data not shown).

To determine the functional significance of PKCη in breast cancer cells we depleted PKCη using siRNA since a selective inhibitor of PKCη is currently not available. Silencing of PKCη by siRNA decreased the growth of both DCIS and T47D cells. Knockdown of PKCη also reduced the long-term clonogenic survival of MCF-7 cells, which express very high level of PKCη. Moreover, since PKCη is regulated by PKCε and PDK1 that play critical roles in breast cancer, PKCη may serve as an important target for breast cancer therapy.

Figure 1. Effects of kinase inhibitors on PKCη levels. MCF-7 (A) or T47D (B) cells were treated with or without 10 μM BIM, 10 μM rottlerin, 1 μM Gö 6976, 25 μM Ly294002, 50 nM rapamycin, 50 μM PD98059, 10 μM U0126 or 2 μM KT5720 for 15 h. Western blot analysis was performed with total cellular extracts using the indicated antibodies. Actin was used as a loading control.

Figure 1.

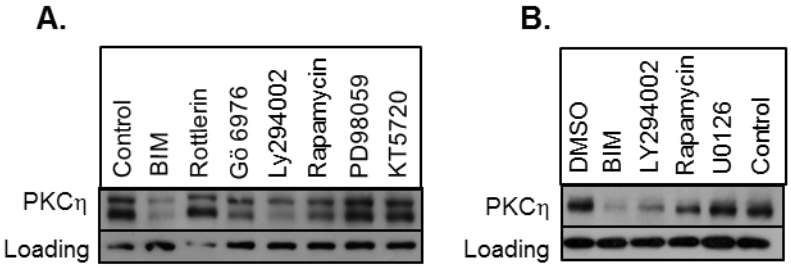

A.
Control
BIM
Rottlerin
Gö 6976
Ly294002
Rapamycin
PD98059
KT5720
PKCη
Loading
B.
DMSO
BIM
LY294002
Rapamycin
U0126
Control
PKCη
Loading

Figure 2. Effect of PKC activators on PKCη downregulation by PKC and PI3K inhibitors. MCF-7 cells were pretreated with 1 μM PDBu for 15 min, followed by treatment with or without 1μM Gö 6983 or 25 μM Ly294002 for 15 h. Western blot analysis was performed with total cellular extracts using the indicated antibodies. Actin was used as a loading control.

Figure 2.

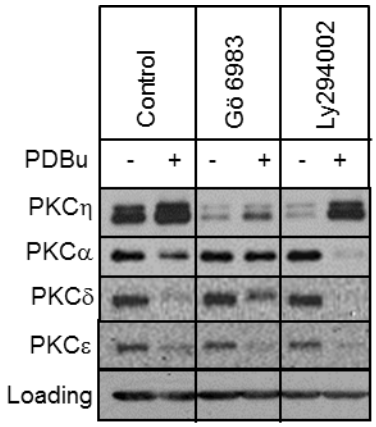


Figure 3. Effect of protease inhibitors on PKCη downregulation in MCF-7 cells. MCF-7 cells were pretreated with or without 50 μM calpeptin or 50 μM cathepsin inhibitor I for 30 min, followed by treatment with 1 μM Gö 6983 or 25 μM Ly294002 for 12 h. Western blot analysis was performed with total cellular extracts using the indicated antibodies. GAPDH was used as a loading control. Results are representative of 2 independent experiments.

Figure 3.

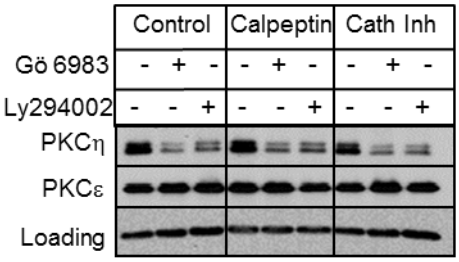

Control
Calpeptin
Cath Inh
Gö 6983
- + - - + - - + -
Ly294002
- - + - - + - - +
PKCη
PKCε
Loading

Figure 4. Effect of proteasome inhibitors on PKCη downregulation. A, MCF-7 cells were pretreated with 10 μM MG-132 for 30 min, followed by treatment with 1 μM Gö 6983 or 25 μM Ly294002 for 12 h. Western blot analysis was performed with total cellular extracts using the indicated antibodies. GAPDH was used as a loading control. B, Densitometric quantification of PKCη protein levels from 3 separate experiments corrected for loading. Data represents the mean +/- s.e.m. The asterisk (*) indicates significant difference of MG-132 treated cells from control or treatment with the inhibitors alone using Student's *t*-test. *, $P<0.05$; **, $P<0.005$ C, T47D cells were pretreated with 10 μM MG-132 or 10 μM lactacystin for 30 min, followed by treatment with 1 μM Gö 6983 or 10 μM Ly294002 for 15 h. Western blot analysis was performed with total cellular extracts using the indicated antibodies. Actin was used as a loading control.

Figure 4.

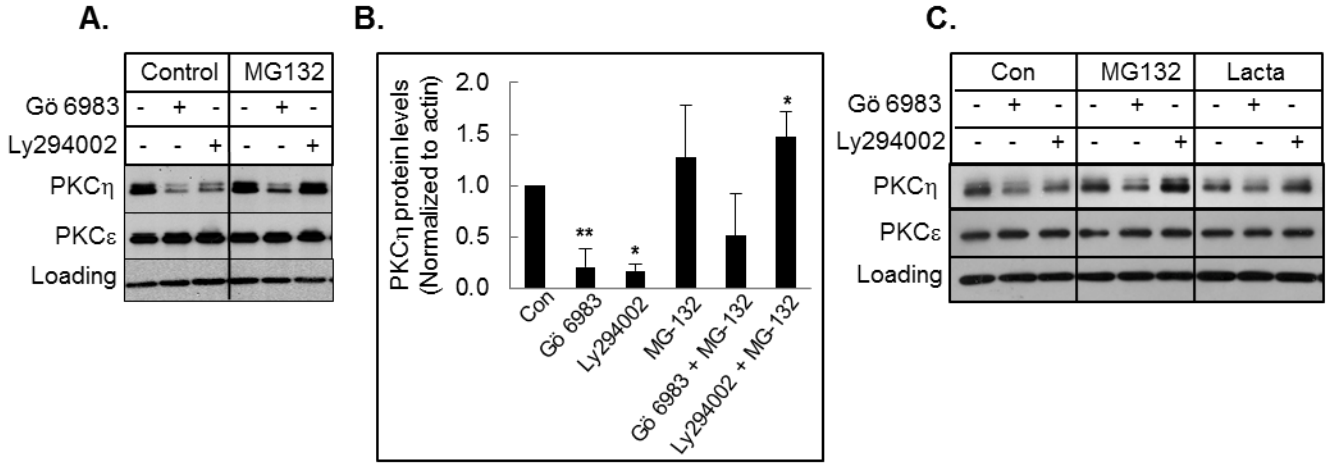

A.
Control
MG132
Gö 6983
Ly294002
PKCη
PKCε
Loading
B.
PKCη protein levels (Normalized to actin)
2.0
1.5
1.0
0.5
0.0
**
*
*
Con
Gö 6983
Ly294002
MG-132
Gö 6983 + MG-132
Ly294002 + MG-132
C.
Con
MG132
Lacta
Gö 6983
Ly294002
PKCη
PKCε
Loading

Figure 5. Effect of PDK1 and PKCε on PKCη downregulation. A, MCF-7 cells were transfected with control non-targeting siRNA, PDK1 siRNA or were left non-transfected (NT). Cells were then pre-treated with either 10 μM MG-132 or 10 μM lactacystin, followed by treatment with 25 μM Ly294002. Western blot analysis was performed with indicated antibodies. Actin was used as a loading control. B, T47D cells were infected with adenovirus vector containing GFP or PKCε construct and then treated with 1 μM Gö 6983 for 15 h. Western blot analysis was carried out with indicated antibodies.

Figure 5.

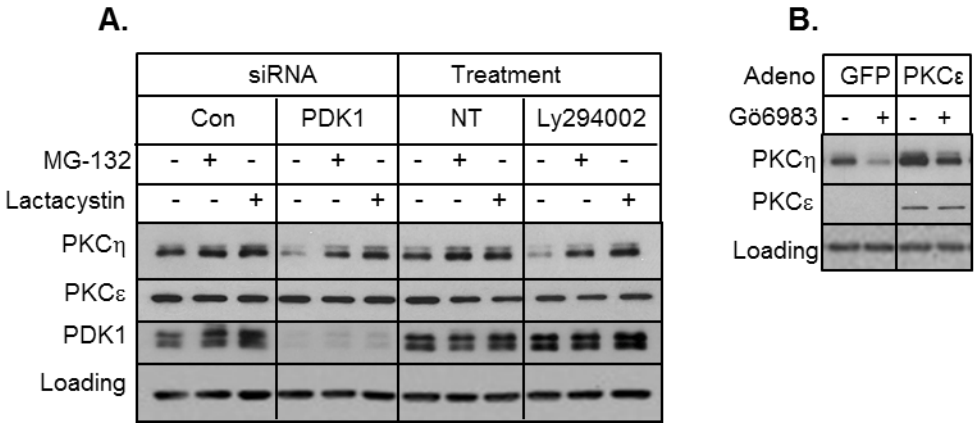

A.
siRNA
Treatment
Con
PDK1
NT
Ly294002
MG-132
Lactacystin
PKCη
PKCε
PDK1
Loading
B.
Adeno
GFP
PKCε
Gö6983
PKCη
PKCε
Loading

Figure 6. Comparison of PKCη levels in MCF10A series. A, Western blot analysis was performed with total cellular extracts from 10A, AT1, DCIS, CA1d and MCF-7 cells and probed with the indicated antibodies. B, CA1d cells were treated with or without 1 μM Gö 6983 or 25 μM Ly294002 for 16 h. Western blot analysis was performed with total cellular extracts using the indicated antibodies. Actin was used as a loading control.

Figure 6.

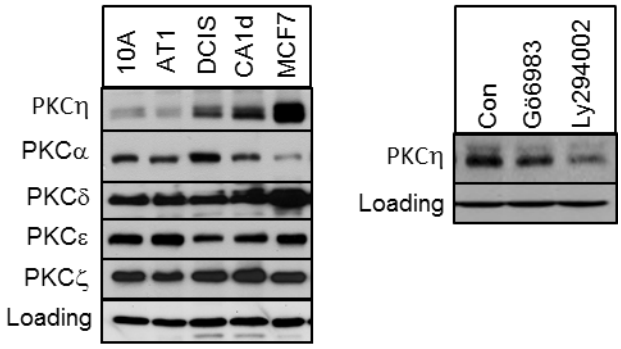
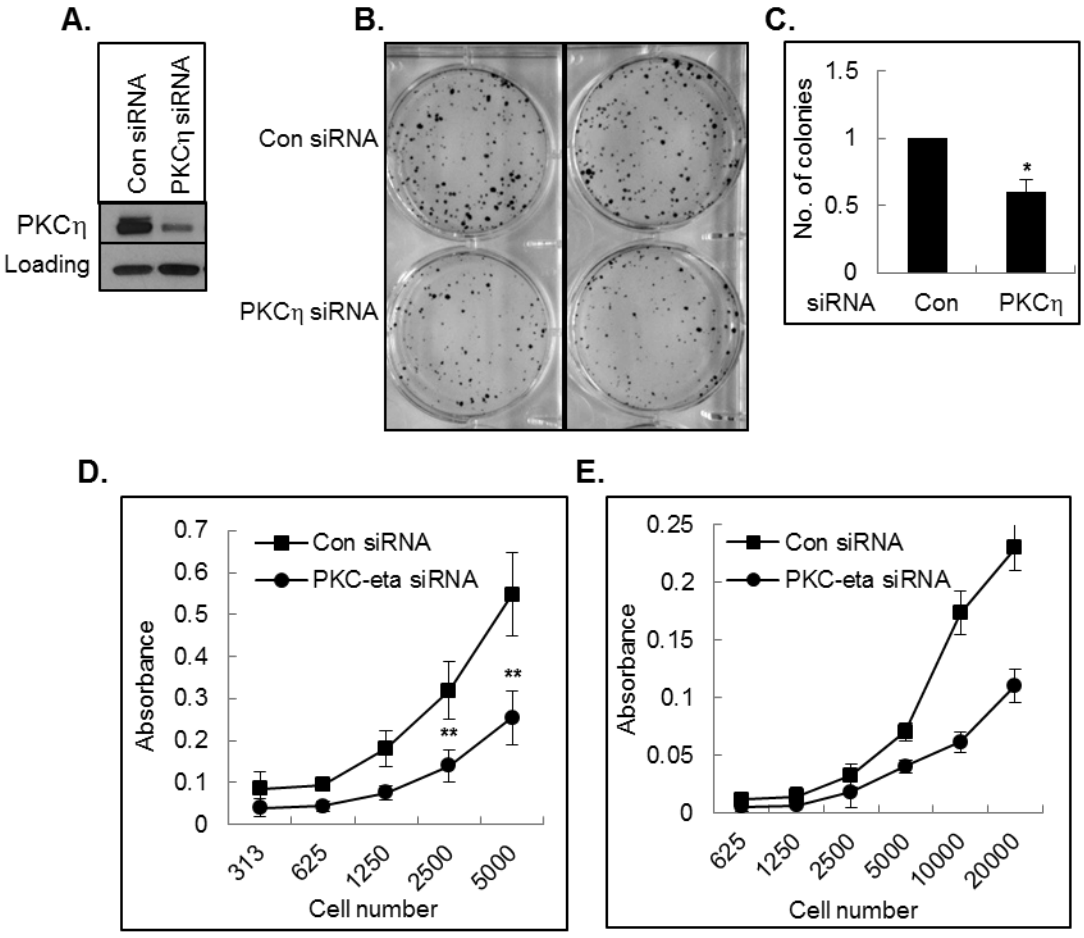

10A
AT1
DCIS
CA1d
MCF7
PKCη
PKCα
PKCδ
PKCε
PKCζ
Loading
Con
Gö6983
Ly294002
PKCη
Loading

Figure 7. Effect of PKCη on cell growth. A, MCF-7 cells were transfected with control non-targeting siRNA or PKCη siRNA and total cell extracts were used for Western blot analysis. Actin was used as a loading control. B, Clonogenic assay was performed with MCF-7 cells transfected with either control non-targeting siRNA or PKCη siRNA as described in the Materials and Methods. C, Quantification of the number of colonies as determined by the clonogenic assay. The results are representative of 3 independent experiments. $*P < 0.05$ using paired Student *t* test. D, MTT assay was performed with T47D cells transfected with control, non-targeting siRNA or PKCη siRNA as described under Materials and Methods. The results are representative of 3 independent experiments. $**P < 0.01$ using paired Student *t* test. E, MTT assay was performed with DCIS cells transfected with control non-targeting siRNA or PKCη siRNA as described under Materials and Methods.

Figure 7.


A.
Con siRNA
PKCη siRNA
PKCη
Loading
B.
Con siRNA
PKCη siRNA
C.
No. of colonies
1.5
1
0.5
0
*
siRNA
Con
PKCη
D.
Absorbance
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Con siRNA
PKC-eta siRNA
**
**
313
625
1250
2500
5000
Cell number
E.
Absorbance
0.25
0.2
0.15
0.1
0.05
0
Con siRNA
PKC-eta siRNA
625
1250
2500
5000
10000
20000
Cell number

References

1. Griner EM, Kazanietz MG. Protein kinase C and other diacylglycerol effectors in cancer. Nat Rev Cancer. 2007;7:281-94.

2. Steinberg SF. Structural basis of protein kinase C isoform function. Physiol Rev. 2008;88:1341-78.

3. Pontremoli S, Melloni E, Damiani G, Salamino F, Sparatore B, Michetti M, et al. Effects of a monoclonal anti-calpain antibody on responses of stimulated human neutrophils. Evidence for a role for proteolytically modified protein kinase C. J Biol Chem. 1988;263:1915-9.

4. Savart M, Letard P, Bultel S, Ducastaing A. Induction of protein kinase C down-regulation by the phorbol ester TPA in a calpain/protein kinase C complex. Int J Cancer. 1992;52:399-403.

5. Lee HW, Smith L, Pettit GR, Smith JB. Bryostatin 1 and phorbol ester down-modulate protein kinase C-alpha and -epsilon via the ubiquitin/proteasome pathway in human fibroblasts. Mol Pharmacol. 1997;51:439-47.

6. Lee HW, Smith L, Pettit GR, Vinitsky A, Smith JB. Ubiquitination of protein kinase C-alpha and degradation by the proteasome. J Biol Chem. 1996;271:20973-6.

7. Lu Z, Liu D, Hornia A, Devonish W, Pagano M, Foster DA. Activation of protein kinase C triggers its ubiquitination and degradation. Mol Cell Biol. 1998;18:839-45.

8. Basu A. The potential of protein kinase C as a target for anticancer treatment. Pharmacol Ther. 1993;59:257-80.

9. Basu A, Pal D. Two faces of protein kinase Cdelta: the contrasting roles of PKCdelta in cell survival and cell death. ScientificWorldJournal. 2010;10:2272-84.

10. Bacher N, Zisman Y, Berent E, Livneh E. Isolation and characterization of PKC-L, a new member of the protein kinase C-related gene family specifically expressed in lung, skin, and heart. Mol Cell Biol. 1991;11:126-33.

11. Pal D, Outram SP, Basu A. Novel regulation of protein kinase C-eta. Biochem Biophys Res Commun. 2012;425:836-41.

12. Chida K, Hara T, Hirai T, Konishi C, Nakamura K, Nakao K, et al. Disruption of protein kinase Ceta results in impairment of wound healing and enhancement of tumor formation in mouse skin carcinogenesis. Cancer Res. 2003;63:2404-8.

13. Kashiwagi M, Ohba M, Chida K, Kuroki T. Protein kinase C eta (PKC eta): its involvement in keratinocyte differentiation. J Biochem. 2002;132:853-7.

14. Krasnitsky E, Baumfeld Y, Freedman J, Sion-Vardy N, Ariad S, Novack V, et al. PKCeta is a novel prognostic marker in non-small cell lung cancer. Anticancer Res. 2012;32:1507-13.

15. Hussaini IM, Carpenter JE, Redpath GT, Sando JJ, Shaffrey ME, Vandenberg SR. Protein kinase C-eta regulates resistance to UV- and gamma-irradiation-induced apoptosis in glioblastoma cells by preventing caspase-9 activation. Neuro Oncol. 2002;4:9-21.

16. Lu HC, Chou FP, Yeh KT, Chang YS, Hsu NC, Chang JG. Analysing the expression of protein kinase C eta in human hepatocellular carcinoma. Pathology. 2009;41:626-9.

17. Cabodi S, Calautti E, Talora C, Kuroki T, Stein PL, Dotto GP. A PKC-eta/Fyn-dependent pathway leading to keratinocyte growth arrest and differentiation. Mol Cell. 2000;6:1121-9.

18. Brenner W, Farber G, Herget T, Wiesner C, Hengstler JG, Thuroff JW. Protein kinase C eta is associated with progression of renal cell carcinoma (RCC). Anticancer Res. 2003;23:4001-6.

19. Ohba M, Ishino K, Kashiwagi M, Kawabe S, Chida K, Huh NH, et al. Induction of differentiation in normal human keratinocytes by adenovirus-mediated introduction of the eta and delta isoforms of protein kinase C. Mol Cell Biol. 1998;18:5199-207.

20. Santner SJ, Dawson PJ, Tait L, Soule HD, Eliason J, Mohamed AN, et al. Malignant MCF10CA1 cell lines derived from premalignant human breast epithelial MCF10AT cells. Breast Cancer Res Treat. 2001;65:101-10.

21. Kim SH, Miller FR, Tait L, Zheng J, Novak RF. Proteomic and phosphoproteomic alterations in benign, premalignant and tumor human breast epithelial cells and xenograft lesions: biomarkers of progression. Int J Cancer. 2009;124:2813-28.

22. Basu A, Lu D, Sun B, Moor AN, Akkaraju GR, Huang J. Proteolytic activation of protein kinase C-epsilon by caspase-mediated processing and transduction of antiapoptotic signals. J Biol Chem. 2002;277:41850-6.

23. Basu A. The involvement of novel protein kinase C isozymes in influencing sensitivity of breast cancer MCF-7 cells to tumor necrosis factor-alpha. Mol Pharmacol. 1998;53:105-11.

24. Basu A, Sridharan S, Persaud S. Regulation of protein kinase C delta downregulation by protein kinase C epsilon and mammalian target of rapamycin complex 2. Cell Signal. 2009;21:1680-5.

25. Balendran A, Hare GR, Kieloch A, Williams MR, Alessi DR. Further evidence that 3-phosphoinositide-dependent protein kinase-1 (PDK1) is required for the stability and phosphorylation of protein kinase C (PKC) isoforms. FEBS Lett. 2000;484:217-23.

26. Gorin MA, Pan Q. Protein kinase C epsilon: an oncogene and emerging tumor biomarker. Mol Cancer. 2009;8:9.

27. Basu A, Sivaprasad U. Protein kinase Cepsilon makes the life and death decision. Cell Signal. 2007;19:1633-42.

28. Miller TW, Rexer BN, Garrett JT, Arteaga CL. Mutations in the phosphatidylinositol 3-kinase pathway: role in tumor progression and therapeutic implications in breast cancer. Breast Cancer Res. 2011;13:224.

29. Masso-Welch PA, Winston JS, Edge S, Darcy KM, Asch H, Vaughan MM, et al. Altered expression and localization of PKC eta in human breast tumors. Breast Cancer Res Treat. 2001;68:211-23.

30. Nomoto S, Watanabe Y, Ninomiya-Tsuji J, Yang LX, Nagai Y, Kiuchi K, et al. Functional analyses of mammalian protein kinase C isozymes in budding yeast and mammalian fibroblasts. Genes Cells. 1997;2:601-14.

31. Hussaini IM, Karns LR, Vinton G, Carpenter JE, Redpath GT, Sando JJ, et al. Phorbol 12-myristate 13-acetate induces protein kinase ceta-specific proliferative response in astrocytic tumor cells. J Biol Chem. 2000;275:22348-54.

32. Reyland ME. Protein kinase C isoforms: Multi-functional regulators of cell life and death. Front Biosci (Landmark Ed). 2009;14:2386-99.

33. Rybin VO, Sabri A, Short J, Braz JC, Molkentin JD, Steinberg SF. Cross-regulation of novel protein kinase C (PKC) isoform function in cardiomyocytes. Role of PKC epsilon in activation loop phosphorylations and PKC delta in hydrophobic motif phosphorylations. J Biol Chem. 2003;278:14555-64.

34. Gould CM, Newton AC. The life and death of protein kinase C. Curr Drug Targets. 2008;9:614-25.

35. Parekh DB, Ziegler W, Parker PJ. Multiple pathways control protein kinase C phosphorylation. Embo J. 2000;19:496-503.

CHAPTER IV

SUMMARY

The development of effective therapeutics for breast cancer warrants a comprehensive understanding of the signaling pathways that are deregulated in breast cancer. PKCs have served as attractive targets for cancer therapy for decades owing to their crucial roles in several cellular processes (1, 2). However, the selective targeting of PKCs has been difficult due to the diverse functional responses of individual PKC isozymes and their complex signaling. PKCη is a novel PKC isozyme that is overexpressed in breast cancer (3) and has been implicated in chemotherapeutic resistance (4-7). Thus far, there has been limited understanding of the regulation of PKCη in breast cancer.

Findings from our study reveal that the regulation of PKCη is unique. While PKCs serve as receptors for tumor promoting phorbol esters which are potent activators of PKCs (2), persistent activation of PKCs leads to their downregulation and subsequent degradation (8, 9). We have shown that in contrast to other PKC isozymes, PKCη is the only isozyme that resists phorbol ester-induced downregulation and is upregulated by several structurally and functionally distinct PKC activators.

Our results further suggest that PKCη is upregulated at the post-transciptional level, and the PKC activator-induced upregulation of PKCη correlates with its phosphorylation status. Since, PKCs can undergo transphosphorylation by PDK1 or by other members of the PKC family (9, 10); we speculated the role of these kinases in the regulation of PKCη. We found that PKCη is transphosphorylated by PDK1 and novel PKCs, especially PKCε but not by conventional PKCs. Moreover, PDK1 and nPKCs had distinct effects on PKCη regulation. While transphosphorylation of PKCη by novel PKCs was responsible for activator-induced upregulation of PKCη, PDK1 affected PKCη basally and had no effect on the upregulation of PKCη by activators. While we have shown that PKCε is involved in PKCη upregulation, the mechanism of their interaction remains to be understood. Since both PKCη and PKCε localize to the Golgi (11, 12), it is possible that their Golgi translocation drives their interaction. Besides PKC isozymes, other kinases and chaperone proteins have also been implicated in the regulation of novel PKCs (13, 14). Future studies should help discern the role of these proteins in the regulation of PKCη. Moreover, potential autophosphorylation sites were identified in the structure of PKCη (15); hence, it is plausible that upon activation by PKC activators, PKCη can mediate autophosphorylation culminating in its upregulation.

We also made a novel observation that inhibition of PDK1 or nPKCs led to PKCη downregulation via two different mechanisms. While inhibition/knockdown of PDK1 caused PKCη downregulation via the proteasomal pathway, the downregulation of PKCη caused by the depletion of PKCε or by nPKC inhibitors was independent of the proteasome-mediated pathway.

Upon examining the functional consequences of PKCη downregulation in breast cancer cells, we found that PKCη knockdown led to a significant decrease in the growth and survival of breast cancer cells. We further demonstrated that the levels of PKCη increased with the aggressiveness

of breast cancer in the progressive MCF10A series. This progressive increase in protein levels with increased malignancy was not observed with the other PKC isozymes. Taken together, these results implicate PKCη in the regulation of growth and survival of breast cancer cells. It would thus be worthwhile to identify novel targets of PKCη which mediate its role in breast cancer.

We have shown that PKCη levels are regulated by PDK1 and PKC pathways, both of which are critical for breast cancer development and progression (16-18). It has been previously reported that PKCη plays a crucial role in the regulation of tight junctions (19). Loss of integrity in the tight junction structure leads to invasion and eventually cancer metastasis (20). These reports coupled with our observation that PKCη levels correlate with breast cancer aggressiveneness implicates PKCη in breast cancer progression. This suggests the possible use of PKCη as a prognostic biomarker for breast cancer. Consistent with our *in vitro* findings, another study reported that invasive tumors associated with significant lymph node metastases maintained high levels of PKCη thereby implicating PKCη in distal invasion (3). Contrary to this, decreased PKCη expression was observed in invasive breast tumor tissues compared to the surrounding normal epithelium suggesting that PKCη is decreased during breast cancer progression (3). Thus, the role of PKCη in progression of breast cancer remains controversial. Although our study has revealed the functions of PKCη in breast cancer cell growth, future studies focused on cell migration and invasion should help discern the role of PKCη in breast cancer progression.

While our work has focused on the post-transcriptional mechanism of PKCη regulation, the transcriptional control of PKCη could also contribute to its role in cancer. The promoter region of PKCη contains multiple binding sites for transcription factors which have been implicated in breast cancer proliferation and progression (21-23). Understanding the interplay of these transcription factors and PKCη in breast cancer could yield interesting results.

Based on our findings, we propose a hypothetical model to account for the unique regulation of PKCη (Figure 1). Phosphorylation of PKCη by both PDK1 and nPKCs protect it from downregulation. Ly294002, a PI3K inhibitor, inhibits phosphorylation of PKCη by PDK1 and leads to its downregulation via a proteasome-dependent pathway whereas Gö 6983, a general PKC inhibitor, inhibits nPKC-mediated phosphorylation of PKCη making it susceptible to downregulation via a proteasome-independent pathway. We have further demonstrated that the regulation of PKCη by PDK1 and PKCε promotes breast cancer cell growth and survival. The unique regulation of PKCη suggests that this pathway could be selectively targeted for breast cancer.

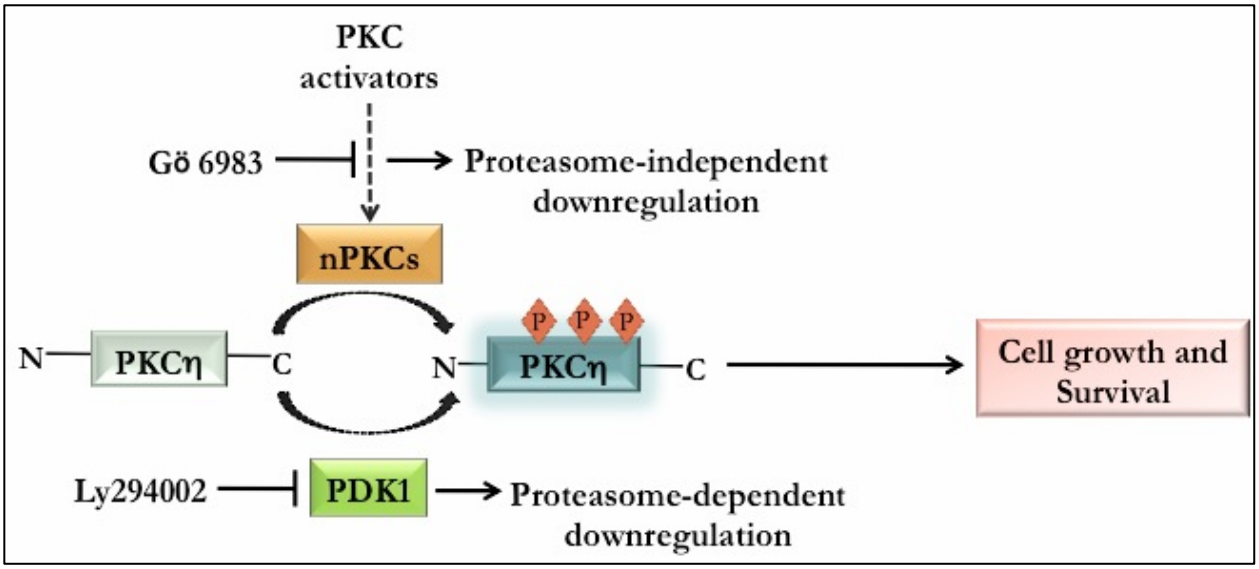


Figure 1. Hypothetical model. *PKCη is regulated by PDK1 and PKCε which maintain PKCη in a stable form thereby protecting it from degradation. Inhibition of PDK1 by Ly294002 leads to PKCη downregulation via the proteasomal pathway while Gö 6983-mediated inhibition of PKC leads to PKCη downregulation which is proteasome-independent. The upregulation of PKCη by PDK1 and PKCε contributes to the growth and survival of breast cancer cells.*

References

1. Griner EM, Kazanietz MG. Protein kinase C and other diacylglycerol effectors in cancer. Nat Rev Cancer. 2007;7:281-94.

2. Blumberg PM. Protein kinase C as the receptor for the phorbol ester tumor promoters: sixth Rhoads memorial award lecture. Cancer Res. 1988;48:1-8.

3. Masso-Welch PA, Winston JS, Edge S, Darcy KM, Asch H, Vaughan MM, et al. Altered expression and localization of PKC eta in human breast tumors. Breast Cancer Res Treat. 2001;68:211-23.

4. Basu A. The involvement of novel protein kinase C isozymes in influencing sensitivity of breast cancer MCF-7 cells to tumor necrosis factor-alpha. Mol Pharmacol. 1998;53:105-11.

5. Akkaraju GR, Basu A. Overexpression of protein kinase C-eta attenuates caspase activation and tumor necrosis factor-alpha-induced cell death. Biochem Biophys Res Commun. 2000;279:103-7.

6. Rotem-Dai N, Oberkovitz G, Abu-Ghanem S, Livneh E. PKCeta confers protection against apoptosis by inhibiting the pro-apoptotic JNK activity in MCF-7 cells. Exp Cell Res. 2009;315:2616-23.

7. Raveh-Amit H, Hai N, Rotem-Dai N, Shahaf G, Gopas J, Livneh E. Protein kinase Ceta activates NF-kappaB in response to camptothecin-induced DNA damage. Biochem Biophys Res Commun. 2011;412:313-7.

8. Steinberg SF. Structural basis of protein kinase C isoform function. Physiol Rev. 2008;88:1341-78.

9. Parekh DB, Ziegler W, Parker PJ. Multiple pathways control protein kinase C phosphorylation. Embo J. 2000;19:496-503.

10. Gould CM, Newton AC. The life and death of protein kinase C. Curr Drug Targets. 2008;9:614-25.

11. Maissel A, Marom M, Shtutman M, Shahaf G, Livneh E. PKCeta is localized in the Golgi, ER and nuclear envelope and translocates to the nuclear envelope upon PMA activation and serum-starvation: C1b domain and the pseudosubstrate containing fragment target PKCeta to the Golgi and the nuclear envelope. Cell Signal. 2006;18:1127-39.

12. Schultz A, Ling M, Larsson C. Identification of an amino acid residue in the protein kinase C C1b domain crucial for its localization to the Golgi network. J Biol Chem. 2004;279:31750-60.

13. Cameron AJ, Linch MD, Saurin AT, Escribano C, Parker PJ. mTORC2 targets AGC kinases through Sin1-dependent recruitment. Biochem J. 2011;439:287-97.

14. Gould CM, Kannan N, Taylor SS, Newton AC. The chaperones Hsp90 and Cdc37 mediate the maturation and stabilization of protein kinase C through a conserved PXXP motif in the C-terminal tail. J Biol Chem. 2009;284:4921-35.

15. Littler DR, Walker JR, She YM, Finerty PJ, Jr., Newman EM, Dhe-Paganon S. Structure of human protein kinase C eta (PKCeta) C2 domain and identification of phosphorylation sites. Biochem Biophys Res Commun. 2006;349:1182-9.

16. Gorin MA, Pan Q. Protein kinase C epsilon: an oncogene and emerging tumor biomarker. Mol Cancer. 2009;8:9.

17. Miller TW, Rexer BN, Garrett JT, Arteaga CL. Mutations in the phosphatidylinositol 3-kinase pathway: role in tumor progression and therapeutic implications in breast cancer. Breast Cancer Res. 2011;13:224.

18. Basu A, Sivaprasad U. Protein kinase Cepsilon makes the life and death decision. Cell Signal. 2007;19:1633-42.

19. Suzuki T, Elias BC, Seth A, Shen L, Turner JR, Giorgianni F, et al. PKC eta regulates occludin phosphorylation and epithelial tight junction integrity. Proc Natl Acad Sci U S A. 2009;106:61-6.

20. Martin TA, Mason MD, Jiang WG. Tight junctions in cancer metastasis. Front Biosci (Landmark Ed). 2011;16:898-936.

21. Lincoln DW, 2nd, Bove K. The transcription factor Ets-1 in breast cancer. Front Biosci. 2005;10:506-11.

22. Shen Q, Uray IP, Li Y, Krisko TI, Strecker TE, Kim HT, et al. The AP-1 transcription factor regulates breast cancer cell growth via cyclins and E2F factors. Oncogene. 2008;27:366-77.

23. Quan T, Fisher GJ. Cloning and characterization of the human protein kinase C-eta promoter. J Biol Chem. 1999;274:28566-74.