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The Pericentrion: Identification, Characterization, and Function of a Novel cPKC-Dependent Compartment

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Abstract

Members of the protein kinase C (PKC) superfamily transduce a myriad of transmembrane signals initiated by the formation of *sn*-1,2-diacylglycerol (DAG). As the primary physiological agonist for C1 domain containing PKC isoenzymes, DAG functions to regulate the spatial and temporal parameters of PKC activity. It is now recognized that cellular DAG levels can vary from minutes to hours and even days. These observations have suggested a division of PKC functions that correlate to the duration of the cellular DAG signal. In their classical role, cPKCs respond acutely to the short-lived (1-2 min) DAG generated at the plasma membrane by the phospholipase C-mediated turnover of phosphoinositides. In contrast, a more persistent DAG signal can be produced through a variety of physiologic as well as pathologic mechanisms, and this DAG has also been proposed to regulate PKC activity. Currently, there are conflicting data on this issue and the specific subcellular localization of cPKC during prolonged DAG is unknown. To investigate the target of PKC during sustained activation, green fluorescent protein (GFP) technology was utilized to monitor the subcellular traffic of various PKC isoenzymes in response to treatment with either DAG-mimicking phorbol esters or DAG-generating agonists (e.g. platelet-derived growth factor). In response to a persistent elevation of DAG, cPKC isoenzymes, α and βII, translocated to the plasma membrane and to a juxtanuclear location in a variety of cell lines examined. Characterization of this compartment revealed that it overlapped/co-localized with a rab11-positive subcompartment of recycling endosomes concentrated around the MTOC/centrosome. The cPKC compartment was distinguish from the rab11-positive compartment by several features including a requirement for kinase and phospholipase D activity, an enrichment of lipid raft components, and the independence of microtubules and temperature for maintenance of the structure. Investigation into the significance of this compartment revealed that cPKC translocation coincided with the attendant sequestration of membrane recycling components. Given these distinctions, it was proposed that this novel cPKC-compartment be named the *pericentrion* in order to

distinguish it from the rab11 subcompartment of recycling endosomes. Subsequent analysis of a disease model wherein cPKC is maintained in a persistently active state, in this case chronic hyperglycemia associated with type I and type II diabetes, revealed a possible role for cPKC in the dysregulation of GLUT4 trafficking in response to insulin. These studies identify a novel site for cPKC translocation and function in response to a physiological and pathological elevation of DAG.

Dedication

To my family and friends, for their guidance, support, and patience.

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Abbreviations

Cellular Proteins/Enzymes

AKAP	A-kinase anchoring protein
GLUT4	glucose transporter-4
MAPK	mitogen-activated protein kinase
MARCKS	myristoylated alanine-rich C-kinase substrate
PDGFβ-R	platelet-derived growth factor-beta
PDK-1	phosphoinositide-dependent kinase-1
PICK	protein that interacts with C-kinase
PKC	protein kinase C
PKM	protein kinase M
PLA	phospholipase A
PLC^2	phospholipase C^2
PLD	phospholipase D
RACK	receptor for activated C-kinase
SMS	sphingomyelin synthase

Cellular Lipids

DAG	sn-1,-2diacylglycerol
IP	inosito-1,4,5-trisphosphate
LPA	lyso-phosphatidic acid
PA	phosphatidic acid
PC	phosphatidylcholine
PI	phosphoinositide
PIP_2	phosphatidylinositol-4,5-bisphosphate
PIP_3	phosphatidylinositol-3,4,5-trisphosphate
Ptd-butanol	phosphatidylbutanol
PS	phosphatidylserine
SM	sphingomyelin

Chemical Compounds

ATP	adenosine 5'-triphosphate
DMSO	dimethyl sulfoxide
Gö 6976	indolocarbazole; cPKC-specific kinase inhibitor

Bioactive Compounds

ACh	acetylcholine
Ca^{2+}	calcium
CTx-B	cholera toxin B subunit
PDGF-BB	platelet-derived growth factor b chain dimer
PMA	phorbol-12-myristate-13-acetate
Tf	transferrin

Miscellaneous

AF-CTx-B	Alexa Fluor conjugated cholera toxin B subunit
AF-Tf	Alexa Fluor conjugated transferrin
CNS	central nervous system
GFP	green fluorescent protein
HEK293	human embryonic kidney-293
kDa	kilodalton
MEF	mouse embryonic fibroblast
MTOC	microtubule-organizing center

NLS	nuclear localization signal
SH2	src homology domain-2
TNF-α	tumor necrosis factor-alpha

Chapter 1: General Introduction and Overview of Protein Kinase C (PKC)

1.1 Historical Significance

In 1953, Hokin and Hokin reported that application of acetylcholine (ACh) to pigeon pancreatic tissue slices resulted in a rapid incorporation of radioactive ^{32}P-orthophosphate into membrane phosphoinositides (PIs) and phosphatidic acid (PA) (Hokin and Hokin, 1953). From these observations, they hypothesized that the effect of ACh was due to the rapid degradation and resynthesis (turnover) of membrane PI lipids (which involves the transient formation of *sn*-1,2-diacylglycerol; DAG), and that this 'PI cycle' could be stimulated through the binding of the ACh agonist to it's cell surface receptor. Subsequent investigations revealed that a large number of extracellular agonists could also induce a 'PI response' including other neurotransmitters, growth factors, and hormones. Remarkably, despite the mention of DAG in the original PI cycle schema, the physiologic significance of the receptor-mediated elevation of DAG went unknown for close to three decades.

In 1977, while studying the biochemical properties of partially purified nucleotide-dependent protein kinases, Nishizuka and coworkers identified a novel nucleotide-independent protein kinase activity they named protein kinase M (PKM) (Inoue et al., 1977; Takai et al., 1977). The 'M' denoted the relatively high concentrations of magnesium that were required for catalytic activity toward some artificial substrates. Two years later, this same group reported the landmark observation that PKM was in fact a constitutively active, proteolytic cleavage product of a larger proenzyme (PKC) that exhibited calcium, phosphatidylserine, and DAG-dependent sensitivity (Takai et al., 1979a; Takai et al., 1979c). Although there was an immediate recognition that this newly identified kinase may functionally couple to extracellular signals that promote PI lipid hydrolysis and DAG generation, a mechanism for how a soluble protein such as PKC and a membrane lipid interacted was initially unclear. A mechanism was revealed three years later when PKC was identified as the long sought cellular receptor for tumor-promoting phorbol esters, and that phorbol esters, which structurally resemble DAG and partition into membranes, could induce a shift in subcellular localization or 'translocation' of soluble PKC from the cytosol

to the plasma membrane (Castagna et al., 1982; Kraft and Anderson, 1983). The model of PKC translocation and activation that emerged from these observations has served as a paradigm for the study of lipid-dependent signal transduction for more than 20 years.

1.2 Classification of the PKC Superfamily

The mammalian PKC superfamily is a large group of serine/threonine kinases organized together on the basis of structural homology and cofactor requirements (table 1). To date, the PKC family consists of eleven isoenzymes divided up into three subfamilies: the classical PKCs (cPKC α, βI, βII, γ), which are calcium-dependent and activated by DAG and *sn*-1,2-phosphatidylserine (PS); and two calcium-independent subfamilies; the novel PKCs (nPKC δ, ε,η,θ) activated by DAG and PS, and the DAG/phorbol ester-insensitive atypical PKCs (aPKC λ/ι ,ζ), which are activated *in vitro* by phosphatidylinositiol-3,4,5-trisphosphate (PIP_3), phosphatidic acid (PA), and ceramide (Liyanage et al., 1992; Mellor and Parker, 1998; Nishizuka, 1992) (Table 1). Currently little is known regarding the lipid regulation of nPKCθ and aPKCλ/ι. In addition, there has been the recent assignment of a newly identified kinase, PKD or PKCμ, to the novel PKC subfamily but given that it contains a catalytic domain with homology to calmodulin kinase, a transmembrane sequence, a pleckstrin homology domain, no autoinhibitory pseudosubstrate, and weak phorbol ester sensitivity, this classification is open to debate (Johannes et al., 1995; Johannes et al., 1994).

1.3 Structural Domains - Functional Organization

Common to all PKC family members is a protease-sensitive hinge region that when cleaved generates two distinct fragments; a 45-49 kDa carboxy-terminus fragment that contains a constitutively active catalytic domain, and a 20 to 40 kDa amino-terminus regulatory domain. It was the carboxy-terminus, catalytic domain fragment (PKM) that Nishizuka and coworkers first purified from bovine cerebellum and subsequently identified as part of a larger proenzyme (PKC) (Inoue et al., 1977; Takai et al., 1977; Takai et al., 1979a). Although the

catalytic domain is common to all PKCs, it is the regulatory domain, with its heterologous orientation and distribution of lipid binding modules, that determines a subfamily's cofactor requirements (Table 1 and Fig. 1.1).

1.3.1 Regulatory Domain.

Protein sequence analysis of the all PKC family members has revealed the existence of four conserved domains (C1-C4) and five variable regions (V1-V5). The amino-terminus regulatory domain contains the cysteine-rich C1 and C2 conserved domains and variable regions V1-V3:

A) *The C1 domain.* The C1 domain is the site of DAG and phorbol ester binding (Ono et al., 1989a). This domain is present in the classical and novel PKC isoenzymes as a tandem repeat (C1a, C1b) of 50-51 amino acids with the sequence H-X_{12}C-X_2C-$X_{13/14}$C-X_2C-X_4H-X_2C-X_7C, where H is histidine and C is cysteine. It is the presence of the cysteine-rich zinc fingers that determines a subfamily's responsiveness to the second messenger DAG and phorbol esters. *In vitro* studies suggest that there are two functional consequences of DAG/phorbol ester binding to cPKC: 1) DAG binding increases the affinity of the enzyme for membrane lipids and calcium. This is achieved through an alteration of the physical properties of the C1 lipid binding module itself. Binding of DAG/PMA to the C1 domain places a hydrophobic cap on top of what is typically, in an unbound state, a hydrophilic surface area. The effect of this physical alteration is to create a hydrophobic surface that is more likely to interact with membranes (Newton, 2001; Zhang et al., 1995); 2) DAG binding leads to an increase in the kinase activity of the enzyme. Maximal activation of cPKC occurs when DAG is bound in the presence of calcium and phosphatidylserine. Using a mixed micellar kinase assay, it has been demonstrated that DAG can bind and activate PKC on a 1:1 molar ratio in the presence of less than 10 but more than 4 phosphatidylserine molecules (Hannun et al., 1986; Hannun, 1985).

The binding of DAG to the C1 domain of cPKC isoenzymes is highly stereospecific and

there is an absolute requirement for *sn*-1,2-DAG; the *sn*-1,3- and *sn*-2,3- configurations are inactive. Within the DAG structure, it is the oxygen esters of the fatty acyl chains and *sn*-3 hydroxyl group on the glycerol backbone that are essential for DAG activation of PKC (Cabot and Jaken, 1984; Ganong et al., 1986; Hannun, 1985). In contrast, the atypical PKC isoenzymes, which contain an abbreviated C1 domain with only a single cysteine-rich zinc finger (most similar to the C1b of the cPKCs), are insensitive to DAG and phorbol esters (Ono et al., 1989b; Ways et al., 1992). *In vitro* studies suggest that the aPKC are activated by PA and PIP_3 but presently there is little *in vivo* data to support this (Limatola et al., 1994; Nakanishi et al., 1993).

B) *The C2 Domain.* The C2 domain of the classical PKC isoenzymes binds anionic phospholipids in a calcium-dependent manner (Shao et al., 1996). The C2 domain is located immediately following the C1 domain in cPKC's, but precedes the C1 and is truncated in the nPKC's (Fig. 1.1). Biochemical and crystallographic analyses have revealed that the C2 domain contains a calcium-binding pocket that is formed by aspartate residues flanking the C2 domain at the amino and carboxy ends. An intracellular elevation in calcium leads to the coordination of two calcium ions by the flanking aspartate residues and this drives a conformational change that permits the anionic phospholipid head group (e.g. phosphatidylserine) to enter and bind (Grobler et al., 1996). It is the calcium-coordinating aspartate residues that are missing from the nPKC isoenzymes and their absence is thought to be responsible for this subfamily's lack of calcium dependence (Newton, 1995). The atypical PKCs do not contain a C2 domain and there is no evidence for calcium-dependent activation of these isoenzymes.

C) *Pseudosubstrate Site.* Immediately amino-terminal to the C1 domains of all PKC isoenzymes (except PKCμ) is the autoinhibitory pseudosubstrate site (Fig. 1.1). The pseudosubstrate is an autoinhibitory module that contains a sequence of basic amino acid residues that mimics a PKC substrate but with an alanine in place of the predicted serine/ threonine (House and Kemp, 1987). A model for PKC autoinhibition proposes that in an

unstimulated state, the pseudosubstrate domain folds back into the catalytic domain and blocks substrate binding through physical occupation. The subsequent binding of an activating lipid second messenger, such as DAG, will then induce a conformational change that causes the displacement of the pseudosubstrate from the active site and the binding a specific substrate. This model is supported with data from molecular studies in which deletion of the pseudosubstrate domain results in an enzyme that exhibits lipid-independent kinase activity (Orr et al., 1992).

D) *The V3 region.* Also known as the hinge region, the V3 region contains the calcium-dependent (calpain I or II) protease-sensitive cleavage site that functionally separates PKC into regulatory and catalytic domains (Kishimoto et al., 1989; Takai et al., 1979b). In addition, there is emerging evidence that the hinge region may contain non-traditional nuclear localization signals (bipartite NLS) that target both PKCα and λ/ι to the nucleus under certain conditions (Malviya and Block, 1992).

1.3.2 The Catalytic Domain.

The catalytic domain consists of the C3 and C4 conserved subdomains, and the variable regions V4-V5.

A) *The C3 domain.* The C3 domain contains a putative ATP-binding site found in many other protein kinase enzymes (Kemp and Pearson, 1990). It consists of the conserved motif Gly-X-Gly-X-X-Gly-X_{14}-Lys. Site-directed mutagenesis of the conserved lysine residue to an arginine results in a full length enzyme that is unable to bind ATP and therefore lacks kinase activity. The use of PKC kinase-defective (K-R) mutants, which appear to function as dominant negative genes, has proven invaluable to cell biologists studying the cellular functions of PKC. Interestingly, the C3 domain of the aPKC isoenzymes is slightly different than other PKC family members due to the replacement of the third glycine with an alanine. The functional significance of this difference is presently unknown.

B) *The C4 domain.* The C4 domain is the most proximal of all conserved PKC domains

(Fig. 1.1). This region contains the substrate binding site and phosphotransfer site (Nishizuka, 1988b).

C) *The V5 region* is a key site for cellular regulation. The V5 region is approximately 50 amino acid long and located at the carboxy terminus end of all PKCs (Fig. 1.1). This region, which was identified through sequence alignment of all the cPKCs isoenzymes, was initially assumed to be unimportant for activity. Identification of alternative splice products of the cPKCβ gene, which leads to the production of two gene products that are identical except for the V5 region, suggested that this region may confer specific properties and functions. It is now recognized that conserved residues contained within the V5 region are required for a variety of cellular regulatory functions (Blobe et al., 1996a; Kiley and Parker, 1995; Mellor and Parker, 1998; Stebbins, 2001) (see below).

1.4 Cellular Regulation of PKC

Biological activity of PKC is dependent upon the ability of individual isoenzymes to gain access to activating lipid signals and compartment-restricted substrates. As it is currently understood, the cellular regulation of PKC involves a complex interplay of acute structural modifications (trans- and cis-phosphorylation events), and lipid-protein and protein-protein interactions that combine to determine the spatial parameters of PKC activity (Kiley et al., 1995; Mochly-Rosen and Gordon, 1998; Nishizuka, 1992).

1.4.1 Regulation of PKC through phosphorylation The allosteric modulation of signaling enzymes through the addition and removal of phosphate groups has emerged as a major mechanism in which to control intrinsic enzymatic activity (Wera and Hemmings, 1995). Recent data suggest that the activation state of PKC is highly dependent upon exogenous and endogenous phosphorylation events at three conserved serine/threonine residues found in the carboxy terminus half of the protein (Keranen et al., 1995). In the case of cPKCβII, newly synthesized enzyme is phosphorylated by phosphoinositide-dependent-

kinase-1 (PDK-1) at a conserved threonine residue (T500) within the activation loop of the catalytic domain (Fig. 1.2). The phosphorylation of the T500 is the rate-limiting step in the maturation of PKC into a fully active enzyme. Mutational analysis of this site has revealed that the inability to phosphorylate T500 can lead to an unstable and dephosphorylated enzyme devoid of kinase activity (Orr and Newton, 1994). Phosphorylation at T500 is necessary for autophosphorylation at the more proximal threonine 641 (T641) and serine 660 (S660). In turn, autophosphorylation of T641 is the key regulatory switch that locks PKC into a catalytically competent conformation and without it PKC is insensitive to activating lipid signals. Once autophosphorylation has occurred at T641, the phosphorylation status of T500 becomes less critical to the overall activity of the enzyme (Keranen et al., 1995). Whereas T641 is required for activity, phosphorylation at S660 appears to modulate protein stability, and interactions with protein substrates and cofactors (Mellor and Parker, 1998; Newton, 2001). Previous data from our laboratory have demonstrated the requirement of phosphorylation at S660 for regulation of subcellular localization. Using a GFP-tagged serine to alanine mutant of cPKCβII, we found that phosphorylation at S660 is necessary for the reverse translocation of cPKC back to the cytosol following recruitment to the plasma membrane with DAG-generating agonists such as angiotensin II (Feng et al., 2000). In support of these data, we also observed an increase in phosphorylation at S660 following phorbol ester treatment suggesting that the phosphorylation of S660 may be subjected to dynamic regulation in response to agonists (unpublished results).

1.4.2 Lipid regulation of cPKC isoenzymes. In cells, DAG produced in the cell membrane can regulate the subcellular localization of DAG/PMA sensitive cPKC and nPKC isoenzymes. This form of PKC regulation was first described for agonists coupled to PI-specific phospholipases but any elevation of intracellular DAG may represent a potential source of lipid regulation for C1 domain containing PKC isoenzymes.

A) DAG derived from PI lipid hydrolysis: The classical model of PKC activation.

The PI-PLC family, which acts on PI lipids and especially phosphatidylinositol-4,5-bisphosphate (PIP_2), consists of eleven isoenzymes that can be arranged into four subfamilies (β, δ, ε, γ) on the basis of structural homology and regulatory mechanisms. Members of the PLC family have been implicated in the cellular response of more than 100 different cell surface receptors. Activation of PI-PLC occurs through several receptor-directed mechanisms including heterotrimeric G-proteins, receptor tyrosine kinases and non-receptor tyrosine kinases (Rhee, 2001). In the case of the PLC-β subfamily, binding of an extracellular agonist to a G-protein-coupled receptor leads to the dissociation of heterotrimeric G-proteins into separate $G_q\alpha$ and $G\beta\gamma$ subunits. Both $G_q\alpha$ and $G\beta\gamma$ have been shown to activate PLC-β isoenzymes. On the other hand, growth factor binding to receptor tyrosine kinases stimulates intrinsic kinase activity and the autophosphorylation of tyrosine residues on the cytoplasmic ends of the receptor. These phosphorylated residues serve as docking sites for many SH2 domain-containing proteins including PLC-γ.

Activated PI-PLC hydrolyzes the phosphodiester bond of the inositol phosphate head group of PIP_2 to liberate the second messengers, inositol-1,4,5-trisphosphate (IP_3) and DAG (Fig. 1.3). Soluble IP_3 diffuses into the cytoplasm and binds receptors on the endoplasmic reticulum, leading to the release of internal calcium stores and an elevation in free intracellular calcium. In addition to activation of calcium/calmodulin-regulated targets, the increase in intracellular calcium acts in concert with membrane-bound DAG to shift the equilibrium of inactive, soluble PKC from the cytosol to the inner face of the plasma membrane where it can bind DAG and acidic phospholipids such as phosphatidylserine. The binding of DAG at the plasma membrane leads to a conformational change that displaces the autoinhibitory pseudosubstrate from the active site.

DAG formed from the hydrolysis of PI lipids first appears in the membrane within seconds of receptor occupancy, but levels drop rapidly thereafter and basal levels are restored within 1-2 minutes (Cook et al., 1990; Hughes et al., 1984; Nishizuka, 1992; Wright et al., 1988). The duration of the DAG signal is determined by the availability of PIP_2 in the cell, which

typically represents less than 1% of the mass of all phospholipids, and by rapid metabolic clearance of DAG by DAG kinases and hydrolases. The presence of PKC at the plasma membrane closely parallels cellular DAG levels.

B) DAG derived from PC hydrolysis. Many of the extracellular ligands that stimulate PI-PLC will also activate other lipases. This was first recognized in 1985 when it was noted that the agonist-induced accumulation of DAG in hepatocytes did not correlate with the formation of IP_3 (Bocckino et al., 1985). When the DAG mass was analyzed it was found that stimulation of cells resulted in two peaks of DAG accumulation. The first peak, which appeared rapidly after agonist stimulation and lasted 1-2 minutes, was attributed to the actions of PI-specific PLC. This early peak was then followed by a second, more sustained elevation of DAG that reached a maximal peak within 5-10 minutes and remained sustained over basal levels for several hours. Molecular analysis of the fatty acid composition of this sustained peak of DAG revealed that it contained relatively high amounts of saturated and mono-unsaturated (e.g. palmitic, oleic, and linoleic) acyl chains at both the *sn*-1 and *sn*-2 positions. The chemical composition of these fatty acyl chains was indicative that the sustained phase of DAG was derived from the hydrolysis of PC (Pettitt et al., 1997; Pettitt and Wakelam, 1993).

PC-specific phospholipase D (PC-PLD). Although long appreciated to exist in plants, the first direct evidence of a receptor-regulated mammalian PC-specific phospholipase D (PC-PLD) activity was reported in differentiated HL-60 cells stimulated with N-formyl-Met-Leu-Phe (fMLP) (Pai et al., 1988a; Pai et al., 1988b). There is now ample biochemical data to support a role for an agonist-induced PC-PLD activity in the generation of DAG from PC. The mammalian PC-PLD family consists of two genes, PLD1 and PLD2, as well as several gene products produced through alternative splicing events. PC-PLD1 can be stimulated by calcium-dependent PKCα and βII, and the small GTPases Rho and ARF. The PC-PLD2 isoenzyme, which is maintained with a relatively high basal activity, exhibits only a modest increase in activity in responses to ARF and PKC, and requires PIP_2 as a lipid

cofactor. Once activated, PC-PLD cleaves the phosphodiester bond of PC to generate PA and free choline. Although it has been demonstrated *in vitro* that PA can modulate the activity of several proteins, this remains to be conclusively established *in vivo*. In most cells, PA generated from the hydrolysis of PC is rapidly metabolized to DAG through the actions of PA phosphohydrolase (PAP) which appears to be variably active in different cells, thus accounting for variable accumulation of PA versus DAG from the action of PLD (Mullmann et al., 1990).

The critical question arises as to whether DAG derived from PC hydrolysis can activate PKC analogous to PI-derived DAG. Presently, this is a hotly debated issue and data exist supporting both contentions. Numerous *in vitro* studies have indicated that polyunsaturated DAG or DAGs derived from PI lipids can effectively activate PKC but that saturated and monounsaturated DAG species were less effective (Marignani et al., 1996; Martin et al., 1990; Schachter et al., 1996). These conclusions are not definitive for two main reasons: 1) there is an inherent difficulty in presenting lipids with structural variations in their acyl chains with similar consistency *in vitro*. Thus, differences in saturated DAG and polyunsaturated DAG may not be due to true affinity differences but actually due to inconsistencies in the incorporation of these lipids into vesicles or micelles; 2) the cellular regulation of PKC is much more complex than can be reproduced in an *in vitro* environment. Cellular activity of PKC is influenced by a large variety of cofactors and spatially defined parameters that would be difficult to mimic *in vitro*. It is quite possible that a localized concentration of saturated DAGs, along with certain cofactors (e.g. PS), may be sufficient to overcome the lower affinities measured relative to polyunsaturated DAGs *in vitro*. In order to draw accurate conclusions of events at the cellular level, a greater understanding is needed of the bioactivity of individual molecular species of DAG and the subcellular compartments in which they arise/reside.

The ability to examine the relationship of PKC and PC-derived DAGs *in vivo* is further complicated by the lack of a well-defined model system wherein a single agonist will activate

one phospholipase and not another (PI-PLC versus PC-PLD). Agonists that are typically utilized include platelet-derived growth factor (PDGF), bombesin, and carbachol. The disadvantage of each of these agonists is that they produce a biphasic elevation of DAG through simultaneous activation of PI-PLC and PC-PLD. Similar to the *in vitro* studies with saturated DAGs and PKC, there are also conflicting data from these studies. Whereas some groups have found no activation (as measured with translocation to a particulate fraction) of PKC with PC-PLD activation (Leach et al., 1991), others have found some persistent membrane association (Ha and Exton, 1993).

One system that has been utilized to address this issue is the porcine aortic endothelial (PAE) cells where stimulation with lyso-phosphatidic acid (LPA) induces the activation of PLD without a concomitant stimulation of PI-PLC (Pettitt et al., 1997). Data from this model suggested that saturated-monounsaturated DAGs were unable to activate PKC; however, these results must be interpreted with some caution since there was significant basal membrane translocation of PKC in the absence of LPA stimulation which was possibly due to a large amount of polyunsaturated DAGs that were evident in these cells.

At present the question of whether DAG derived from PC can activate PKC is still open, but this issue may be particularly amendable to emerging technologies such as mouse embryo fibroblasts (MEF's) that can be generated devoid of complicating lipases, and RNA Interference (RNAi) technology.

PC-specific phospholipase C (PC-PLC). There are reports that DAG can also be derived from PC through the actions of a PC-PLC which would generate DAG directly as well as choline phosphate (Sheikhnejad and Srivastava, 1986; Wolf and Gross, 1985). Several studies have suggested the presence of a PC-specific PLC activity which was stimulated by TNF-α and cross-linking antibodies to the B-cell receptor. Unfortunately, progress in this area has suffered from a lack of purified PC-PLC and from lack of molecular cloning of such an enzyme from mammals. Conclusions from these studies have been further complicated by the use of the pharmacological inhibitor D609 which was discovered to

inhibit PC-PLC from bacterial species *in vitro*, and used to implicate the existence of a mammalian PC-PLC. Unfortunately, D609 has never been shown to inhibit a mammalian PC-PLC. On the other hand, D609 has been shown to inhibit mammalian-PC:ceramide phosphocholine transferase (sphingomyelin synthase; SMS) (Luberto and Hannun, 1998) and PC-PLD (Kiss and Tomono, 1995) at similar concentrations used to inhibit bacterial PC-PLC. Given the underdetermined specificity of the D609 inhibitor, the existence of a genuine PC-PLC is unclear, and the possibility exists that the activity characterized in these studies may have been misidentified.

DAG produced during sphingomyelin synthesis. Evidence of a sphingomyelin cycle (SM cycle) was first described in 1989 when it was reported that the application of 1α,25-dihydroxyvitaminD$_3$ could stimulate the hydrolysis of SM with the subsequent generation of choline-phosphate and the lipid second messenger, ceramide (Hannun, 1994; Okazaki et al., 1989). During the resynthesis phase of the SM cycle, SM synthase utilizes ceramide and PC to generate SM and DAG. A possible physiological role for DAG derived from SM synthesis has been proposed for the regulation of NF-κB (Luberto et al., 2000). Similar to PC-PLC, a detailed molecular understanding of this enzyme and its lipid products awaits molecular cloning.

1.4.3 Regulation of PKC through membrane translocation. The groundbreaking discovery by Nishizuka and colleagues that unsaturated DAG was the agonist for PKC did not yield an immediate understanding of how DAG and PKC interacted (Takai et al., 1979a; Takai et al., 1979c). A subsequent report by Castagna et al. also from Nishizukas' group, identified PKC as the cellular receptor for tumor promoting phorbol esters, but provided only slight clues as to the mechanism (Castagna et al., 1982). At that time, it was known that phorbol esters mediated their cellular responses by primarily acting on receptors at the cell membrane. Thus, it was inferred that the target of PMA was PKC at the plasma membrane. In 1983, Kraft and Anderson demonstrated that treatment of parietal yolk sac

(PYS) cells with PMA could induced a shift in the subcellular distribution of PKC from the cytosol to the particulate or membrane fraction (Kraft and Anderson, 1983). These data provided a mechanism for the interaction of PKC and DAG/PMA, whereby soluble PKC responds to the presence of DAG or PMA in the membrane by "translocating" to the membrane surface from the cytosol. These observations served to establish the translocation of PKC to the membrane fraction as the conceptual and biochemical hallmark of PKC activation that still stands today.

Just how PKC interacts with DAG and other lipids at the membrane has been the subject of intense investigation. Using a novel mixed micellar method to investigate the specificity and stoichiometry of PKC-lipid interactions, Hannun et al. reported that full activation of PKC occurred when a single molecule of DAG was presented in the context of several molecules of phosphatidylserine (PS) (Hannun, 1985). Further, with this approach they demonstrated the remarkable specificity for the interaction of PKC with both DAG and PS (Hannun et al., 1986).

The model that arose out of these *in vitro* findings was that four molecules of PS bind Ca^{2+} at the membrane surface and that this creates a PS-Ca^{2+} complex that initiates the binding of inactive PKC to the membrane. The subsequent interaction of DAG with PKC bound at the membrane to the PS-Ca^{2+} complex leads to the full activation of the PKC activity (Hannun et al., 1986). This model fits very well with the mechanics of PI lipid turnover and generation of DAG which has been shown to be preceded by an intracellular elevation of Ca^{2+}

For more than 15 years, the plasma membrane was conceptualized as the primary target for PKC translocation without the confirmation. Although the use of subcellular fractionation provides information regarding whether PKC has moved from a soluble location to a membrane (particulate) fraction, this approach has limitations that include the inability to resolve specific compartments, and the possibility of artifactual (e.g. false negative) results due to *ex vivo* effects. This changed with the development of green fluorescent protein

(Cubitt et al., 1995). Beginning in the mid 1990's, several groups took advantage of GFP to tagged PKC so as to monitor PKC localization in live cells following agonist stimulation. Specifically, their approach demonstrated and confirmed that PKC does in fact translocate to the plasma membrane, and that this occurs in a highly dynamic manner involving multiple lipid binding domains and kinase activity (Feng et al., 1998; Oancea and Meyer, 1998; Sakai et al., 1997). Future investigation of PKC with a similar approach has the potential to take our understanding of the cellular regulation of PKC to the next level.

1.4.4 Regulation of PKC subcellular localization through protein-protein interactions. Although there is little debate that lipid-dependent translocation/activation of PKC at the plasma membrane represents a fundamental form of PKC regulation, there have been subsequent discoveries that suggest that this model may not be applicable to all forms of PKC regulation. In particular, identification, molecular cloning, and sequence analysis has revealed variations in the complement and composition of conserved domains (C1 and C2) found distributed throughout the PKC family members (Coussens et al., 1986; Knopf et al., 1986; Ono et al., 1986b; Parker et al., 1986). The implications of these findings is that different forms of regulation may exist (Nishizuka, 1988a). Further, biochemical studies have revealed that there is little *in vitro* substrate discrimination amongst different PKC isoenzymes (Kazanietz et al., 1993). This lack of substrate selectivity *in vitro* suggested that mechanisms must exist within cells that would function to coupled PKC to specific substrates. Evidence in support of this proposal has come from immunofluorescence studies which revealed differential subcellular localization of PKC isoenzymes (Goodnight et al., 1995). In addition to the cytosol and plasma membrane, PKC has now been localized to the golgi (Goodnight et al., 1995), cytoskeleton (Blobe et al., 1996a; Ito et al., 1989; Mochly-Rosen et al., 1990), mitochondria (Majumder et al., 2000), nuclear envelope (Hocevar and Fields, 1991; Leach et al., 1989), and nucleus interior (Beckmann et al., 1994; Neri et al., 1994). These findings have been used to support the hypothesis that substrate selectivity

i.e. isoenzyme-specific functions of PKC at the cellular level may be mediated by targeting PKC to distinct regions of local substrates, and that this targeting can be carried out be lipid second messengers, such as DAG, as well as more recently, through PKC binding proteins.

In the early and mid 1990's several groups undertook an effort to identify PKC binding proteins. From these studies, several classes of proteins were identified including STICKS or substrates interacting with C-kinase, RICKS or receptors for inactive C-kinase, RACKs or receptors for activated C-kinase, PICKs or proteins that interact with C-kinase, and scaffolding proteins including 14-3-3 and AKAP (Jaken and Parker, 2000; Mochly-Rosen et al., 1991; Staudinger et al., 1995). As it turns out, many of these proteins are nonspecific and bind multiple PKCs (scaffolding proteins e.g. AKAP), but others have been reported to bind to specific PKC isoenzymes (RACK1 to cPKCβII). Moreover, it has been reported that PKC binding proteins can recognize a variety of PKC states including an unphosphorylated-inactive form, a phosphorylated-inactive, or a phosphorylated-active form (Jaken and Parker, 2000). Importantly, many of these protein-protein interactions are lipid dependent and most of these PKC binding proteins are themselves lipid binding proteins. Nonetheless, disruption of these interactions leads to the mislocalization of PKC and the disruption of PKC signaling, suggesting a significant contribution of these protein-protein interactions in PKC regulation (Jaken and Parker, 2000).

1.5 Cellular Functions of PKC

Activated PKC catalyzes the transfer of a terminal γ phosphate of ATP to serine and threonine residues on protein components of signaling cascades. Many of the biological functions proposed for PKC have been derived from: 1) cellular responses elicited by agonists coupled to the generation of DAG and which were sensitive to PKC inhibitors; 2) application of cell-permeable DAG analogues or phorbol esters; 3) overexpression studies; 4) *in vitro* kinase assays with recombinant or immunoprecipitated protein; and 5) knockout or transgenic mouse models.

The general picture that has emerged from these studies suggests that there is enormous heterogeneity in the cellular functions of PKC. Currently, at least 110 PKC substrates have been identified, and these span the range of tissue types and physiological functions. Some examples of PKC substrates include neuromodulin, MARCKS protein, several of the classes of G-protein coupled receptors (e.g. cholinergic, catecholinergic, tryptaminergic), growth factor receptors (e.g. EGF), metabolic enzymes (e.g. glycogen synthase), signaling enzymes (e.g. MAPK), cytoskeletal proteins (e.g. adducin), nuclear proteins (e.g. lamin B), and the proto-oncogene (e.g. pp60c-src) (Dempsey et al., 2000b). The breadth of these putative substrates is indicative of the evolutionary advantage of PKC activity that can be found conserved throughout biology.

1.6 Acute versus long term PKC activity.

It had been proposed by Nishizuka and colleagues, and more recently by Blobe et al. that the cellular functions of PKC can be divided into two general types based upon the persistence of the DAG signal: 1) those cellular functions that require an acute activation of PKC (occurs on the order of minutes) and primarily target lipids signals generated at the cell membrane; and 2) functions that require long term PKC activity (hours-days) (Blobe et al., 1996b; Nishizuka, 1986; Nishizuka, 1995).

1.6.1 Role of PKC as mediator of acute signal transduction events. Translocation and activation of PKC at the plasma membrane is a key event in PKC-dependent signal transduction. This classical role of PKC was first described for the release of serotonin from platelets during platelet activation. A similar mechanism of activation has since been demonstrated for a large number of other cellular functions including neurotransmitter release, secretion, vascular smooth muscle contraction and relaxation, lipogenesis, and glycogenolysis. All these events have in common that they involve a transient (1-2 min) elevation of lipid second messengers and the reversible translocation of cPKCs to the plasma

membrane.

1.6.2 Role of sustained PKC activity in cell physiology and disease states. Sustained PKC activity is required for normal physiological functions such as cell differentiation, mitogenesis, and gene expression. Persistent PKC activity has also been implicated in a variety of diseases and disease models.

A) Role of DAG-PKC in tumor progression and cancer. A major role for DAG in cell growth and tumorigenesis was established when it was discovered that tumor-promoting phorbol esters could functionally and structurally recapitulate DAG generated at the plasma membrane (Castagna et al., 1982; Niedel et al., 1983; Yamanishi et al., 1983). These observations implicated a direct role for DAG and its primary cellular target, PKC, in the cellular changes that occur during tumor progression. Further, a role for protein kinase-mediated phosphorylation in tumor promotion is also supported by the observation that another tumor promoter, okadaic acid, is an inhibitor of protein phosphatases.

Extensive investigation into the mechanism of tumor-promotion by phorbol esters suggest that they induce their effects through a wide variety of biochemical changes including increases in reactive oxidative stress, secretion of proteases and collagenases, alteration of gene expression, protein phosphorylation, and cytoskeletal changes. In many cases these cellular alterations lead to uncontrolled cell proliferation associated with tumorigenesis but in other cases they may result in cell differentiation or even cell death depending upon cell type (and complement of DAG/phorbol ester-sensitive PKC isoforms expressed). PKC has been directly implicated through overexpression studies which found PKCα, βII, ε, and ζ all associated with either tumor promotion, increased cell survival or suppression of apoptosis. In contrast, nPKCδ exhibited pro-apoptotic functions (Blobe et al., 1994; Dempsey et al., 2000a).

A role for sustained DAG is supported with molecular studies that have correlated the overexpression of the small GTPase oncogenes *ras* or *sis* with the elevation of DAG and the

transformed state of the cells (Fleischman et al., 1986; Preiss et al., 1986; Wolfman and Macara, 1987). The increase in DAG was concluded to be derived from an enhanced rate of phospholipid turnover (phospholipase-mediated) and not a *de novo* synthesis of glycerophospholipids (Fleischman et al., 1986). These data implicate a role for the abnormal activity of glycerophospholipid metabolizing enzymes in the progression toward the transformed phenotype.

B) Role of DAG-PKC in diabetes. Chronic hyperglycemia is a major initiator of the pathological complications associated with type 1 and type 2 diabetes (Sheetz and King, 2002). Many of the effects of hyperglycemia are mediated through a sustained elevation of DAG and the activation of DAG/PMA-sensitive PKC isoenzymes (Way et al., 2001). Sustained elevations of DAG and PKC activity have been detected in the endothelial tissues of the heart, aorta, retina, brain, nerves, and kidney of diabetic animal models and diabetic patients. Indeed there are clinical trials underway evaluating the efficacy of cPKCβ specific inhibitors (e.g. LY333531) in blocking the complications of diabetic nephropathy and neuropathy. The hyperglycemia-induced elevation of DAG has been attributed to an enhanced flux through the glycolytic pathway and the de novo biosynthesis of glycerolipids but data supporting these contentions is incomplete. At present, there is a great need to identify the mechanism(s) of hyperglycemia-induced DAG elevation for future therapeutic development.

C) Role of DAG-PKC in neurological diseases Abnormal enzymatic activities of PI-PLC, PC-PLD, and PLA_2 have been detected in a variety of neurological disease states including Alzheimer's disease, Huntington disease, schizophrenia, and in some neurodegenerative disease models. Although the key neurochemical mechanisms underlying these enzymatic abnormalities are unknown, it is predictable that DAG levels, and subsequently PKC activity, will be abnormal as a result. Further, PKC may also have a role in the control of glial cell regulation. The characteristic molecular feature of many primary tumors of the CNS is an overexpression or hyperactivity of select PKC isoenzymes (Bredel and Pollack., 1997; Sharif and Sharif, 1999).

D) Role of DAG-PKC in cardiac disease. The link between DAG-PKC and cardiac disease was established through the analysis of several mouse models of cardiac hypertrophy. Utilizing a transgenic mouse model, Lefkowitz and colleagues noted that the constitutive expression of the α_1-adrenergic receptor resulted in a cardiac hypertrophy phenotype and an accumulation of DAG which they attributed to the prolonged and aberrant activation of receptor-coupled phospholipases (Milano et al., 1994). DAG signaling has also been implicated with data from transgenic mice that expressed a constitutively active cPKCβII. This model exhibited a hypertrophic phenotype similar to the α_1-adrenergic mouse model which could be alleviated with PKC inhibitors (Wakasaki et al., 1997).

1.7 Rationale/preliminary data.

Unlike the classical model of PKC activation where the primary site of PKC translocation and activity is the plasma membrane, little is known about the subcellular localization of PKC during more sustained activation. Data that are available come from studies that have primarily assessed the activation state of PKC and not the specific subcellular target of translocation. Most of these studies employed a generalized biochemical approach wherein the cellular movement or translocation (i.e. activation) of PKC was monitored by subcellular fractionation and immunoblotting for PKC. More recently, the use of confocal microscopy and GFP technology has provided a complementary approach to fractionation studies but to date they have been used primarily for the examination of the acute activation of PKC activation/translocation. Very little microscopic information is available addressing the cellular behavior of PKC under physiological and pathological conditions of sustained activation.

Using the PDGFβ receptor (PDGFβ-R) as an endogenous source of sustained DAG, we investigated the subcellular localization of GFP-tagged cPKC isoenzymes in live cells with confocal imaging. Overexpression of the PDGFβ receptor in the presence of serum led to a modest translocation of GFP-cPKCα to the plasma membrane. In addition, translocation to a secondary, juxtanuclear location that was also evident. Importantly, this translocation was not observed in the absence of serum, and juxtanuclear translocation could be restored with the addition of PDGF-BB to the serum-free culture media. To confirm that PC-PLD was activated through PDGF-BB stimulation, a transphosphatidylation assay was performed in parallel with the microscopic studies. PC-PLD activation occurred in the presence of PDGF and with serum. Thus, confocal imaging and the use of the PDGF receptor coupled to PC-PLD allowed the visualization of an membrane compartment distinct from the plasma membrane that serves as a target for PKC translocation during sustained activation.

This juxtanuclear accumulation of PKC stimulated with PDGF-BB was similar to a novel site of translocation which we have recently identified using DAG-mimicking phorbol esters

Thus, long term (1 hr) PMA can recapitulated the juxtanuclear compartment that was observed with PDGF stimulation. This suggested that PMA could be used to probe the properties and mechanism of PKC translocation to this novel compartment.

What follows are the data that describe the identification, characterization, and function of this novel site of cPKC translocation during long term activation. Moreover, cPKC translocation is investigated and discussed within the context of glucose transport in the diabetic cell model.

Mammalian PKC Superfamily

subtype	Mw (KDa)	Activators	Tissue Expression
grp A, classical			
α	76.8	PS, Ca, DG, FFA, LPC	ubiquitous
βI	76.8	PS, Ca, DG, FFA, LPC	various
βII	76.8	PS, Ca, DG, FFA, LPC	various
γ	78.4	PS, Ca, DG, FFA, LPC	brain
grp B, Novel/New			
δ	77.5	PS, DG, PIP_2	ubiquitous
ε	83.5	PS, DG, FFA, PIP_2	brain
η	78.0	CS, PIP_2	lung, skin, heart
θ	81.6	?	skeletal muscle
grp C, atypical			
ζ	67.7	PS, FFA, PIP_3, CER	ubiquitous
λ/ι	67.2	?	ovary, testis

Table 1. The Mammalian PKC Superfamily. The mammalian PKC superfamily is currently made up of 11 isoenzymes that are grouped together on the basis of lipid cofactor requirements and structural homology. All PKC isoenzymes are single polypeptide phosphoproteins with molecular weights that range between 67-84 kDa and representatives of each subfamily are found in all eukaryotic cells. There is an absolute requirement for phospholipids for PKC activity.

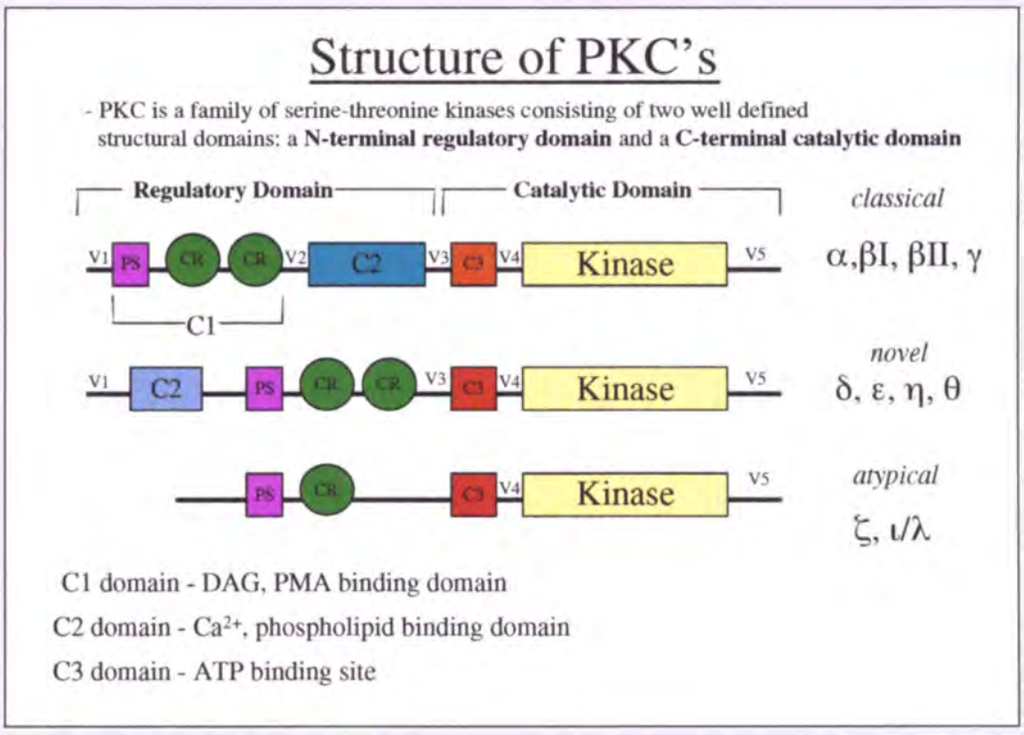


Figure 1.1 Structure of the PKC superfamily. All PKC family members contain the highly conserved (approx. 85% homology) carboxy terminus catalytic domain and an amino terminus regulatory domain that is made up of various complements and compositions of lipid binding modules.

- PKC βII has three major phosphorylation sites: **Thr^{500}**, **Thr^{641}**, and **Ser^{660}**

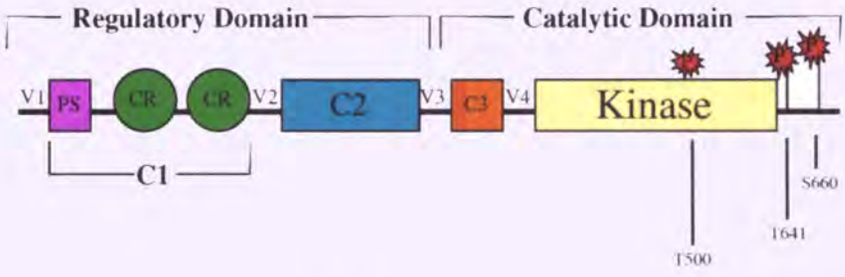


Figure 1.2 Scheme for regulation of cPKC through phosphorylation at conserved serine-threonine residues. Nascent cPKC beta2 is phosphorylated at T500 by phosphoinositide-dependent kinase- 1 and this is required for autophosphorylation at threonine 641 and serine 660. Phosphorylation at T641 is necessary for catalytic competency whereas p660 modulates cPKC-protein interactions and subcellular localization.

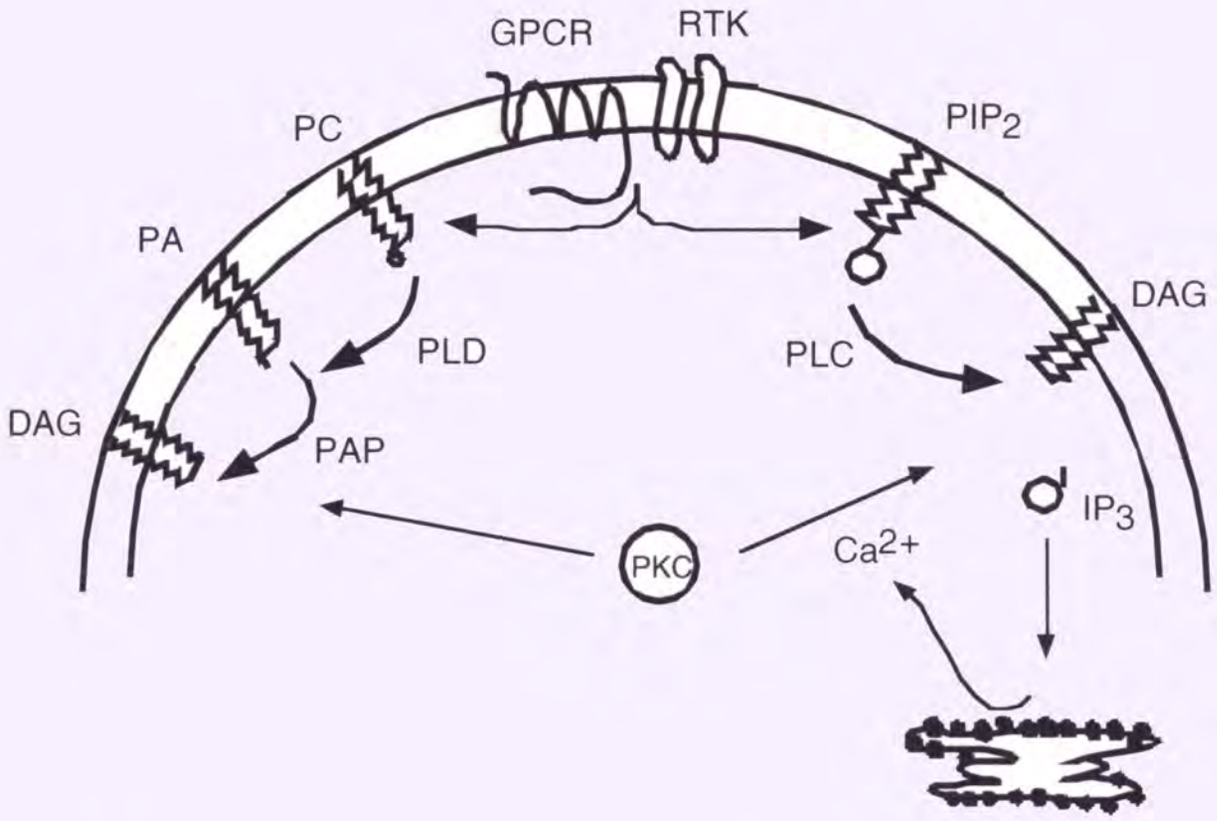


Figure 1.3 Lipid dependent translocation of PKC to the cell membrane. Agonist binding to a cell surface receptor (e.g. G-protein coupled receptor, receptor tyrosine kinase) triggers the activation of a PI-specific phospholipase C. The hydrolysis of PIP2 leads to the formation of membrane-bound DAG and soluble IP3. IP3 diffuses to the endoplasmic reticulum where is binds receptors that release incellular stores of calcium. The increase elevation of calcium along with DAG act in concert to recruit PKC to the plasma membrane. DAG-derived from PI lipid hydrolysis is rapidly metabolized and PKC reverses translocation within 1-2 min. Agonist binding can also activate PC-specific phospholipase D. DAG, which can be sustained for hours, is proposed to recruit PKC to the membrane in a mechanism analogous to PI-derived DAG but with a more persistent association with the membrane.

Chapter 2: Role of a Novel cPKC-Dependent Compartment in the Sequestration of Recycling Membrane Components

2.1 Introduction

Members of the Protein Kinase C (PKC) superfamily of lipid-dependent, serine-threonine kinases function as critical signaling intermediates in numerous signal transduction pathways and cellular regulatory processes. Currently, PKC consists of 11 distinct isoenzymes grouped into three subfamilies (classical, novel, and atypical) on the basis of lipid cofactor requirements and structural homology (Ohno and Nishizuka, 2002). Each PKC isoenzyme can be divided into two functional domains; a highly conserved carboxy terminus domain that encodes the subdomains required for catalytic activity; and a less conserved, amino terminus which contains the lipid binding modules that determine subfamily-specific lipid requirements. The first PKC kinases to be identified were the calcium-dependent or classical PKC (cPKC) isoenzymes (α, βI, βII, and γ). Members of this subfamily contain a cysteine-rich zinc finger C1 domain which is the site of *sn*-1,2-diacylglycerol (DAG) and phorbol ester binding, and a C2 domain which confers calcium-dependent phospholipid binding properties. The novel PKC (nPKC) isoenzymes (δ, ε, η, θ) contain a C1 domain similar to the classical isoenzymes but are calcium-independent due to a truncated, non-functional C2 domain. Lipid regulation of the atypical PKC (aPKC) subfamily (λ/ι, ζ) is the most divergent due to a truncated, DAG-insensitive C1 domain and no C2 domain. (Mellor and Parker, 1998; Newton and Johnson, 1998; Ohno and Nishizuka, 2002).

A key determinant of cellular PKC activation lies in the ability of individual isoenzymes, especially the cPKC's and less so the nPKC's, to 'translocate' to plasma membrane in response to DAG and tumor-promoting phorbol esters. At the plasma membrane, PKC is activated and gains access to specific substrates (Nishizuka, 1995). Although this classical model of acute PKC activation (seconds to minutes) stands today as a paradigm for the study of lipid-dependent signal transduction, there is additional biochemical and immunocytochemical evidence to suggest that PKC may translocate to sites other than the plasma membrane (Mochly-Rosen and Kauvar, 1998). Using a variety of PKC agonists, including extracellular agonists and phorbol esters, PKC has been localized to the Golgi complex (Goodnight et

al., 1995), nuclear envelope (Hocevar and Fields, 1991; Leach et al., 1989), nucleus (Beckmann et al., 1994; Neri et al., 1994) mitochondria (Majumder et al., 2000), and/or cytoskeleton (Blobe et al., 1996a; Ito et al., 1989; Mochly-Rosen et al., 1990). Translocation of PKC into the nucleus occurs in response to the agonist-mediated turnover of nuclear PI lipids and the subsequent generation of DAG (for a recent review on nuclear inositol signaling and PKC in the nucleus see (Martelli et al., 2002) and (Martelli et al., 2001))

In the course of investigating the mechanisms of PKC translocation, we observed that following relatively long term treatments (>45mins) select PKC isoenzymes translocated. to a singular, juxtanuclear location. In this study, we present data describing the translocation of members of the cPKC subfamily to a novel, temperature- and kinase activity-dependent derivative of the endosomal recycling system, which was found to be closely associated with the Golgi complex and microtubule-organizing center (MTOC). These data also suggest that translocation of PKC to this compartment coincides with an enrichment of this compartment in lipid raft components and that this compartment functions to sequester recycling components.

2.2 Materials and Methods

Materials

Eagle's minimal essential media, Dulbecco's modified Eagle's medium, and HEPES were from Life Technologies, Inc (Gaithersburg, MD). The HeLa and DLD-1 cell lines were kindly provided by Dr. Dennis Watson (Medical University of South Carolina), and the CHO-K1 cell line was kindly provided by Justin Turner (Medical University of South Carolina). The HEK 293, HT-1080, COS-1, and A549 cell lines were all purchased from American Tissue Culture Collection (Manassa, VA). Primary antibodies: Anti-cPKCα was described previously (Wetsel et al., 1992); Anti-20S Proteasome (cat #539145), 4β-phorbol-12-myristate-13-acetate (PMA) and 4α-phorbol were purchased from Calbiochem (La Jolla, CA.); anti-pericentrin (cat # PRB-432C) and anti-giantin (cat # PRB-114C) were from Covance (Oakland, CA); anti-GM130 (cat # 610822) and anti-p230 (cat # 611280) were purchased from BD Transduction Labs (Lexington, KY); anti-rab11 (cat # 9020) and anti-Ubiquitin (cat # FL-76) were obtained from Santa Cruz (Santa Cruz, CA). Alexa Fluor®-594 Transferrin, Alexa Fluor®-594 Cholera Toxin, anti-mouse and anti-rabbit TRITC secondary antibodies were from Molecular Probes, Inc (Eugene, OR.). All other chemicals were from Sigma (St. Louis, MO).

Cell culture conditions

HeLa, Cos-1, and A549 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), HEK 293 and HT-1080 cells were maintained in Eagle's minimal essential media (MEM), DLD-1 cells was maintained in RPMI-1640, and CHO-K1 cells were maintained in Ham's F12K. All media (GIBCO-BRL) were supplemented with 10% (v/v) fetal bovine serum (Summit), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown at 37°C in a humidified incubator with 5% CO and passaged every 3-4 days to maintained

logarithmic growth.

Constructs

The subcloning of full length wild-type cPKCβII, cPKCβII-mtC1, and cPKCβII-ΔC2 into pBK-CMV-GFP was described previously (Feng et al., 2000; Feng et al., 1998). GFP-fusion constructs of cPKCα and nPKCε were prepared using a similar method; cDNA was amplified with polyermase chain reaction (PCR) and gene specific primers synthesized with a 5' BssH II and 3' Kpn I restriction site. The amplified PCR products were digested and ligated into the pBK-CMV-GFP mammalian expression vector. The GFP-cPKCβII-C1 domain was produced by amplifying the C1 domain of cPKCβII with PCR primers containing restriction sites. Amplified product was ligated into the pEGFP-C3 vector (Clontech). A full length human EST encoding wild-type atypical PKC zeta was purchased from American Type Culture Collection (Rockville, MD) and amplified with PCR. Atypical PKC zeta was amplified with primers encoding a 5' XhoI restriction site and an 3' Eco RI site. The amplified full length, wild-type PCR product was ligated into pEGFP-C3 vector (Clontech) and sequenced at the MUSC DNA Sequencing Facility.

Transient transfection, indirect immunofluorescence, and confocal microscopy

Cells were plated onto 35 mm confocal dishes (MatTek) at a density of 2.5-5.0 x 10^{-5} cells/ dish and grown for 24 hours. Transient transfection of DNA (1.5 μg/dish) was performed with Lipofectamine 2000 (Invitrogen) according to manufacturer's recommendations. Twelve hours post transfection, cells were treated and fixed with 3-3.7% paraformaldehyde with or without 10% methanol for 10 minutes at 37°C or room temperature. Following fixation, cells were permeabilized with –20°C 100% methanol for 10 minutes. The methanol was aspirated and cells were allowed to air dry (approx. 5 minutes). Cells were washed 3 times with 1.5% FBS/PBS for 5 minutes each and then blocked in 2.5% FBS for 1 hour at room temperature. All primary antibody incubations were performed in 1.5% FBS/PBS

with 0.15% saponin at dilutions of 1:50 to 1:100 for 3 hours at room temperature or overnight at 4°C. Following incubation with the primary antibody, cells were washed 3 times with 1.5% FBS/PBS for 5 minutes each. Cells were incubated with secondary antibody diluted in 1.5% FBS/PBS and 0.15% saponin at a dilution of 1:50 to 1:100 for one hour at room temperature. Cells were washed 3 additional times with 1.5% FBS/PBS for 5 minutes each. Confocal images were captured immediately following immunofluorescence processing with an Olympus IX-70 Spinning Disk Confocal Microscope and a PLAN APS 60x (NA. 1.4) oil objective. Each micrograph represents a single image captured at the equatorial plane of the cell. All micrographs were processed for publication in Adobe Photoshop® 5.0.

Disruption of the Golgi complex with nocodazole and brefeldin A

The Golgi dissociating agents, nocodazole and brefeldin A were employed to probe cellular localization of cPKC. Cells were plated in 35 mm confocal dishes (MatTek) at a density of 2.5-5.0 x 10^{-5} cells/dish. After 24 hours of growth, cells were transiently transfected with GFP-cPKCα. Twelve hours post transfection, cells were treated with 100 nM PMA for 1 hour at 37°C to induce the translocation of cPKCα to the pericentrion. Following this initial cPKC stimulation, cells were then treated with concentrations of either nocodazole (5 μM) or brefeldin A (5 μg/ml) that were previously (Cole and Lippincott-Schwartz, 1995; Klausner et al., 1992) determined to cause full Golgi dissociation which was also verified in this study (Fig. 4B and D).

Electron Microscopy

HEK 293 cells were plated onto 35 mm dishes at a density of 2.5-5.0 x 10^{-5} cells/dish. After 24 hour of growth, cells were either treated with 0.01% DMSO or 100 nM PMA for 1 hour. Cells were fixed for electron microscopy in pre-warmed, 2.5% glutraladehyde-0.1 M sodium cacodylate for 30 minutes at 37°C and then rinsed overnight in cacodylate buffer with 7%

sucrose. Cells were post-fixed in 1% osmium tetroxide and 1% potassium ferrocyanide for 1 hour. Potassium ferrocyanide was included along with osmium tetroxide to enhance staining of membrane structures. Cells were then dehydrated in graded ethyl alcohol as follows: 5 min in 50% ethyl alcohol, 5 min in 70% ethyl alcohol, 5 min in 95% ethyl alcohol, and 10 min in 100% ethyl alcohol. Samples were infiltrated with a 1:1 solution of 100% ethyl alcohol and Embed 812 embedding resin for 30 min and then embedded in Embed 812 resin. Samples were polymerized for 24 hour in a 60°C oven with cover on dish and 24 hour at 60°C without dish cover. The hardened resin was separated from the plastic dish and blocks were cut with a Sears electric scroll saw. Blocks were re-embedded for orientation and polymerized for a 24 h at 60°C. Thick sections (0.5 μm) were cut, stained with toluidine blue and examined with light microscopy to determine which blocks were appropriate for thin sectioning. Thin sections (70 nm) were cut with a Riechert Ultramicrotome Ultracut E, picked up on copper grids, and double-stained with uranyl acetate and lead citrate. Thin sections were viewed with a JEOL 1210 Transmission electron microscope at 80 kV. Images represent the average appearance of a minimum of 20 high power magnification fields.

Immunoelectron Microscopy

A pre-embedded immunoelectron microscopic approach was modified from Ohta et al. (Ohta et al., 2002) for these studies. Briefly, cells were plated in 35 mm dishes at a density of 2.5 to 5.0 x 10^{-5} cells/dish. Twenty four hours later cells were treated either with 0.01% DMSO or 100 nM PMA for 1 hour, washed 3 times with PBS, and then fixed with 3% formaldehyde in pre-warmed Pipes pH 6.8 for 20 minutes at 37°C. Cells were permeabilized with 0.5% Triton X-100 in Pipes pH 6.8 for 10 minutes at room temperature and washed 3 times with PBS for 10 minutes each at room temperature. Non-specific binding was blocked with 10% BSA in PBS with 0.05% Tween-20 for 60 minutes at 37°C. The primary antibody (anti-cPKCα) was diluted to 1:50 in 1.5% FBS/PBS with 0.15% saponin and incubated for 2

hours at 37°C. Cells were washed 3 times for 10 minutes each with PBS/0.05% Tween-20 at room temperature. The anti-rabbit IgG secondary antibody conjugated to 12 nm colloidal gold (Jackson ImmunoResearch) was diluted 1:10 in 1.5% FBS/PBS with 0.15% saponin and incubated at 37°C for 5 hours. Cells were washed 3 times for 10 minutes each with 0.05% Tween20/PBS at room temperature and then fixed a second time with 2.5% glutaraldehyde in Pipes pH 6.8 for 60 minutes. Further processing for immunoelectron microscopy was carried out as described above for electron microscopy.

Steady state labeling with transferrin

Cells were plated onto 35 mm confocal dishes at a density of 2.5-5.0 x 10^{-5} cells/dish, grown for 24 hours, and then transiently transfected with GFP-cPKCα. Twelve hours post transfection, visualization of total cellular recycling compartments was achieved by steady state labeling cells with 10 μg/ml Alexa Fluor (594)-conjugated transferrin (Molecular Probes) for 60 minutes in the presence of 0.01% DMSO or 100 nM PMA. All labeling was performed in the presence of complete culture media with 10% FBS.

Recycling assay

Cells were plated onto 35 mm confocal dishes at a density of 2.5-5.0 x 10^{-5} cells/dish, grown for 24 hours, and then were transiently transfected with GFP-cPKCα. Twelve hours later, the recycling of membrane components was visualized by chase labeling of transfected cells with 10 μg/ml of unlabelled transferrin for 30 minutes following a 1 hour steady state labeling of cells with 10 μg/ml Alexa Fluor-conjugated transferrin. The steady state labeling and subsequent chase with unlabelled transferrin were performed either in the presence of 0.01% DMSO or 100 nM PMA. All images represent the average appearance of a minimum of 20 60X fields from 3-5 separate experiments.

2.3 Results

2.3.1 Endogenous and overexpressed PKC translocates to a juxtanuclear location when stimulated in a prolonged manner.

Whereas acute (5-10 mins) stimulation of PKC with its agonist 4β-phorbol-12-myristate-13-acetate (PMA) results in translocation of PKC to the plasma membrane, longer PMA treatments (>45 mins) of Hela cells resulted in the translocation of both endogenous cPKCα and GFP-fused cPKCα to the plasma membrane and, to a singular, juxtanuclear region (Fig. 2.1A). The inactive phorbol ester analogue, 4α-phorbol, did not result in translocation of PKC to either the plasma membrane or the juxtanuclear region (Fig. 2.1B), and translocation of cPKCα was not limited to activation by phorbol esters since the non-phorbol PKC activator 7-octylindolactam, was also able to induce translocation of GFP-PKCα to the juxtanuclear region (data not shown). To investigate whether juxtanuclear translocation was limited to HeLa cells, GFP-cPKCα was transiently transfected into a variety of other cell lines, and the cells were treated with PMA for 60 min. Juxtanuclear cPKC translocation was observed in several cell lines including HEK 293, HT-1080, DLD-1 (Fig. 2.1C), CHO, COS-1, and A549 adenocarcinoma cells (data not shown) indicating that translocation of PKC to this site represents an ubiquitous response to sustained activation. Thus, cPKCα undergoes translocation to a novel, juxtanuclear location upon prolonged stimulation with various PKC agonists.

2.3.2 Translocation of PKC to the juxtanuclear compartment is specific for the classical PKC isoforms, α and βII

To determine if translocation of cPKCα to the juxtanuclear location was isoenzyme-specific or was common to all isoenzymes, representatives of each PKC subfamily (classical - βII, novel - ε, atypicals - ζ) were subcloned into GFP vectors and transiently transfected into HeLa cells. Following 1 hour exposure to PMA, only GFP-cPKCβII was observed to translocate to both the plasma membrane and to the juxtanuclear site (Fig. 2.2A). The

DAG-sensitive and calcium-independent nPKC isoenzyme GFP-nPKCε was found only at the plasma membrane, whereas the DAG-insensitive GFP-aPKCζ did not respond to PMA and exhibited no alteration in cytoplasmic localization. These data suggest that the translocation of cPKC to the juxtanuclear region involves a mechanism that appears restricted to members of the calcium- and DAG/PMA-sensitive cPKC subfamily.

2.3.3 Juxtanuclear translocation of cPKC requires C1 and C2 domains

To investigate the requirement of the C1 and/or C2 domain in translocation of cPKC isoenzymes to the juxtanuclear compartment, a series of cPKC mutants were generated encoding either a mutant of the C1 domain (cPKCβII-mtC1) that disrupts binding of DAG/PMA, a full deletion of the C2 domain (cPKCβII-ΔC2), and an isolated C1 domain (GFP-C1) of cPKCβII. One hour treatment with PMA lead to translocation of wild-type GFP-cPKCβII to both the plasma membrane and juxtanuclear, whereas the diffuse cytoplasmic localization of cPKCβII-mtC1 was unchanged (Fig. 2.2B, panels 1 and 2). This suggests that cPKC translocation to the plasma membrane requires a functional interaction with its agonist at the C1 domain. Importantly, GFP-C1 exhibited translocation to the plasma membrane as has been previously reported (Oancea et al., 1998) but no translocation to the juxtanuclear region was evident (Fig. 2.2, panel 3). This suggested that binding to the phorbol ester alone was not sufficient to induce translocation and that translocation may require full length protein. To that end, similar to the isolated C1 domain, the GFP-cPKCβII-ΔC2 exhibited translocation to only the plasma membrane (Fig. 2.2, panel 4). These results suggest that translocation of cPKC requires both a C1 and C2 domain. In subsequent studies, the cPKCα and βII isoforms were found to behave identical in parallel experiments, and therefore, for extended investigation we chose to focus on the cPKCα isoenzyme.

2.3.4 cPKCα translocates to a singular, juxtanuclear location distinct from the Golgi complex

In an effort to identify the juxtanuclear compartment to which cPKC translocated, an extensive colocalization study was undertaken. As expected, given that cPKC translocates to a singular location in the center of the cell, the mitochondria-specific dye, MitoTracker, the late endosome/lysosomal-specific dye, LysoTracker, and an ER-targeted protein, pDS-Red-ER, all failed to display any overlap with GFP-cPKCα translocated to the juxtanuclear region (data not shown). Translocation of cPKC to a central region of HeLa or HEK 293 cells suggested that, under conditions of prolonged stimulation, cPKCα may associate with an organelle that can also be found as a single compartment such as the Golgi complex. This was thought to be a strong possibility since the cPKCγ isoform was suggested to localize to the golgi complex in the NIH 3T3 cell line following long term activation with PMA (Goodnight et al., 1995). In addition, some DAG/PMA-sensitive, non-kinase proteins, including munc13 (Song et al., 1999) and chimaerins (Caloca et al., 2001) have also been reported to translocate to the Golgi complex following activation with phorbol esters. Using antibodies to GM130 (Nakamura et al., 1995), giantin (Linstedt and Hauri, 1993), and p230 (Kooy et al., 1992), as markers of the cis, medial, and trans compartments of the Golgi complex, respectively, GFP-cPKCα was observed to localize close to the Golgi compartment at low magnification (Fig. 2.3 A-C, rows 1 and 3)). However at higher magnification and resolution, it was evident that the pattern of cPKC translocation was separable from the Golgi complex (Fig. 2.3 A-C, rows 2 and 4). Analysis of high resolution micrographs revealed that GFP-cPKCα translocated to a region of the cell that was surrounded by the Golgi complex but displayed only minimal overlap with that organelle. In order to conclusively rule out any association of cPKCα with the Golgi, the Golgi complex was disrupted with 5 μg/ml Brefeldin A (BFA) (Klausner et al., 1992). Total disruption of the Golgi complex was indicated by dispersal of GM130 visualized by indirect immunofluorescence (Fig. 2.4A). However, BFA had little effect on cPKCα translocated to

the juxtanuclear site (Fig. 2.4B). It has been reported that BFA may incompletely disrupt the Golgi complex and that the cis/medial Golgi dissociates, but the trans-Golgi remains intact (Ladinsky and Howell, 1992). To address this possibility, cells were treated with 5 μM nocodazole which effectively disrupts all aspects of the Golgi complex following 1 hour PMA (Cole and Lippincott-Schwartz, 1995)(Fig. 2.4C). The administration of nocodazole had no effect on the GFP-cPKCα associated with the juxtanuclear site (Fig. 2.4D). Quantitation of the effect of both nocodazole and BFA cPKC translocation showed that there was only modest effects on the number of cells exhibiting juxtanuclear translocation (Fig. 2.4E). Thus, the apparent resistance of this compartment to BFA and nocodazole-induced disruption suggests that cPKCα translocated to an organelle distinct from the Golgi complex and whose structure was independent of microtubules. These results argue against the localization of cPKCα to the Golgi complex upon prolonged activation.

2.3.5 The juxtanuclear accumulation of cPKC is not a result of aggregated or degraded protein.

Recent reports suggest that the excess production of misfolded proteins may result in the aggregation of protein in an MTOC-associated structure which the authors named the 'aggresome' (Garcia-Mata et al., 1999; Johnston et al., 1998). The aggresome consists of aggregated protein encased in a vimentin-supported structure that functions to recruit cytosolic pools of ubiquitin and proteasome subunits to aid in the degradation of misfolded proteins (Wigley et al., 1999). In our studies, no evidence was found that overexpression of GFP-tagged cPKC isoenzymes and/or long term treatment with PKC agonists could modulate the subcellular location of ubiquitin or the 20S proteasome (Fig. 2.5A and B), and thus, cPKCα did not colocalize with either ubiquitin or 20S proteasome. Further, no PKC degradation was detected up to 6 hours for either endogenous (Fig. 2.5C) or overexpressed protein (data not shown), although some loss of PKC was seen at later time points. Thus, within the time frame of the current studies (1 h) there was no detectable degradation of

PKC

Importantly, cPKCα translocation to the juxtanuclear compartment was found to be reversible and temperature dependent such that stimulation at 37°C resulted in the translocation of GFP-cPKCα to the juxtanuclear compartment; however, at lower temperatures (4-30°C), this translocation was not observed (Fig. 2.5D, panel 1-3). Also, juxtanuclear translocation of cPKCα could be fully reversed with subsequent incubation at 4°C and then restored following reincubation at 37 °C (Fig. 2.5D, panel 4-5). These results demonstrate that cPKC translocation and association with the juxtanuclear compartment involves a dynamic and reversible interaction and not the aggregation of protein. Moreover, they emphasize the high dependence of translocation on physiologic temperature.

2.3.6 cPKCα translocates to a compartment that is closely associated with the centrosome.

The centrosome or the 'central body' named by Theodor Boveri more than 100 years ago can be found at the center of interphase cells where it functions as the primary MTOC (Rieder et al., 2001). Due to its central location, the centrosome became a candidate for the novel compartment defined by cPKC translocation. Indirect immunofluorescence with markers of the centrosome demonstrated that GFP-cPKCα translocated to a compartment that was centrally defined by gamma tubulin (data not shown) and pericentrin (Doxsey et al., 1994)(Fig. 2.6A). However, it was evident that GFP-cPKCα localization extended significantly beyond the pericentrin-defined centrosome. These observations suggested that with long term activation, cPKCα translocates to a novel compartment located in a central region of the cell and that this compartment was closely associated with the centrosome.

2.3.7 cPKCα translocates to a subset of transferrin-positive recycling endosomes

Based on the above evidence, we evaluated compartments/cellular functions that show

pericentriolar behavior. Although the endosomal recycling compartment (ERC) usually shows a diffuse tubulo-vesicular morphology, in some cells a subcomponent of the recycling compartment adopts a singular pericentriolar distribution, closely related to the Golgi complex and MTOC (McGraw et al., 1993; Yamashiro et al., 1984); however, this distribution has not been shown to be associated with any known specific functions or regulation of the recycling compartment (McGraw et al., 1993; Mukherjee et al., 1997). To determine if cPKCα localized to any aspect of the endosomal recycling system, we examined the effects of PMA on transferrin recycling and its relationship to PKC. Cells were labeled to steady state with Alexa Fluor® 594-conjugated, fluorescent transferrin (AF-Tf) and treated with phorbol esters. As shown in Fig. 2.6B, sustained activation of PKC resulted in localization of cPKCα to a subset of transferrin-positive recycling endosomes concentrated in a central area of the cell suggesting that cPKC translocated to the component of the recycling system associated with the MTOC/centrosome. Currently, the best markers for the pericentriolar recycling compartment (PCRC) include RME-1 (Lin et al., 2001) and the small GTPase, rab11 (Ullrich et al., 1996). Indeed, GFP-cPKCα was found to have extensive colocalization with rab11 at the juxtanuclear compartment (Fig. 2.6C). These studies suggest that cPKC may associate with a specialized subcompartment of the endosomal recycling system or a derivative of this system.

However, unlike rab11, which was present at the juxtanuclear region and in the membrane fraction independent of temperature or PMA activation (Fig. 2.6D-F), cPKCα was not a resident component of this compartment, but actively translocated to it in a temperature- and activation-dependent process, suggesting that the cPKC-defined compartment is not identical to the rab11-defined one but closely associated with it.

2.3.8 Translocation of cPKCα to the pericentrion is accompanied by membrane lipids and proteins.

Since activation of PKC by phorbol esters involves an association with membrane lipids we

next evaluated if the translocation of cPKCα to the juxtanuclear region was accompanied by membrane lipids. Utilizing a fluorescent analogue of the ganglioside GM1 (bodipy-GM1) as a plasma membrane marker, it was observed that in the absence of PKC stimulation, GM1 was localized predominantly to the plasma membrane (Fig.2.7A, panel 1). Following 1 hour PKC stimulation, GM1 was found in the plasma membrane and also at a juxtanuclear location (Fig. 2.7A, panel 2), and similar to cPKC the trafficking of GM1 to the juxtanuclear site was also highly dependent upon temperature (Fig. 2.7B). Quantitatively, approximately 50% of the Bodipy-GM1 that loaded into the membrane in the absence of PKC stimulation accumulated at the juxtanuclear compartment upon PMA stimulation. To confirm that GM1 accumulated in the cPKC-positive juxtanuclear compartment, cells were labeled with the fluorescent, GM1-binding, cholera toxin B subunit (AF-CTx-B) in the absence or presence of phorbol esters. Following 1 hour stimulation of PKC, AF-CTx-B colocalized with GFP-cPKCα in the juxtanuclear compartment (Fig. 2.7C). Similar to cPKC and GM1, AF-CTx-B also displayed temperature dependent translocation (Fig. 2.7D). Interestingly, CTx-B accumulated at the juxtanuclear compartment independent of stimulation with phorbol esters; however, the addition of CTx-B also caused accumulation of cPKCα at the juxtanuclear compartment (data not shown). Further, treatment with Gö 6976, a cPKC-selective kinase inhibitor, caused the dissociation of AF-CTx-B from the juxtanuclear compartment following accumulation at the juxtanuclear location (Fig. 2.7E). These data show that CTx-B activates cPKC and that cPKC kinase activity is required for maintenance of this compartment.

Thus, cPKCα translocation involves plasma membrane lipids, suggesting the translocation of at least subcompartments of the plasma membrane. This was further evaluated by following the simultaneous localization of caveolin-1, a plasma membrane protein and a key constituent of caveoli (Glenney and Soppet, 1992). Long term stimulation of cells with PMA resulted in the concentration of caveolin-1 at the pericentriolar region and colocalization of cPKCα with caveolin-1 (Fig. 2.7F). These observations suggest that cPKC translocates to a subcompartment of the endosomal recycling system that is enriched in components of

lipid rafts/caveolae and that kinase activity is required for maintenance of this compartment. Lipid rafts have recently emerged as distinct subdomains of the plasma membrane that function as platforms for the signal transduction of raft-associated receptors and enzymes (for a recent review see (Simons and Toomre, 2000)). The above results demonstrate dynamic regulation of plasma membrane rafts by cPKC.

2.3.9 Translocation of cPKCα to the juxtanuclear compartment coincides with the formation of a pericentriolar membranous structure.

Despite data describing the pericentriolar component of the recycling compartment (Mukherjee et al., 1997; Yamashiro and Maxfield, 1987; Yamashiro et al., 1984), little ultrastructural information is available defining this compartment. Moreover, the active translocation of PKC, caveolin, membrane lipids, and presumably other membrane constituents to this compartment and the requirement for PKC activity in this process suggest that a distinct compartment, containing membrane structures should exist. In an effort to better visualize this compartment, an electron microscopic approach was undertaken. Noting the exquisite dependence of cPKCα association on temperature we suspected that this compartment may disperse at room temperature (or temperatures lower than 30°C). Thus, special care was used to maintain the temperature at 37°C during preparation of cells for electron microscopy. In addition, the presence of a membranous structure also suggested the need to optimize preparatory conditions for the best visualization of internal membranes. To that end, the membrane enhancing stain potassium ferrocyanide was included along with osmium tetroxide to accentuate membranes (Karnovsky, 1971). In untreated cells, the centrosome was seen as the expected 400 nm structure surrounded by 50-80 nm vesicles and tubular structures with golgi complex seen in the surrounding periphery (Fig. 2.8A). Stimulation of PKC with PMA resulted in a reorganization of the centrioles, with an extension of the centriolar appendages and an appearance of a membranous structure that seemed to radiate from these appendages in multiple concentric membranes (Fig. 2.8B). Immunogold

experiments localized cPKCα to these centriolar-associated membrane structures (Fig. 2.8C) and in a perimeter surrounding each centriole (Fig. 2.8D). To our knowledge, this is the first time that a membranous structure has been visualized associated with the centrosome. This may have previously been missed due to lack of potassium ferrocyanide usage, failure to maintain appropriate temperature, and to the requirement for prolonged cPKC activation.

2.3.10 Long term activation of cPKC results in the sequestration of membrane recycling components.

Next we explored the biological consequences of the translocation of cPKCα to this compartment. The endosomal system is known to function as a sorting center for proteins and lipids destined for degradation in lysosomes, trafficking to other organelles, or recycling back to the plasma membrane (Mukherjee et al., 1997; Mukherjee and Maxfield, 2000). Since the trafficking of transferrin has been the subject of extensive study (Yamashiro et al., 1984), we examined the effects of stimulation and inhibition of PKC on the kinetics of endocytic membrane trafficking using the fluorescent-conjugated transferrin ligand (AF-Tf). Steady state labeling of cells with AF-Tf, in the absence of PKC activation, resulted in the localization of transferrin in a broad, pericentriolar pattern (Fig. 2.9A), which is consistent with previous results (Yamashiro et al., 1984). On the other hand, when cells were steady state labeled with transferrin in the presence of PMA, the pericentriolar localization of transferrin intensified and GFP-cPKCα was observed to colocalize with this pericentriolar subset of transferrin positive compartments (Fig. 2.9B). Since kinase activity is required for the maintenance of the cPKC-dependent juxtanuclear compartment, the effects of kinase inhibitors were evaluated. As seen in Fig. 2.9C, preincubation with 3 μM Gö 6976 did not block uptake of AF-Tf, but there was an absence of any pericentriolar intensity. Gö 6976 also inhibited the translocation of GFP-cPKCα. These data suggest that, under conditions where cPKC activity is sustained for a prolonged period, there may be a reorganization of a subset of recycling endosomes to the pericentriolar region.

To investigate the functional consequences of sustained cPKC activity on membrane recycling, chase experiments were performed with non-fluorescent transferrin. In the absence of PKC stimulation, a 30 minute chase following steady state labeling with AF-Tf resulted in the recycling of all transferrin back to the plasma membrane and a complete loss of fluorescence from the cell (Fig. 2.9D), again consistent with published results (Dunn et al., 1989; Hopkins and Trowbridge, 1983; Mayor et al., 1993). In stark contrast, a 30 minute chase in the presence of sustained cPKC activity resulted in the retention of AF-Tf in the cPKC positive juxtanuclear compartment (Fig. 2.9E). These observations demonstrate that cPKC can effectively sequester recycling components in this juxtanuclear compartment.

2.4 Discussion

This study shows that unlike the classical model of PKC activation, in which PKC responds to the acute elevation of lipid second messengers with a reversible (30-120 seconds) translocation to the plasma membrane, a more prolonged stimulation can induce translocation to a site which is centered on the centrosome but extends beyond it. This compartment is most closely related to a previously recognized variant of the endocytic recycling compartment (ERC) which can be found in some cells concentrated in an area around the MTOC/centrosome. Currently there is no known function associated with this specific distribution of ERC. However, there are several distinctions between the cPKC-positive compartment and the typical ERC: 1) unlike rab11, which in our studies was localized to the center of the cell regardless of PKC stimulation, cPKC was found to translocate to the juxtanuclear compartment only upon long term activation with PKC agonists. Moreover, and unlike rab11, inhibition of PKC activity reverted the translocated PKC off the juxtanuclear compartment (data not shown). Therefore, unlike the rab11 compartment, this structure is dynamically regulated through PKC; 2) Similarly, the juxtanuclear PKC-dependent structure was exquisitely dependent on physiologic temperature and was dynamically and reversibly regulated by temperature changes; again unlike what was seen with rab11. 3) The MTOC/ centrosome distribution of the ERC is sensitive to microtubule-disrupting drugs such as nocodazole (McGraw et al., 1993). In contrast, the cPKC-positive compartment was largely resistant to the effects of microtubule disruption; 4) Translocation of cPKC was coincident with the accumulation of plasma membrane components at this compartment. Accumulation of these components at the juxtanuclear compartment was fully dependent upon PKC activity; 5) Electron and immunoelectron microscopy studies localized cPKC and cPKC-associated membranes to the appendages of the centrioles and centrosome and they revealed distinct morphologic features seen only upon stimulation with PKC and preservation of 37° C temperature. Thus, while cPKC translocated to a pericentriolar compartment that very closely overlaps the rab11/MTOC-defined pericentriolar compartment, the above criteria clearly

indicate that the two compartments are not identical.

Given these distinctions, it is apparent that long term activation of cPKCα results in the formation of a specialized pericentriolar recycling compartment that serves as a novel target for cPKC translocation. In turn, cPKC then functions to regulate the rate of recycling, and thus the partitioning of membrane components between this compartment and the plasma membrane. We propose to name this compartment the *pericentrion* in order to emphasize its unique regulation and functional significance (further discussed below).

Functionally, cPKCα regulates the formation and persistence of the pericentrion with attendant sequestration of membrane components in this compartment. This novel function of PKC may be hypothesized to have relevance to many events dependent on the availability of membrane components (such as receptors, channels, and transporters) and subdomains (such as rafts) at the plasma membrane. For example, it has been shown that activation of PKC regulates the availability of the dopamine transporter at the plasma membrane (Melikian and Buckley, 1999). It would be interesting to determine if these effects are due to retention of the dopamine transporter at the pericentrion, in a mechanism similar to that seen with transferrin.

cPKC may also regulate the dynamics of rafts and their function. Rafts are glycosphingolipid- and cholesterol-enriched subdomains of the plasma membrane that function to segregate the biochemical activity of associated proteins. A role for lipid rafts has been proposed in membrane trafficking and signal transduction (Simons and Toomre, 2000) From the results in this study, it is obvious that stimulation of cPKC can result in movement of key components of caveoli and rafts (caveolin and glycosphingolipids) to the pericentrion,. This may result in sequestration of these components with significant consequences to the function of rafts and raft-related activities. This should be the subject of further investigation.

In addition to such potential consequences of cPKC-regulated sequestration on the function of protein targets at the plasma membrane, it is also possible the concentration /

sequestration of specific bioactive lipids and membrane proteins at the pericentrion may serve to exert compartment-specific effects.

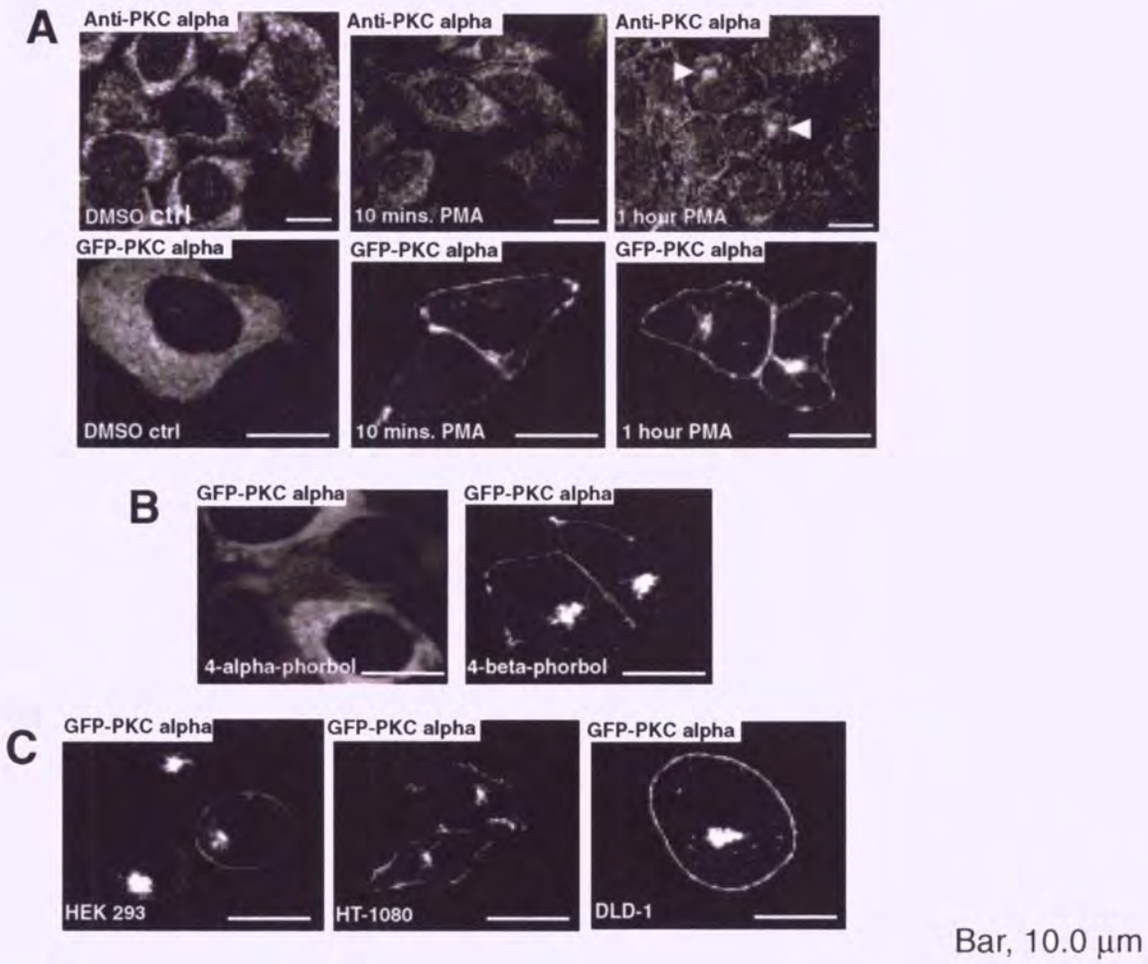


Figure 2.1. Juxtanuclear translocation of endogenous and GFP-tagged classical PKC isoforms in various cell lines. **(A)** Indirect immunofluorescence of endogenous cPKCα and GFP-tagged cPKCα in HeLa cells treated with DMSO alone (0.01%) for 1 hour or 100 nM PMA for 10 minutes and 1 hour. Indirect immunofluorescence was performed with anti-cPKCα antibody as described in Materials and Methods. Arrows indicate site of juxtanuclear compartment. For overexpression studies, HeLa cells were transiently transfected with GFP-cPKCα and 24 hours post transfection were treated with DMSO alone or 100 nM PMA. **(B)** HeLa cells transiently transfected with GFP-cPKCα were treated 12 hours post transfection with either 100 nM 4β-phorbol or the inactive 4α phorbol ester. **(C)** Transient transfection of various cell lines including HEK 293, HT-1080, and DLD-1 with GFP-cPKC alpha. Cells were treated 12 hours post transfection with 1 hour, 100 nM PMA. All fluorescent images were captured on an Olympus Ultra View Spinning Disk Confocal Microscope IX-70 and each panel is representative of a minimum of 20 fields. All experiments were repeated three to five times each with similar results. Bar represents 10.0 µm.

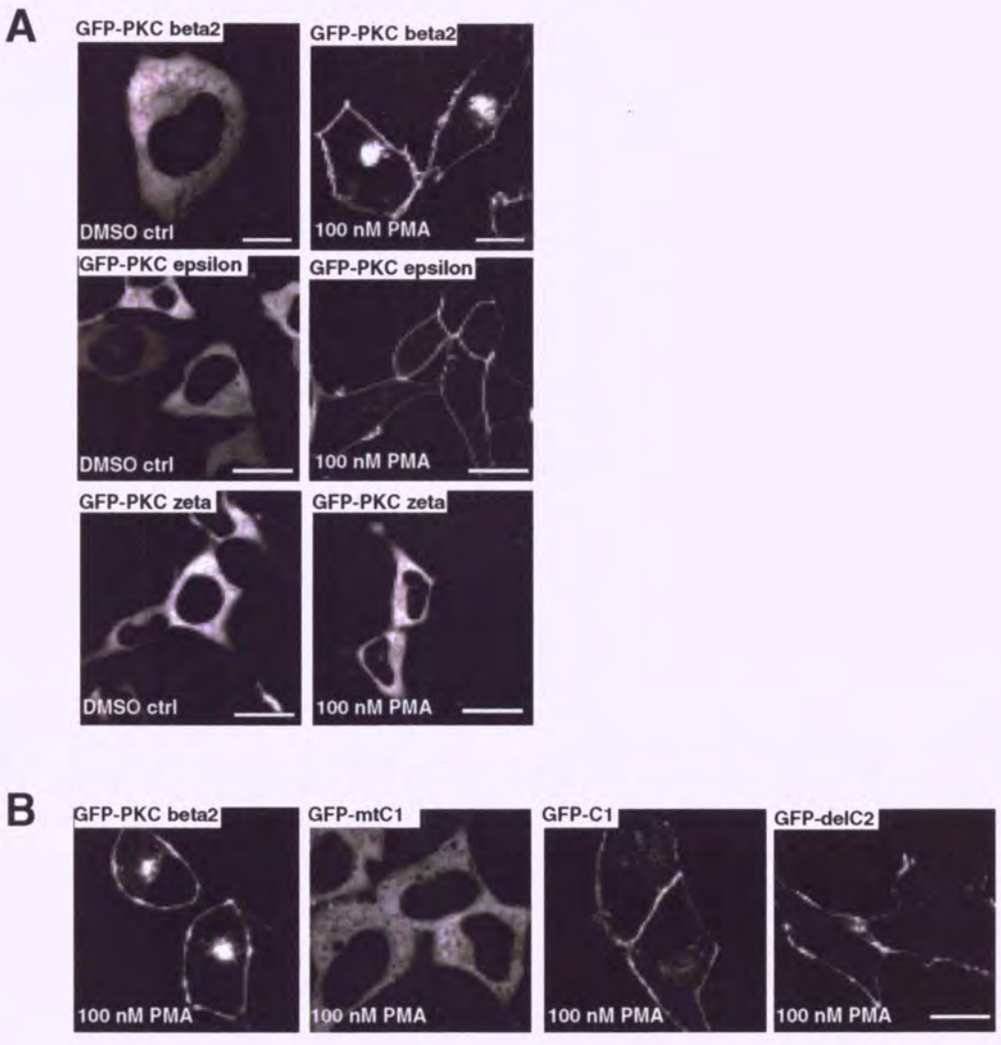


Figure 2.2. Translocation of PKC to the juxtanuclear compartment is specific for cPKC isoenzymes and requires the C1 and C2 domains. **(A)** Representative isoenzymes from each PKC subfamily (classical, novel, and atypical) were subcloned into pEGFP vector and Hela cells were transiently transfected. Twelve hours post transfection, cells were treated with 100 nM PMA for 1 hour. **(B)** GFP tagged wild type and mutants of cPKCβII; GFP-cPKCβII, GFP-cPKCβII-mtC1, GFP-C1 domain, GFP-cPKCβII-ΔC2, were transiently transfected into HeLa cells and treated with 100 nM PMA for 1 hour. Each panel is the average representative of a minimum of 20 fields. All experiments were repeated three to five times each with similar results. Bar, 10 μM.

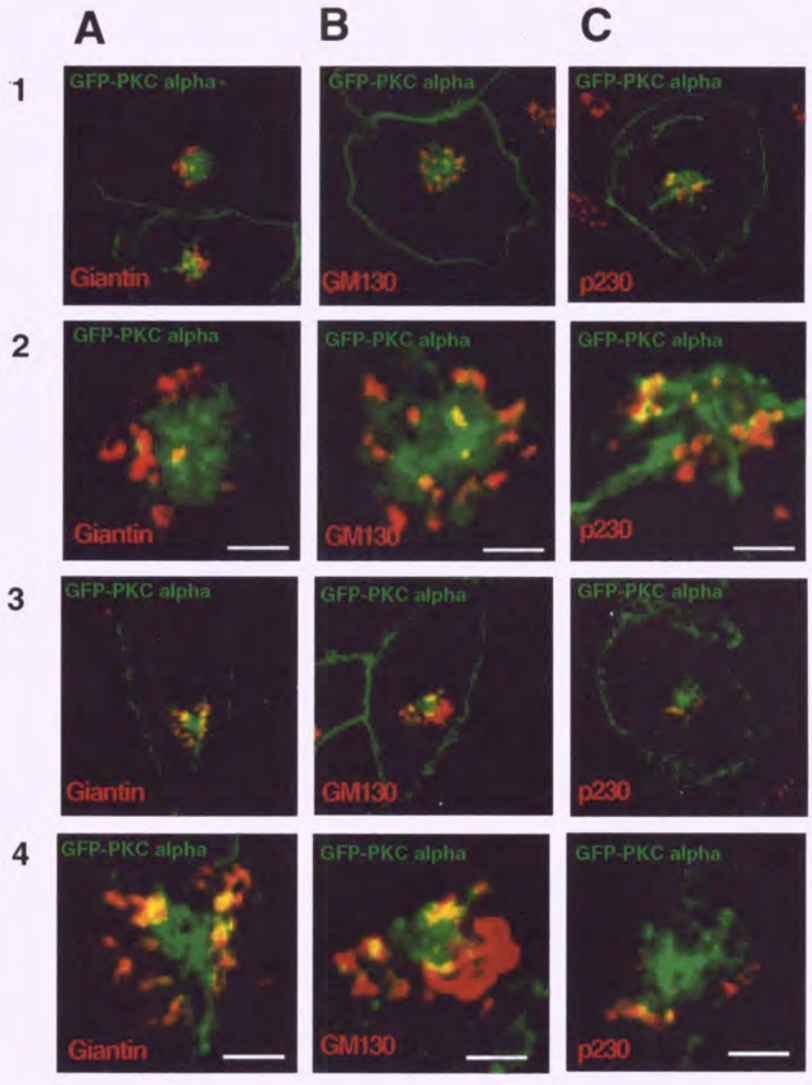
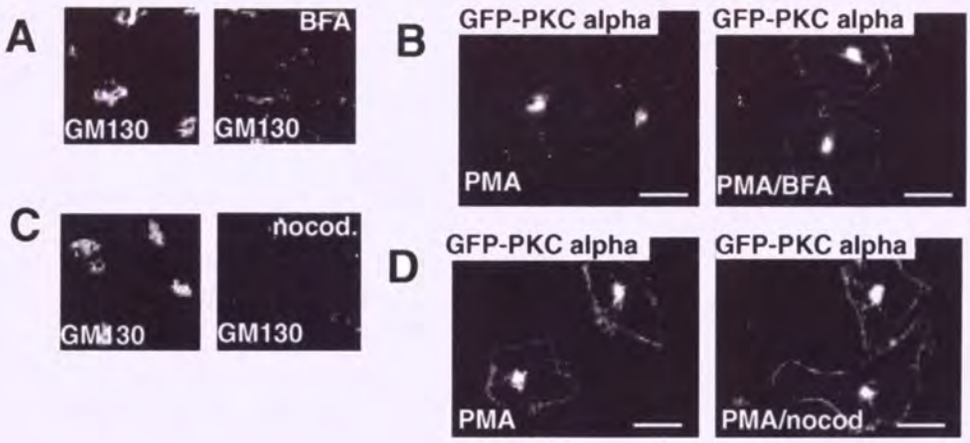


Figure 2.3 Translocation of cPKC isoforms to a juxtanuclear compartment closely associated with Golgi complex and MTOC/centrosome. (A-C, rows 1 and 3) Low magnification images of HeLa cells transiently transfected with GFP-cPKCα. 12 hours post transfection, cells were treated with 100 nM PMA and subjected to indirect immunofluorescence with anti-Giantin (medial), anti-GM130 (cis), and anti-p230 (trans). Primary antibodies were detected with TRITC-conjugated secondary antibody. **(A-C, rows 2 and 4)** High magnification images of juxtanuclear compartment found in cells of rows 1 and 3. Represents indirect immunofluorescence for Golgi markers Giantin, GM130, and p230. Bar represents 1.0 μm.



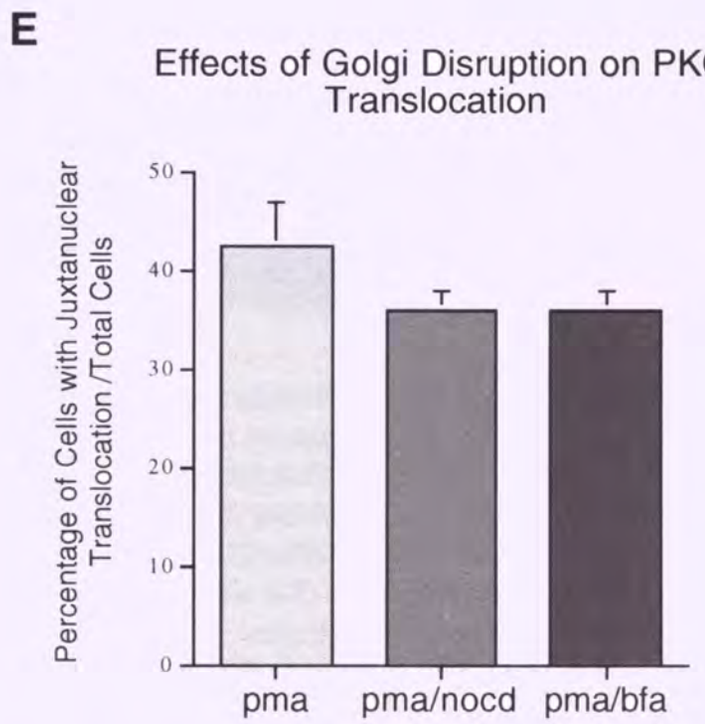


Figure 2.4. The juxtanuclear compartment is resistant to Golgi disrupting drugs (A) Indirect immunofluorescence for GM130 in HeLa cells treated with 5 μg/ml Brefeldin A for 1 hour. **(B)** HeLa cells transiently transfected with GFP-cPKCα were stimulated with 100 nM PMA for 1 hour to induce translocation of GFP-cPKCα to the juxtanuclear compartment. Cells were then treated with 5 μg/ml BFA for 1 hour to dissociate the golgi complex. **(C)** Indirect immunofluorescence for GM130 in HeLa cells treated with 5 μM nocodazole for 1 hour. **(D)** Translocation of GFP-cPKCα was induced with 1 hour PMA and followed by 1 hour treatment with 5 μM nocodazole to dissociate golgi complex. **(E)** Percentage of total cells exhibiting juxtanuclear translocation of GFP-cPKCα following stimulation with 1 hour of 100 nM PMA, 1 hour of 100 nM PMA followed by 1 hour 5 μM nocodazole or 1 hour 5 μg/ml BFA. Quantification was obtained from 20 random fields at 60X and repeated three separate times. Bar represents 10.0 μm.

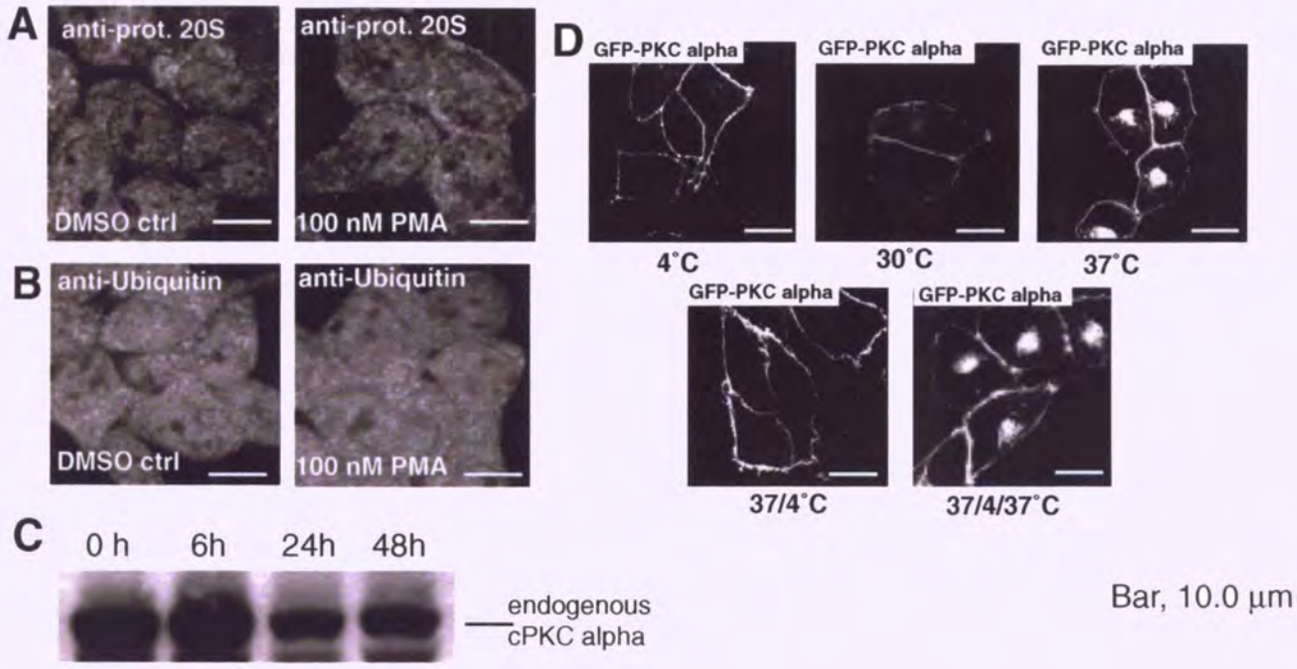


Figure 2.5 Accumulation of GFP-cPKCα at the juxtanuclear location is not due to aggregation of PKC. **(A)** Indirect immunofluorescence for proteasome 20S and **(B)** ubiquitin in HeLa cells following 1 hour DMSO (0.01%) or 100 nM PMA. **(C)** Immunoblot for endogenous cPKCα in cells treated for 0, 6, 24, and 48 hours with 100 nM PMA. **(D, panels 1-3)** HeLa cells transiently transfected with GFP-cPKCα were incubated with 100 nM PMA at either 4°C, 30°C or 37°C for 1 hour. **(D, panels 4-5)** Cells with juxtanuclear translocation of GFP-cPKCα induced with 1 hour PMA at 37°C were then incubated at 4°C for 1 hour or 4°C 1 hour and then 37°C 1 hour. Bar represents 10 µM.

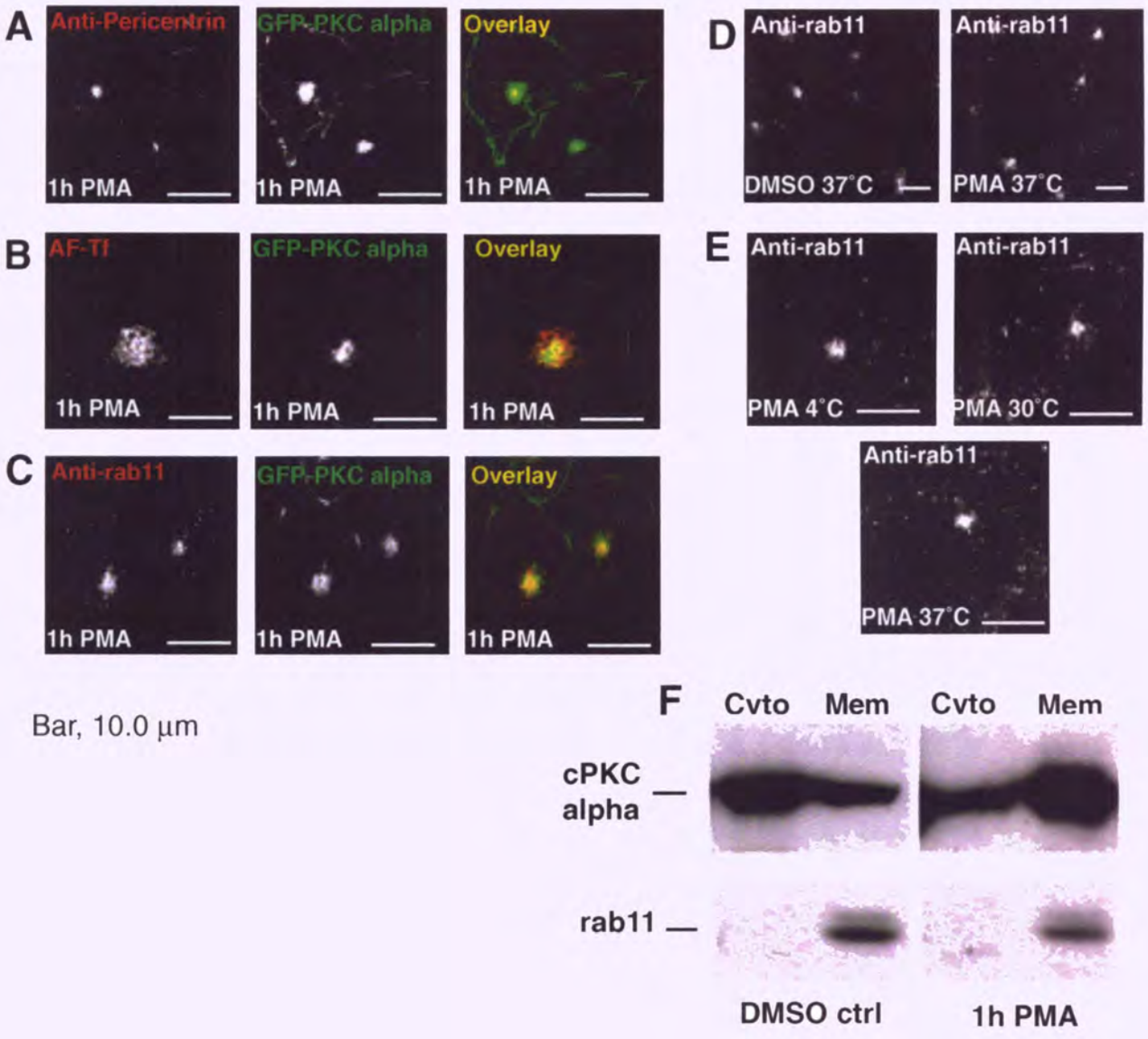


Figure 2.6 cPKCα translocates to a subset of transferrin-positive endosomal recycling compartments concentrated around the MTOC/centrosome. **(A)** Indirect immunofluorescence with anti-pericentrin in HEK 293 cells transiently transfected with GFP-cPKCα and treated with 1 hour PMA. **(B)** Cells were steady-state labeled with Alexa Fluor-conjugated transferrin (A.F.-Tf) for 1 hour in the presence of 100 nM PMA. **(C)** Indirect immunofluorescence for endogenous rab11 in cells transiently transfected with GFP-cPKCα and treated with 100 nM PMA for 1 hour. **(D)** Indirect immunofluorescence for endogenous rab11 in cells treated with 100 nM PMA for 1 hour at either 4°C, 30°C, or 37°C. **(E)** Indirect immunofluorescence for endogenous rab11 in cells treated with either DMSO (0.01%) or 100 nM PMA for 1 hour. Bar represents 10.0 μm. **(F)** Immunoblot of subcellular fractions from cells treated with or without 100 nM PMA for 1 hour. Cytosolic ('cyto') and membrane ('mem') fractions were probed for endogenous cPKCα and rab11.

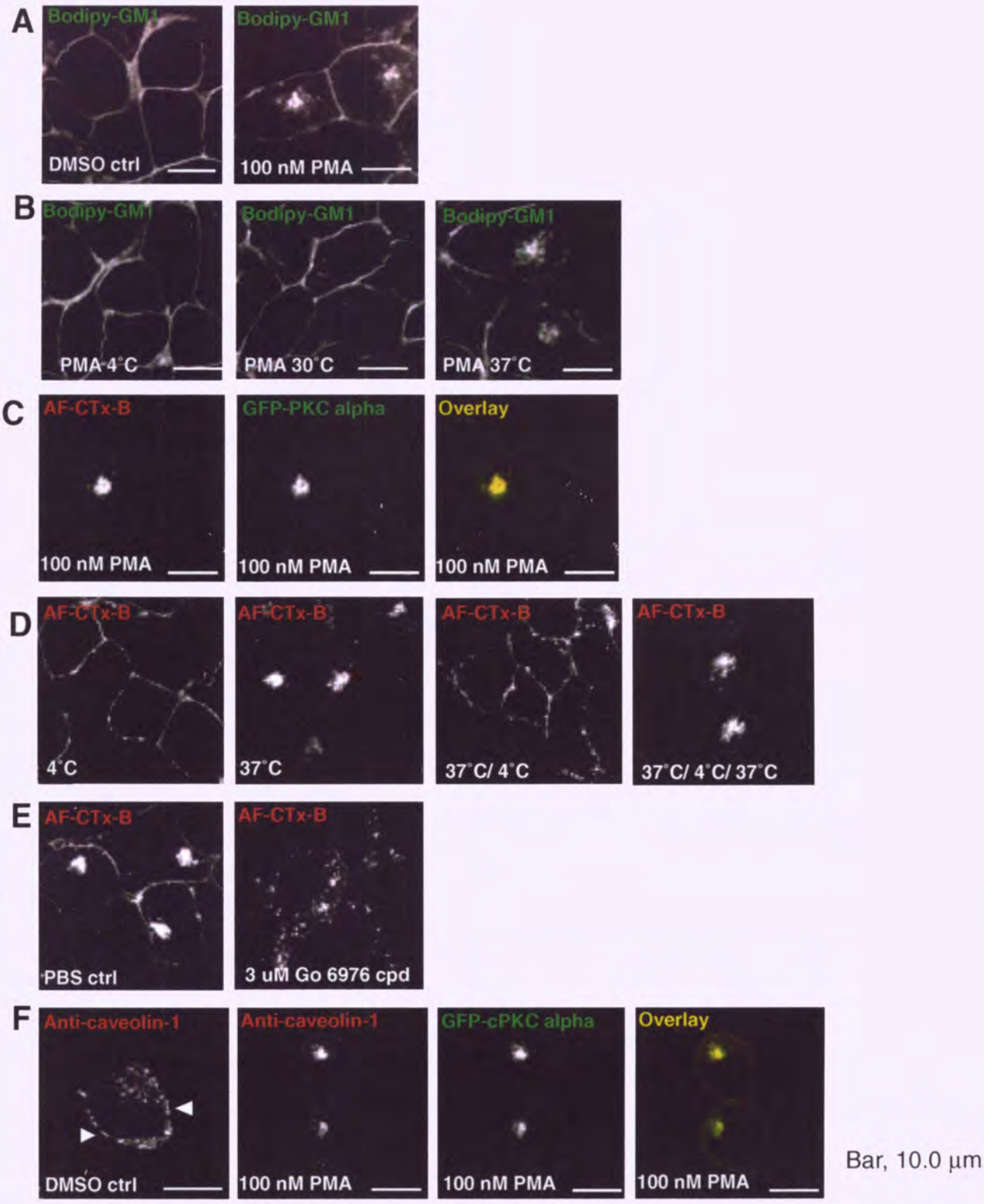


Figure 2.7 PKC translocates to a juxtanuclear compartment enriched in plasma membrane lipids and lipid raft components. **(A)** HEK 293 cells were treated with 5 µM bodipy-GM1 complexed to BSA for 30 mins at 4°C, washed with ice-cold PBS and incubated at 37°C for 1 hour in the presence of 0.01% DMSO or 100 nM PMA. **(B)** Cells were labeled with bodipy-GM1 as described above, washed with ice-cold PBS, and either incubated at 4° C, 30° C, or 37° C in the presence of 100 nM PMA for 1 hour. **(C)** Cells were transiently transfected with GFP-cPKCα and 12 post transfection, treated with 1 µg/ml of Alexa Fluor® (594)-cholera toxin-B for 1 hour at 37°C in the presence of 100 nM PMA. **(D)** Cells were treated with 1 µg/ml CTx-B for 1 hour at 4°C, 37°C, 37°C followed by 1 hour at 4°C, or 37°C followed by 1 hour at 4°C and finally 1 hour at 37°C. **(E)** Cells were treated with 1 µg/ml CTx-B for 1 hour to induce accumulation at juxtanuclear compartment and then incubated for an additional 1 hour in the presence of the cPKC selective kinase inhibitor, 3 µM Gö 6976. **(F)** Indirect immunofluorescence for endogenous caveolin-1 in cells transiently transfected with GFP-cPKCα and treated with 100 nM PMA for 1 hour. Bar represents 10.0 µm.

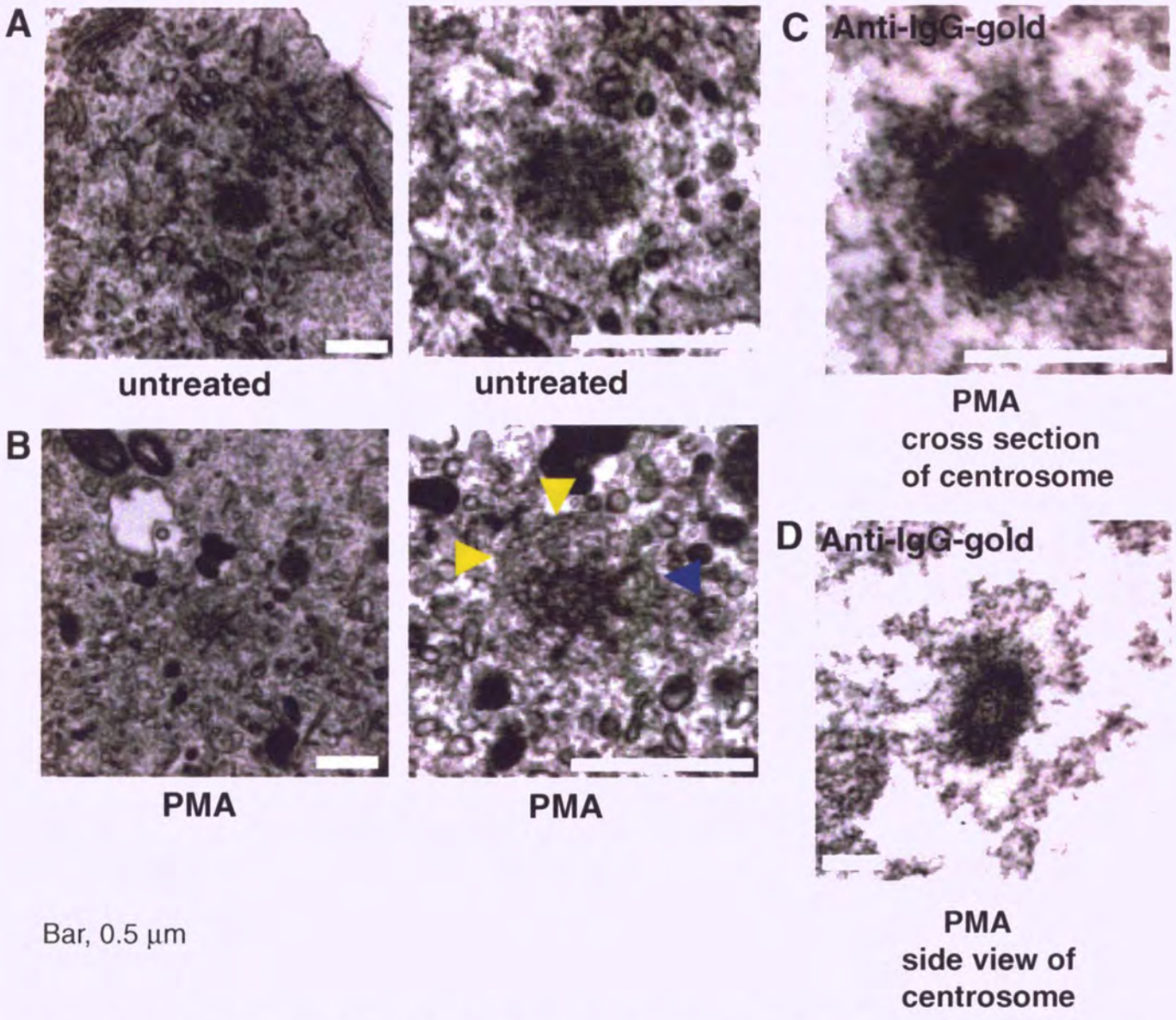


Figure 2.8 Translocation of cPKCα to the juxtanuclear compartment coincides with the formation of a pericentriolar membranous structure. Cells were treated with **(A)** DMSO (0.01%) or **(B)** 100 nM PMA for 1 hour. **(B)** Treatment of cells with PMA results in the reorganization of centrosome structure and the appearance of circumspect membranes (yellow arrowheads) associated with the appendages of centrioles, and membranous spokes found between appendages (denoted blue arrowhead). In these studies, potassium ferrocyanide was included along with the osmium tetroxide fixative to enhance appearance of membranes, and 37°C was maintained throughout preparation of samples. **(C and D)** Pre-embedded immunoelectron microscopy. Cells were treated with PMA for 1 hour and then probed for endogenous cPKCα with anti-cPKCα antibodies followed by incubation with anti-IgG-12 nm colloidal gold as described in the Material and Methods. Bar represents 0.5 µm.

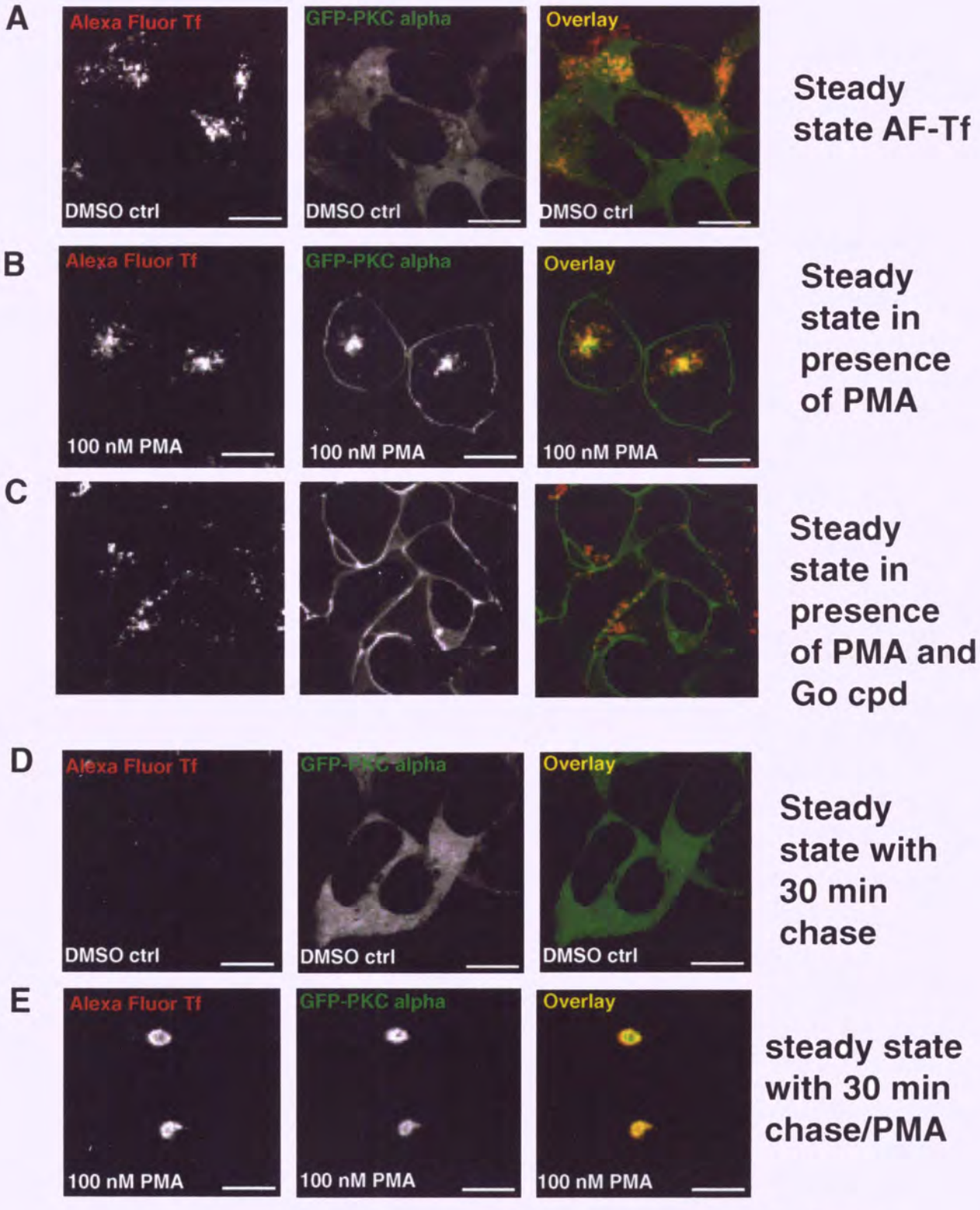


Figure 2.9 PKC regulates the sequestration of recycling components in a juxtanuclear compartment. Steady state labeling of HEK 293 cells with Alexa Fluor® (594)-conjugated transferrin (AF-Tf) in **(A)** absence or **(B)** presence of 100 nM PMA. **(C)** Cells were transiently transfected with GFP-cPKCα. Twenty four hours post transfection, total recycling endosomes were steady state labeled with 10 µg/ml AF-Tf for 1 hour in the presence of 3 µM Gö 6976. **(D)** 30 min chase with unlabeled transferrin following 1 hour AF-Tf steady-state labeling in the absence of PMA. **(E)** 30 min chase with unlabeled transferrin following 1 hour AF-Tf steady-state labeling in the presence of 100 nM PMA. Micrographs are representative images from 3 independent experiments. Bar represents 10.0 µm.

Chapter 3: Isoenzyme-Specific Translocation of PKC to the Pericentrion Involves Activation of Phospholipase D

3.1 Introduction

Members of the protein kinase C (PKC) superfamily of lipid-dependent, serine-threonine kinases function as integral signaling intermediates in the transmission of numerous extracellular signals. PKC currently consists of 11 closely-related isoenzymes that can be grouped into 3 subfamilies (classical, novel, atypical) on the basis of lipid cofactor requirements and structural homology (Mellor and Parker., 1998). A major form of cellular regulation of PKC involves the dynamic redistribution or 'translocation' of PKC isoenzymes from cytosol to the plasma membrane. Membrane translocation of PKC is regulated through receptor-coupled lipid hydrolases that act on phosphoinositides (PI) and phosphatidylcholine (PC) to generate diacylglycerol (DAG) lipid second messengers. The recruitment and activation of PKC at membranes links activation of the enzyme to the proximity of membrane protein substrates (Nishizuka, 1995).

One of the major targets of activated PKC is PC-specific phospholipase D (PC-PLD). Activated PC-PLD hydrolyzes the phosphodiester bond of PC to generate free choline and phosphatidic acid (PA) which is converted to DAG through the actions of lipid phosphate phosphatase (LPP). DAG derived from PC hydrolysis has been proposed to mediate the reciprocal regulation of PKC, but there are incomplete and conflicting data on this topic (Ha and Exton, 1993; Leach et al., 1991; Pettitt et al., 1997). The functional significance of PC-PLD-derived DAG is that it is present in the membrane for extended periods of time and in relatively high concentrations in comparison to DAG derived from PI-PLC, and this PC-derived DAG has been proposed to mediate long term cellular processes that require sustained PKC activation (Nishizuka.,1988; Billah and Anthes, 1990).

The implication of specific PKC isoenzymes in distinct pathological processes has led to an intense effort to identify and elucidate the mechanisms that regulate isoenzyme-specific functions of PKC. To date, these efforts have been complicated by the large number of highly homologous PKC family members, expression of multiple PKC isoenzymes in each cell type, and the finding that there are only minor differences in substrate selectivity amongst

different PKCs.

One the best opportunities to study the mechanisms of isoenzyme specificity arises from the alternative splice gene products of the classical PKC (cPKC) β gene. Alternative splicing of the cPKCβ gene creates two proteins, cPKCβI and βII, which display 100% identity over their first 621 amino acids but then diverge in the last 50-51 carboxy terminal residues (Ono et al., 1986a). Importantly, these divergences are conserved across rat, rabbit, and human suggesting that there are distinct functions encoded by both gene products. To that end, Blobe et al. identified residues in the carboxy terminus of cPKCβII that are homologous to actin binding proteins (Blobe et al., 1996b). They demonstrated that with long term stimulation with phorbol esters, cPKCβII differentially interacts with the actin cytoskeleton where it becomes activated, and is then protected from PMA-induced down-regulation.

In another line of investigation, we have recently reported that prolonged activation of PKC with DAG-mimicking phorbol- and non-phorbol PKC agonists can induce the formation of a specialized pericentriolar recycling compartment that serves as a novel target for the isoenzyme-specific translocation of cPKCα and βII isoforms, and which we have named the *pericentrion*. Analysis has revealed that cPKC translocation to the pericentrion was coincident with an enrichment of the compartment with some plasma membrane proteins and lipids, including caveolin-1 and ganglioside-GM1. Kinetic studies with a fluorescent-conjugated transferrin ligand as a marker of membrane trafficking demonstrated that the cPKC-dependent pericentrion functions to sequester membrane recycling components.

In this study, we investigated the isoenzyme specificity of this novel translocation and found that while cPKCα and βII translocated to the pericentrion, cPKCβI did not. Further analysis showed that cPKCβII translocated to the pericentrion in a time- and microtubule-dependent manner, first accumulating at the plasma membrane within 5 min of stimulation, and then at the pericentrion within 30-40 min. The specificity for cPKCβII versus βI and the requirement for kinase activity raised the possibility that PC-PLD may be involved in the process, and further experiments did indeed implicate PLD in the translocation of cPKCβII

to the pericentrion. These results define a novel pathway of translocation of cPKCβII requiring differential activation of PLC. These data suggest that cPKCβII differentially activates PLD and that this is required for the mechanism of translocation to the pericentrion. The implications of these results are discussed.

3.2 Materials and Methods

Materials

Eagle's minimal essential media, Dulbecco's modified Eagle's medium, and HEPES were from Life Technologies, Inc (Gaithersburg, MD). HeLa cells were kindly provided by Dr. Dennis Watson (Medical University of South Carolina) and the HEK 293 cell line was purchased from American Tissue Culture Collection (Manassa, VA). [^{3}H] palmitic acid was from New England Nuclear Life Sciences Products (Boston, MA). 4β-phorbol-12-myristate-13-acetate (PMA) was purchased from Calbiochem (La Jolla, CA.). Mouse monoclonal anti-HA antibody was from Covance (Oakland, CA). Phospholipid standards were from Avanti Polar Lipids, Inc (Alabaster, AL). Anti-mouse TRITC secondary antibodies were from Molecular Probes, Inc (Eugene, OR.). Whatman silica gel 60 TLC plates were from Fisher Scientific (Pittsburgh, PA). All other chemicals were from Sigma (St. Louis, MO).

Cell Culture

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and HEK 293 cells were maintained in Eagle's Minimal Essential Media (MEM) supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 incubator at 37°C. Cells were passaged every 3-4 days to maintain cells in logarithmic growth.

Plasmid construction

All recombinant DNA procedures were carried out following standard protocols. The wild type pBK-CMV-GFP-cPKC-βII and kinase-defective pBK-CMV-GFP-K371R-βII cPKC constructs were previously described (Feng et al., 1998). PDGF-β receptor was amplified from a human fetal kidney cDNA library with PCR primers containing a 5' ECO RI and

3'XbaI restriction sites. Amplified PDGF-β-R was ligated into pcDNA3.1-FLAG. HA-tagged KR-hPLD1 was kindly supplied by Dr. Michael Frohman (SUNY-Stony Brook). All DNA sequencing was performed at the MUSC DNA Sequencing Facility.

Indirect Immunofluorescence

HEK 293 cells were plated onto MatTek (Ashland, MA) confocal dishes at a density of 2.5-5.0 x 10^{-5} cells/dish and 24-48 hours later were co-transfected with either HA-tagged KR-PLD1 or GFP-cPKC βII or HA-tagged KR-PLD1 and GFP-cPKC βII using Lipofectamine® 2000 according to manufacturer's recommendations. Twelve hours post transfection, cells were either treated with DMSO (0.01%) or 100 nM PMA for 1 hour and fixed with 3.7% paraformaldehdye/10% methanol for 10 minutes at room temperature. Following fixation, cells were permeabilized at –20°C with 100% methanol for 5 min. Cells were then washed with 1.5%FBS/PBS 3 times for 5 min each and then blocked with 2.5%FBS/PBS for 1 hour. Primary antibodies were incubated at a dilution of 1:50 or 1:100 in 1.5% FBS/PBS with 0.15% saponin for 2 hours at room temperature or overnight at 4°C. Following incubation with primary antibody, cells were washed 3 times for 5 min each with 1.5% FBS/PBS. TRITC-conjugated secondary antibody (Molecular Probes) was incubated at a dilution of 1:50 or 1:100 in 1.5% FBS/PBS and 0.15% saponin for 1 hour. Cells were washed a final 3 times for 5 min each with 1.5% FBS/PBS and visualized immediately with confocal microscopy.

Confocal Microscopy

Preparation of samples for immunofluorescence is described above. Cells expressing green fluorescent (GFP)-fusion proteins alone were viewed live in 10 mM HEPES-buffered media or viewed immediately following 10 min fixation with 3.7% paraformaldehyde/10% methanol pre-warmed to 37°C. All confocal images were taken with an Olympus UltraView Spinning Disk Confocal IX-70 system with an Olympus 60X 1.4 NA lens and Krypton/

Argon laser line. Each microscopic image is representative of 20 fields over a minimum of three experiments and all images are taken at the equatorial plane of the cell. GFP fusion proteins were excited at 488 nm and TRITC was excited at 543. All fluorescent images were captured sequentially and combined within the UltraView software (Perkin-Elmer). Raw data images were cropped in Adobe Photoshop® 5.0 for publication.

Transphosphatidylation assay

The cellular activity of PLD was assessed with a transphosphatidylation assay modified from Yeo et al (Yeo and Exton, 1995). Twelve hours post transfection, HEK 293 cells were labeled overnight (12-16 hours) in 35 mm dishes with 3.0 μCi/ml [^{3}H]-palmitic acid in MEM supplemented with 10% FBS and 0.1% delipidated bovine serum albumin (BSA). Cells were then washed 3 times with PBS and serum-free MEM/0.1% BSA media was added. Cells were allowed to recover in serum free media at 37°C for 30 mins prior to stimulation. Ten minutes prior to stimulation, 0.4% 1-butanol was added to the cells. Cells were stimulated with 100 nM PMA for the specified times and at the noted concentrations. All incubations were performed at 37°C. Following stimulation, the culture media were removed, and the cells were washed rapidly 3 times with 1 ml ice-cold PBS. Total cellular membranes were extracted via the method of Bligh and Dyer (Bligh and Dyer, 1959). Lipids were dried down and resuspended in 75 μl chloroform:methanol (2:1). 50 μl of the 75 μl volume was loaded per lane. The thin layer chromatography (TLC) solvent system consisted of ethyl acetate:iso-octane:acetic acid (9:5:2). A phosphatidylbutanol (Ptd-But) standard (Avanti) was included in parallel to confirm lipid species. The plate was sprayed with EN^{3}HANCE Spray Perkin-Elmer (Boston, MA.) to amplify the tritium signal and exposed for autoradiography for 24 hours. The Ptd-But band and the total remaining lipids were scraped and separately counted. The [^{3}H]-Ptd-But band was compared to total labeled lipids to generate percent increase over total labeled lipid. Each experiment was repeated 3-5 times.

3.3 Results

3.3.1 Isoenzyme-specific translocation of cPKCβII to the pericentrion

To investigate the isoenzyme specificity of cPKCβ translocation to the pericentrion, cells were transiently transfected with a GFP-tagged-cPKC βI or GFP-cPKC βII and stimulated with PMA. Indirect immunofluorescence and confocal microscopic imaging revealed that in the absence of phorbol ester stimulation both GFP-tagged cPKC isoenzymes displayed a diffuse cytoplasmic localization with exclusion from the nucleus (Fig. 3.1A and B). Addition of 100 nM PMA for 1 hour induced translocation of GFP-cPKC βII to both the plasma membrane and to a juxtanuclear location (Fig. 3.1A, panel 2) that colocalized with the GM1binding, Alexa Fluor-cholera toxin. (AF-CTx-B) (data not shown). In previous studies, we demonstrated that translocation of cPKCα and βII coincided with an enrichment of the pericentrion in components of lipid rafts including caveolin-1 and the ganglioside GM1, and that the GM1-binding AF-CTx-B could function as a marker of this compartment. In contrast, stimulation of GFP-cPKCβI with 1 hour PMA resulted in the translocation of βI to the plasma membrane only as no translocation to the pericentrion was evident (Fig. 3.1B, panel 2). These results suggest that amino acid residues encoded within the carboxy terminus of cPKCβI and βII determine the specificity for translocation of cPKC βII to the pericentrion.

In order to control for the possibility that there may be different rates of translocation for the two cPKC isoenzymes, a time course experiment was performed. Translocation of both the cPKCβI and βII isoenzymes was evident at the plasma membrane within 5 min of PMA stimulation, suggesting that translocation to the plasma membrane occurs independent of carboxy terminus differences (Fig. 3.1C, panel 1). Beginning at 30-40 min of PMA stimulation, cPKCβII appeared at the pericentrion and displayed maximum translocation by 60 min (Fig. 3.1C, panels 3 and 4). On the other hand, the cPKCβI enzyme remained at the plasma membrane at all time points examined out to 3 hours (data not shown). These results confirmed that the differences in translocation observed for cPKCβI and βII were

not due to different rates of translocation. Of note, during the time course of cPKCβII translocation, filamentous structures appeared in a time dependent manner. Preincubation with nocodazole, a microtubule depolymerizing agent (Cole and Lippincott-Schwartz, 1995) had no effect on the translocation of cPKC βII to the plasma membrane but nearly totally blocked translocation to the pericentrion (Fig. 3.1C, panel 5). Thus, cPKCβII translocation to the pericentrion occurs in a time-dependent manner and appears to require microtubules.

3.3.2 Kinase activity is required for translocation of cPKC βII to the pericentrion.

To investigate the role of kinase activity in the translocation of cPKC βII to the pericentrion, cPKC βII was stimulated in a prolonged manner with PMA, with or without a 30 minute pre-incubation with 3 μM Gö 6976, a specific inhibitor of the calcium-dependent PKC isoenzymes (Martiny-Baron et al., 1993). As seen in Figure 3.2A, when the kinase activity of cPKCβII was inhibited with Gö 6976, cPKCβII translocated to the plasma membrane but there was no translocation to the pericentrion. This requirement for kinase activity was further probed using a kinase-defective GFP-tagged cPKC βII mutant K371R which is unable to bind ATP and phosphorylate cPKC substrates. When stimulated with PMA, GFP-K371R-βII was observed to translocate to the plasma membrane, but no pericentrion accumulation was evident (Fig. 3.2B). These data suggest that cPKC βII translocation to the plasma membrane occurs independent of kinase activity but that kinase activity is required for translocation to the pericentrion.

3.3.3 PC-PLD is activated in a time- and PKC-dependent manner during the prolonged stimulation of PKC with phorbol esters

PC-PLD1 is a major downstream target for calcium dependent, DAG/PMA-sensitive cPKC isoenzymes. Currently there are some data regarding isoenzymes-specific differences in the cPKC regulation of PLD, including differences between the two PKCβI and cPKCβII. In addition, very recently Exton and colleagues reported that the full length rat cPKCα

isoenzyme binds and activates PC-PLD1 dependent upon the presence of a phenylalanine reside (phe 663) in the V5 region of cPKCα (Hu and Exton, 2003). These data implicate a possible role for the V5 variable region in the PKC-PLD interaction. These observations, coupled with the requirement for kinase activity for cPKCβII translocation to the pericentrion, suggested the hypothesis that the mechanism of this translocation may involve the activation of PLD1. To begin to explore this possibility, it was important to first establish whether PLD was activated in this system. Using a transphosphatidylation assay with butanol to evaluate cellular PLD activity, HEK 293 cells were treated with 100 nM PMA. As seen in Figure 3.3A, Ptd-butanol accumulation, corresponding with PLD activity, was evident starting at 30-40 mins of PMA stimulation and this could be completely blocked with preincubation with 3 μM Gö 6976 compound. Interestingly, this time frame of PLD activation correlated closely with the translocation of cPKCβII to the pericentrion.

To determine if cPKCβI and βII regulated PLD differentially, cells were transiently transfected with each PKC isoenzyme and the transphosphatidylation reaction was repeated. The efficiency of transfection was similar for both isoenzymes and ranged between 40-50% of total cell population. Interestingly, in cells that were transfected with cPKCβII there was a higher basal level of PLD activity in the absence of phorbol stimulation when compared to cPKCβI. Upon stimulation with PMA, cells that expressed cPKCβII demonstrated a 50% increase in Ptd-butanol accumulation above cells transfected with cPKC βI (Fig. 3.3B). These data suggest the possibility that the isoenzyme specificity in translocation of cPKC β isoenzymes to the pericentrion may be due to the differential activation of PLD.

3.3.4 Translocation of cPKCβII to the pericentrion is dependent upon PLD activity

To investigate whether PLD activity is required for translocation of PKC to the pericentrion, 1-butanol was utilized as an inhibitor of PLD activity in these cells. HEK 293 cells were preincubated with 0.4% 1-butanol for 10 min prior to long term PMA stimulation. Pre-incubation with 1-butanol had no effect on the translocation of cPKCβII to the plasma

membrane, but there was a complete inhibition in the translocation of cPKC to the pericentrion (Fig. 3.4A, panels 1-2). This inhibition was specific for the primary alcohol since the secondary alcohol 2-butanol did not block translocation (data not shown). To confirm this pharmacological inhibition, a catalytically deficient mutant PLD, KR-PLD1, was cotransfected along with GFP-cPKCβII into HEK 293 cells and trafficking of cPKCβII to the pericentrion was evaluated. When GFP-cPKCβII was stimulated in a prolonged manner with PMA, the co-expression of KR-PLD1 had no effect on the translocation cPKCβII to the plasma membrane but there was a total block of translocation to the pericentrion (Fig. 3.4A, panel 3). The ability of the KR-PLD1 mutant to inhibit PLD was evaluated in transphosphatidylation assays that was performed in parallel with the confocal studies. Transient transfection of KR-PLD1 reduced endogenous PLD activity by approximately 50% (Fig. 3.4B), thus. confirming that the KR-PLD1 inhibited cellular PLD activity. These data demonstrate that PLD activity is involved in the translocation of cPKC to the pericentrion.

3.3.5 Physiological elevation of DAG derived from the activation of the PDGF-β receptor induces the translocation of cPKCβII to the pericentrion

Stimulation of some cells with angiotensin, bombesin, PDGF, or vasopressin results in a biphasic elevation of DAG with an initial peak at 1-2 minutes generated by hydrolysis of PI lipids and a second more sustained peak that derives from the hydrolysis of PC by PC-PLD. Since cPKC translocation to the pericentrion requires a long term exposure to phorbol esters, it was hypothesized that an agonist that activates PC-PLD and generates a sustained DAG may be sufficient to induce translocation of PKC to the pericentrion. GFP-cPKCβII was transiently transfected into HEK 293 cells that stably expressed a FLAG-tagged PDGF-β receptor in HEK 293. In the absence of expressed receptor, cPKCβII was observed in a diffuse cytoplasmic location with no increase at the juxtanuclear location (Fig. 3.5A, panel 1). In contrast, cells expressing the receptor and cultured in the presence of serum displayed an increase in cPKC concentration at the plasma membrane but more significantly at the

juxtanuclear location (Fig. 3.5A, panel 2). The juxtanuclear accumulation of cPKCβII was observed in approximately 30% of transfected cells cultured in the presence of serum (Fig. 3.5B). Further, cPKCβII accumulation at the juxtanuclear location was not observed in cells that were cultured in serum-free media and this could be restored with the addition of 50 ng/ml PDGF BB (Fig. 3.5A, panels 3 and 4). This translocation of cPKCβII to the plasma membrane and juxtanuclear location correlated with the serum- and PDGF-BB agonist-induced accumulation of Ptd-butanol (Fig. 3.5C). To investigate the role of the PC-PLD-derived DAG, cells were incubated with propranolol, an inhibitor of lipid phosphate phosphatase. Juxtanuclear localization induced with PDGF and serum was lost in the presence of propranolol suggesting that it was the DAG from PLD that was important (data not shown). These data suggest that under conditions where PC-PLD is activated and DAG is elevated for prolonged periods of time, cPKCβII translocates to the pericentrion.

3.4 Discussion

In the preset study, we have identified a role for the carboxy terminal V5 variable region in the differential subcellular localization of cPKCβ isoenzymes to the pericentrion following sustained activation with DAG-inducing PDGF and DAG-mimicking phorbol esters. Investigation into the mechanism, revealed that cPKCβII translocation occurred in a time- and activity-dependent manner, and was due to the differential activation of PLD. These data define an isoenzyme-specific function for cPKC in the regulation of PLD, and in turn, the reciprocal regulation of PKC through the modulation of PKC subcellular localization. Additionally, we also provided evidence for the translocation of cPKCβII that was stimulated through a sustained elevation of DAG generated by PC-PLD coupled to the PDGF-β receptor. These data suggest that cPKC translocates to this novel compartment under physiological conditions.

Isoenzyme-specific differences in the subcellular localization and function of cPKCβI and βII have been previously reported. Using indirect immunofluorescence and monoclonal antibodies, several groups have observed an isoenzyme-specific association of cPKCβII with the actin cytoskeleton (Disatnik et al., 1994; Goodnight et al., 1995). To that end, Blobe and co-workers identified an actin-binding sequence (ABS-1) located within the V5 region of cPKCβII that was absent from cPKCβI (Blobe et al., 1996a). This sequence was found to be necessary for the phorbol ester-induced translocation of cPKCβII to the actin cytoskeleton and isoenzyme-specific functions associated with this translocation including protection from down regulation. Further, Yamamoto et al. identified specific and opposing functional roles for the cPKCβ isoenzymes in vascular smooth muscle cells (Yamamoto et al., 1998). They reported that upon overexpression of cPKCβI there was a stimulation of cell proliferation whereas cPKCβII overexpression was inhibitory to cell proliferation. In addition to these studies, there are also a number of other studies that have proposed specific subcellular localization and/or functions for either one or the other isoenzyme but in most cases there was no direct comparison between βI and βII and it is not known if the proposed

function was isoenzyme-specific (Hocevar and Fields, 1991).

PC-PLD is a major downstream target for PKC. Data from overexpression studies and in vitro experiments with purified or recombinant protein indicate that it is the calcium-dependent cPKC isoenzymes (α and β) that are the primary PKC regulators of PLD in the cell. Additionally, there are some data to suggest possible isoenzyme-specific differences between cPKCβI and βII. For instance, a role for cPKCβI has been suggested through the examination of PLD activity in cells that stably overexpressed cPKCβI and which displayed an enhanced formation of DAG in response to phorbol ester treatment (Pachter et al., 1992; Pai et al., 1991). These results were further supported with studies that used purified PKC and a cell-free system to demonstrate a rank order of potency for PLD activation with cPKC βI>α>γ and βII showing little or no activity (Lopez et al., 1995). Interestingly, these studies are in conflict with the current study as well as a number of *in vitro* studies that have seen little to no differences in the specificity of βI and βII activation of PLD. However, a very recent report by Exton and colleagues who identified residues in carboxy terminus of cPKCα as critical to the activation of PLD by PKC underscores the importance of the variable region in PKC-PLD relationship.

Of note, while researching the literature for this manuscript it was discovered that there is an inconsistency in the nomenclature that has been applied to the cPKCβ isoenzymes very early on. Whereas Nishizuka and co-workers (Ono et al., 1987) proposed to name the 673 amino acid splice product, cPKCβII, and the 670 amino acid protein βI, Coussens et al. (Coussens et al., 1987) used the opposite terminology. Currently, the accepted nomenclature is that the longer gene product is cPKCβII and the shorter one, cPKCβI.

The major findings of this study have important implications for cell signaling through PDGF and other agonists that couple to PC hydrolysis and induce a sustained elevation of DAG. Currently, there are conflicting data regarding the function of the sustained DAG generated by PDGF stimulation and whether it can activate PKC in a manner analogous to DAG derived from PI lipid hydrolysis (Ha and Exton, 1993; Pettitt et al., 1997). To date,

most cell studies that have investigated this issue have failed to detect a translocation event (i.e. activation) of PKC to a particulate/membrane fraction in response to agonist stimulation and sustained DAG. In contrast, Ha et al have reported a persistent translocation of nPKC epsilon to a membrane fraction in cells stimulated with PDGF (Ha and Exton, 1993). The data reported in the present study have demonstrated that stimulation of the PDGF receptor and activation of PC-PLD can lead to an alteration in the subcellular localization of PKC isoenzymes which is mimicked with phorbol esters.

In addition, these results impact on the long term use (30-60 min) of phorbol esters to probe the cellular functions of PKC. The time course of translocation in this study suggests that during phorbol ester stimulation there are isoenzyme-specific differences in subcellular localization of cPKCβI and βII, depending upon length of exposure to PMA. Whereas at short time exposure such as 5 min both isoenzymes will be localized to the plasma membrane, starting at 30 min and longer βII will be found specifically at the pericentrion whereas βI is localized to the plasma membrane. This differential subcellular localization during phorbol ester stimulation may lead to disparate isoenzyme effects such as differential down-regulation or differential access to compartmentalized substrates.

Finally, these studies serve to reinforce the continuing application of GFP technology and confocal imaging to the study of the PKC. The use of these technologies in the present study have allowed the discovery of isoenzyme-specific differences in cPKC subcellular localization that other methods (e.g. subcellular fractionation) may not have had the resolution to detect. Moreover, these results further illustrate the emerging significance of PKC translocation and function at alternate subcellular locations e.g. nucleus and pericentrion.

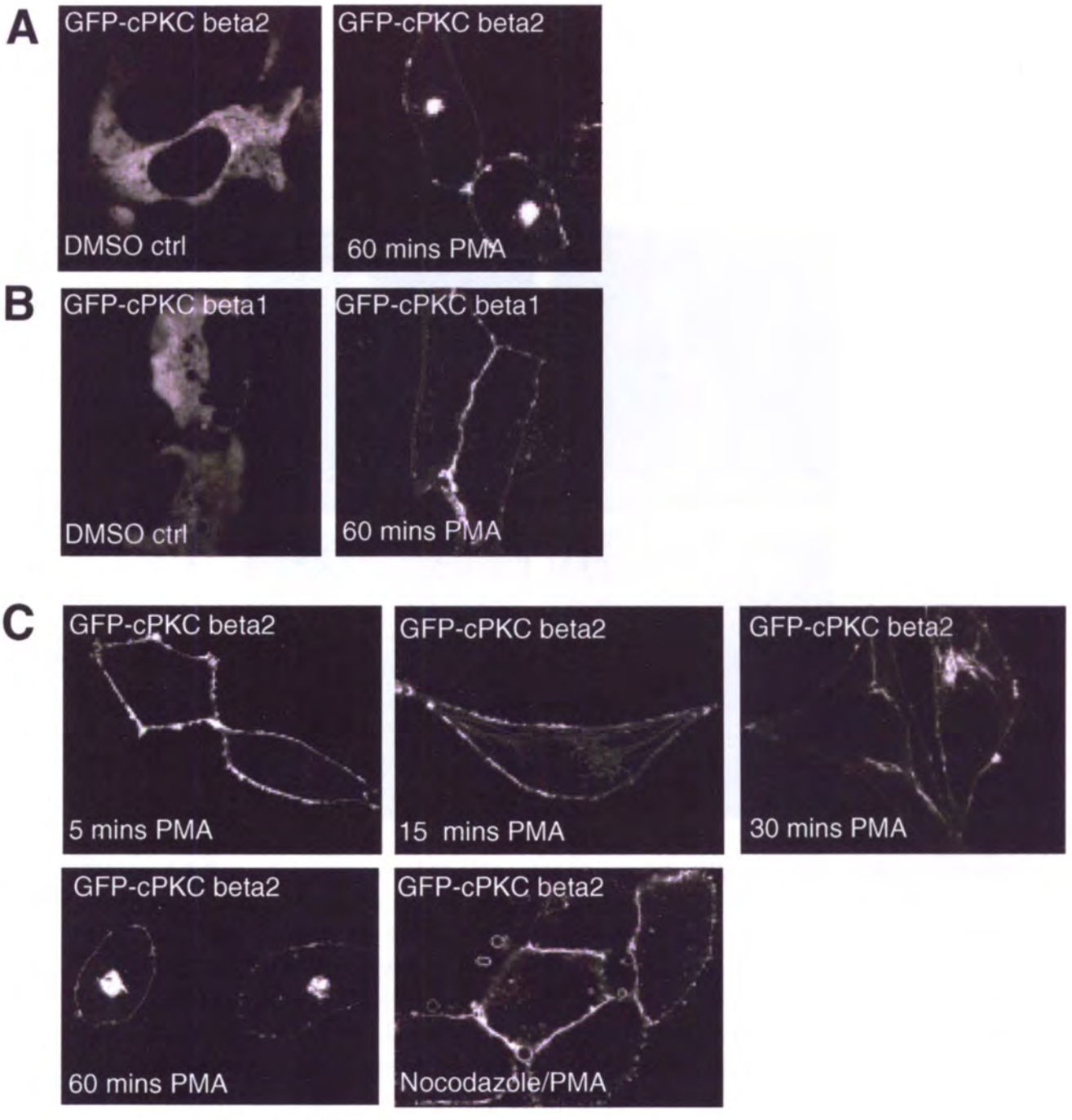


Figure 3.1 Isoenzyme-specific translocation of GFP-cPKCβII to the pericentrion occurs in a time-dependent manner and is sensitive to microtubule disrupting agents. A, shown are representative confocal images of cells transiently transfected with GFP-cPKCβII and treated 12 hours post transfection with either 0.01% DMSO or 100 nM PMA for 1 hour. **B,** confocal images of cells transiently transfected with GFP-cPKCβI and treated with either 0.01% DMSO or 100 nM PMA for 1 hour. **C,** time course of cells transiently transfected with GFP-cPKCβII stimulation with PMA for the indicated time periods. Panel 5 was preincubated with 5 μM nocodazole followed by 100 nM PMA. Each panel is representative of at least 20 60X fields and each micrograph is a single image captured at the equatorial plane of the nucleus.

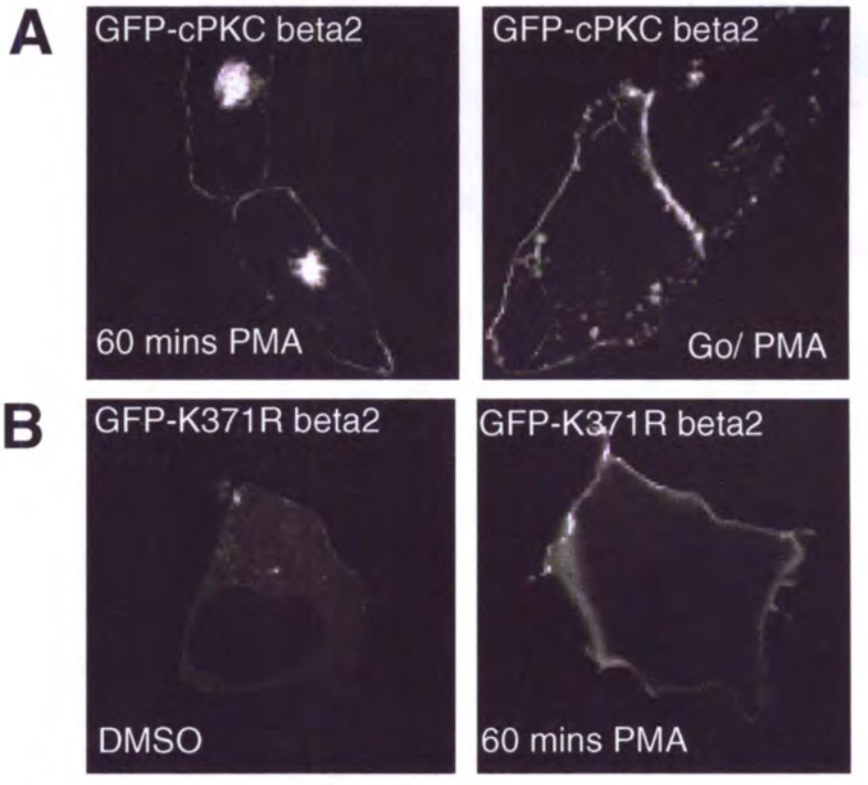


Figure 3.2 Translocation of GFP-cPKCβII requires kinase activity. A, representative confocal images of cells transiently transfected with GFP-cPKCβII and treated 12 hours post transfection with either 1 hour 100 nM PMA or 30 min preincubation with 3 μM Gö 6976 followed by 1 hour 100 nM PMA. **B,** confocal images of cells transiently transfected with GFP-K371R-cPKCβII and treated 12 hours post transfection with either 0.01% DMSO control vehicle or 1 hour PMA. Each panel is representative of at least 20 60X fields and each micrograph is a single image captured at the equatorial plane of the nucleus.

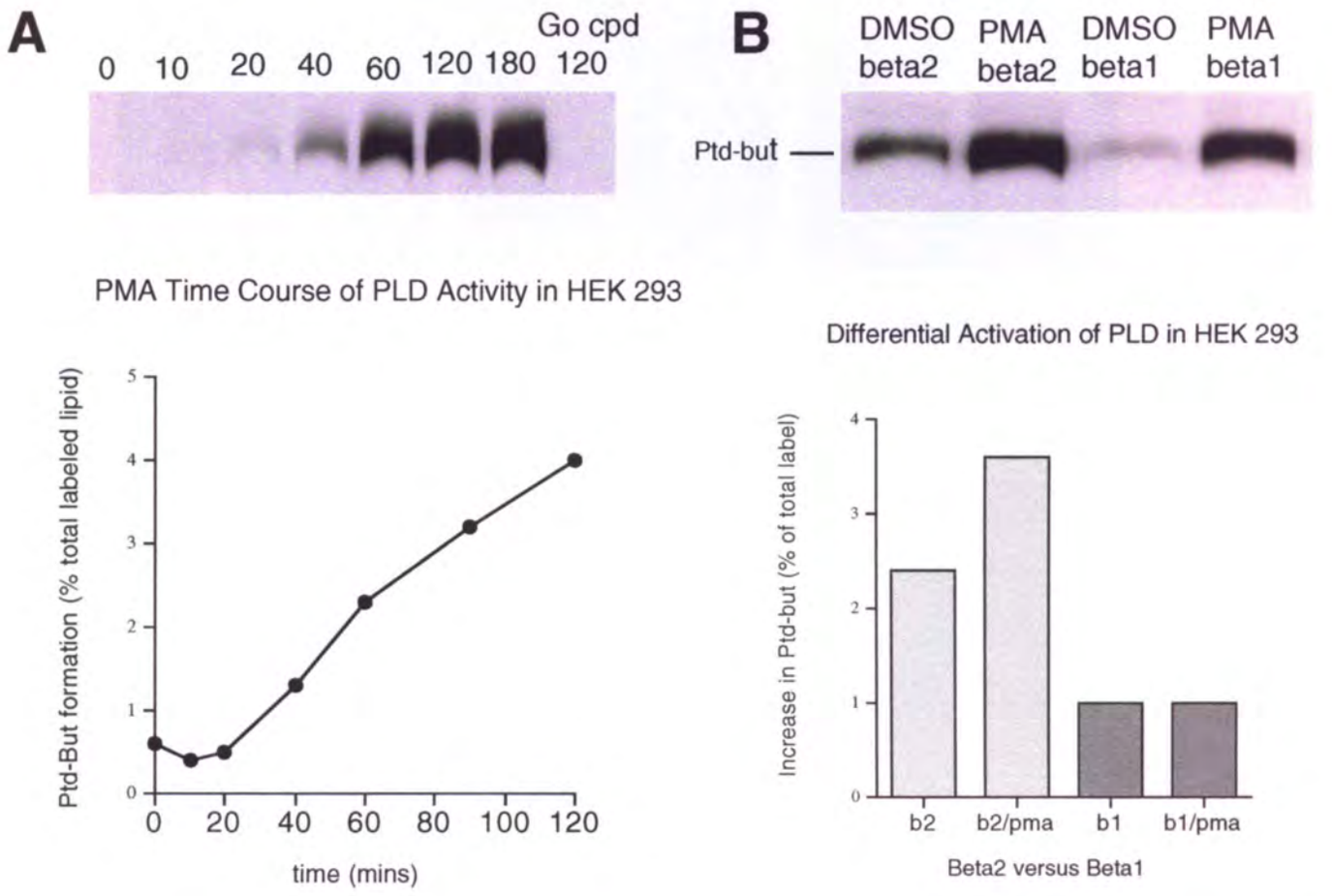
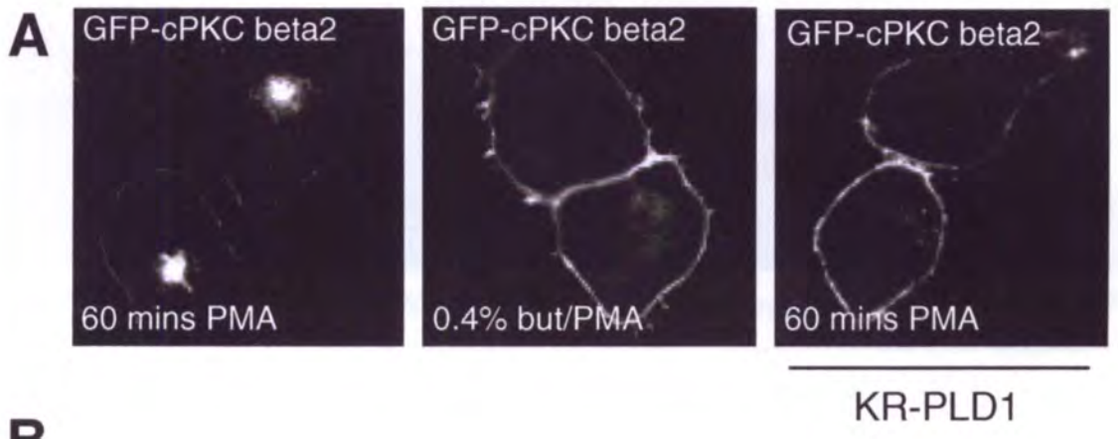


Figure 3.3 Time course of PLD activation and differential activation by cPKCβII versus cPKCβI. **A,** cells were steady state labeled overnight and subjected to a transphosphatidylation assay with 100 nM PMA for the indicated time periods. Represented in the last lane of the autoradiography is the effect 3 μM Gö 6976 on the PMA induced accumulation of Ptd-butanol. **B,** Representative autoradiography and quantitation of [^{3}-H]-Ptd-butanol formation. Cells were transiently transfected with either cPKCβII or cPKCβI and steady state labeled with [3-H] palmitate overnight. Cells were washed and treated with either 0.01% DMSO control vehicle or 100 nM PMA for one hour. Lipids were extracted as described in 'Material and Methods' and then separated by thin layer chromatography and put for autoradiography.



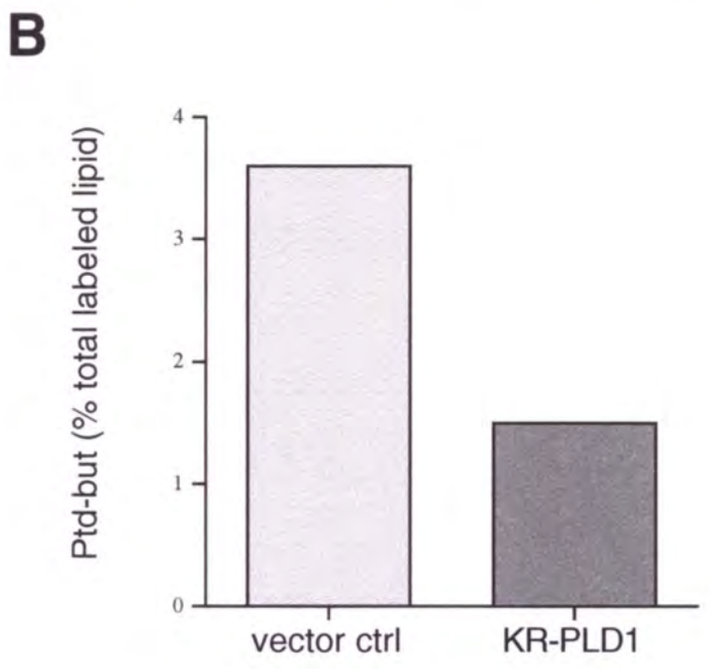


Figure 3.4 Translocation of GFP-cPKC beta2 requires PLD activity. A, representative confocal images of cells transiently transfected with either GFP-cPKCbeta2 **(panels 1 and 2)** or KR-PLD1 and GFP-cPKC beta2 **(panel 3)**. Each panel is representative of at least 20 60X fields, and each micrograph is a single image captured at the equatorial plane of the nucleus. **B,** the effects of KR-PLD1 on endogenous PLD activity. Cells were transiently transfected with either pGCN-vector or KR-PLD1 and 12 hours post transfection subjected to steady state labeled with 3 uCi [3-H] palmitate overnight. Cells were washed and then treated with 100 nM PMA for 1 hour. Ptd-butanol formation was quantitated by scrapping and scintillation counting. This experiment is representative of 3 experiments.

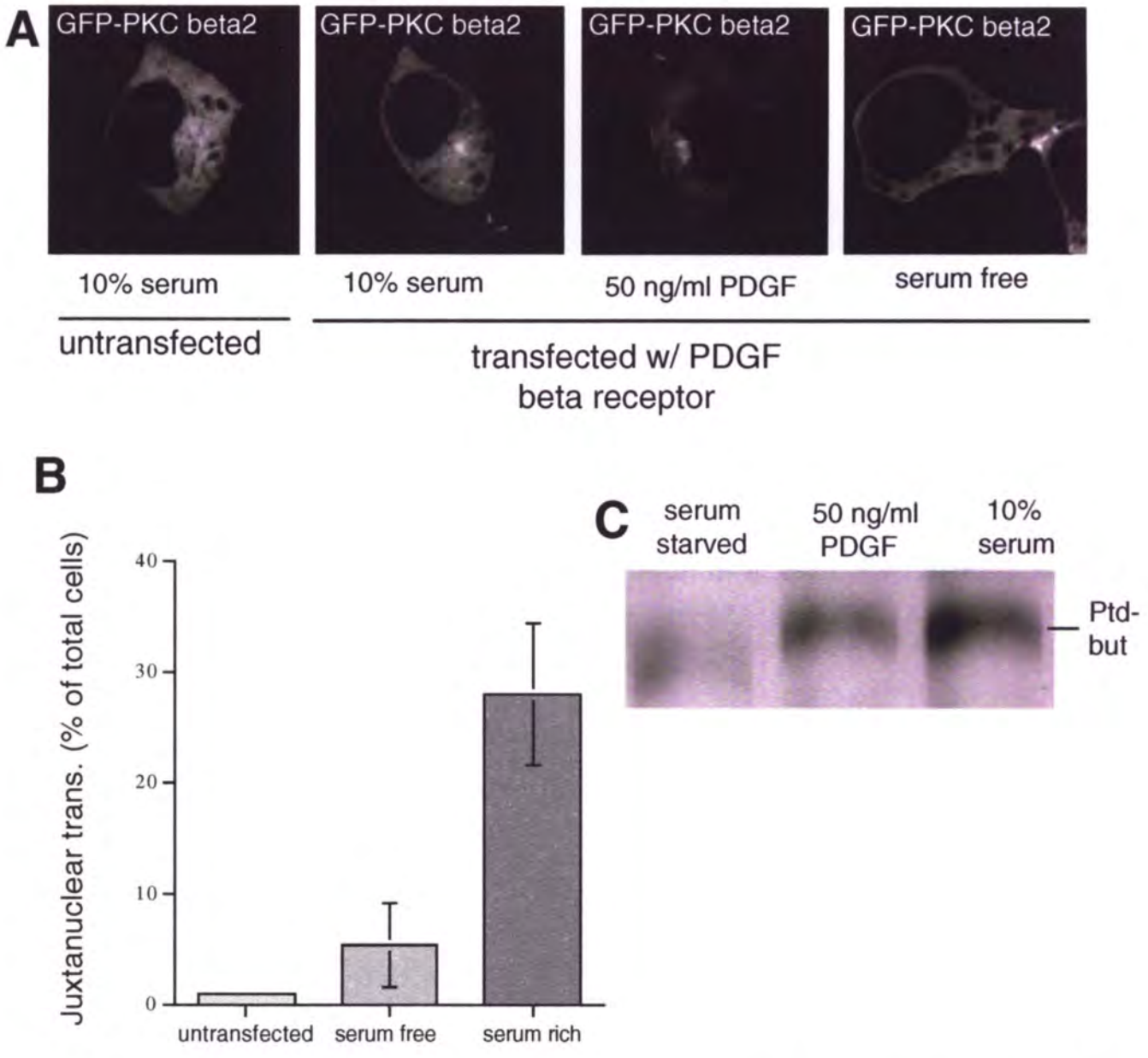


Figure 3.5 Effects of growth factor-induced activation of PLD on cPKC beta2 subcellular localization. **A,** representative confocal images of cells expressing GFP-cPKCbeta2 alone (panel 1) or stably expressing FLAG-PDGF-beta receptor with transiently transfected GFP-cPKCbeta2 (panels 2-4). Cells in panels 1 and 2 are cultured in the presence of 10% serum, panel 3 is serum starved for 3 hours and then stimulated with 50 ng/ml PDGF-BB for 1 hour, and panel 4 was cultured in the absence of serum for 3 hours. Each panel is representative of at least 20 60X fields and each micrograph is a single image captured at the equatorial plane of the nucleus. **B,** quantitation of the number of cells demonstrating juxtanuclear translocation as a percentage of total transfected cells. 20 60X high power fields were examined over 3 separate experiments. **C,** autoradiography of [3-H] Ptd-butanol formation in cells shown in **A**. Cells were transfected and maintained in media as described above and then subjected to steady state labeling with palmitate for overnight. Cells were washed and total lipids extracted as described in 'Materials and Methods' and separate with TLC.

Chapter 4: Hyperglycemia-Induced Sequestration of GLUT4 Through a cPKC-Dependent Mechanism

4.1 Introduction

Members of the protein kinase C (PKC) superfamily mediate a multitude of signaling events initiated by the formation of the lipid second messenger, *sn*-1,2-diacylglycerol (DAG). During acute signal transduction, agonist binding to a cell surface receptor leads to the phospholipase C-mediated hydrolysis of phosphoinositide (PI) with a subsequent elevation in DAG/calcium, and the recruitment and activation of PKC at the membrane. In this capacity, DAG serves as a link between PKC activity and compartment-restricted substrates. Acute activation of cPKC at the membrane is ensured through the rapid metabolic clearance of the DAG signal which leads to the removal of the activating signal and a reversal of membrane translocation within 1-2 min (Nishizuka and Nakamura, 1995).

In contrast to this acute signaling model, DAG can also be generated in a more prolonged manner, and this persistent DAG has been proposed to mediate several long term PKC functions including cell proliferation and differentiation (Nishizuka, 1986; Nishizuka, 1995). The primary physiological source of sustained DAG is derived from the hydrolysis of phosphatidylcholine (PC) by PC-specific phospholipase D (PC-PLD). Additionally, DAG and PKC activity can be elevated in the cell through a variety of pathological mechanisms and sustained PKC activity has been implicated in a number of diseases states (Fleischman et al., 1986; Koya and King, 1998; Milano et al., 1994; Preiss et al., 1986; Wolfman and Macara, 1987). In most cases the subcellular target and function of PKC during long term activation is unknown.

We recently reported that the sustained activation of PKC with PC-derived DAG and DAG-mimicking phorbol esters can lead to the formation of a lipid raft-enriched juxtanuclear compartment that serves as a novel target for cPKC translocation. We further demonstrated that PKC translocation to this compartment was coincident with the dysregulation of membrane recycling and this was found to be due to the cPKC-dependent sequestration of recycling components within the juxtanuclear compartment. These data serve to define a novel site of translocation and function for cPKCs during sustained activity.

Glucose homeostasis is maintained through a coordination of a specialized network of organs/tissues and endocrine hormones that function to preserve a careful balance between glucose utilization and glucose production. A primary metabolic defect associated with type II diabetes is the inability of the peripheral tissues such as muscle and adipose tissue to respond to insulin (insulin resistance) and reduce blood glucose levels (Sheetz and King, 2002). Under normal circumstances, binding of insulin to a cell surface insulin receptor triggers an intracellular response that induces a redistribution of the major insulin-regulatable glucose transporter, GLUT4, from a juxtanuclear location to the plasma membrane where it can then facilitate the transport of glucose across the membrane bilayer. It has been proposed that a lesion anywhere along this pathway, including a reduction or block in translocation of GLUT4 may lead to insulin resistance (Pessin and Saltiel, 2000).

Chronic, uncontrolled hyperglycemia has been linked to the microvascular pathological complications associated with type I and type II diabetes (Sheetz and King, 2002). Presently, significant evidence has emerged to suggest that chronic hyperglycemia mediates at least some of its effects through a persistent elevation of DAG and the activation of DAG-sensitive PKC isoenzymes (Koya and King, 1998). Indeed, cPKCs have been demonstrated through a variety of studies including inhibitor studies and knockout mice to be necessary for many of the vascular dysfunctions associated with retinopathy, nephropathy, neuropathy, and microangiopathy (Ishii et al., 1998; Letiges et al., 2002; Standaert et al., 1999). Further, there are clinical trials currently underway to assess the efficacy of a cPKCβ specific inhibitor in the prevention of microangiopathies of diabetes.

In the present study, we hypothesized that the hyperglycemia-induced elevation of DAG and activation of PKC may not be limited to vascular endothelial tissues but that hyperglycemia may exert a similar effect on cPKCs in peripheral tissues as well. Given our previous data on the functional consequence of sustained PKC activation on membrane recycling, it was further hypothesized that hyperglycemia through DAG-PKC may function to sequester GLUT4 in a cPKC-dependent compartment and that this may lead to a block in

insulin-induced GLUT4 translocation to the membrane.

We now report that hyperglycemia can induced an elevation of DAG in HEK 293 cells and that the hyperglycemia-induced elevation of DAG is sufficient to induce a redistribution of cPKC from a diffuse cytoplasmic location to a juxtanuclear compartment. Further, hyperglycemia induced a sequestration of GLUT4 in a juxtanuclear compartment of HEK 293 cells and this occurred in a cPKC-dependent manner. In 3T3 L1 adipocytes, cPKC colocalizes with GLUT4 and this is also in a kinase activity-dependent manner, suggesting the intriguing hypothesis that sustained PKC activation may have a role in postreceptor insulin resistance.

4.2 Materials and Methods

Materials

Eagle's minimal essential media (MEM), Dulbecco's modified Eagle's medium (DMEM), HEPES, and trypsin-EDTA were from Life Technologies, Inc (Gaithersburg, MD). Fetal bovine serum (FBS) was from Summit Technology. [γ-32P]ATP (10 mCi/ml, 3000Ci/mmol) were from NEN Life Science Products (Boston, MA). Murine 3T3 L1 preadipocytes and HEK 293 cell lines were purchased from American Tissue Culture Collection (Manassa, VA). 4β-phorbol-12-myristate-13-acetate (PMA) was purchased from Calbiochem (La Jolla, CA.). Anti-FLAG M2 monoclonal antibody was from Sigma (St. Louis, MO). Rabbit polyclonal Anti-GLUT4 was from Abcam (England, UK). Silica Gel 60 thin-layer chromatography plates were from Whatman (Clifton, NJ). Anti-mouse TRITC secondary antibodies were from Molecular Probes, Inc (Eugene, OR.). All other chemicals were from Sigma (St. Louis, MO).

Cell Culture

3T3-L1 preadipocytes were maintained in DMEM with 25 mM glucose and supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin until differentiation. HEK 293 cells were maintained in MEM supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 incubator at 37°C. Cells were passaged every 3-4 days to maintain logarithmic growth.

Diacylglycerol measurements

HEK 293 cells were maintained in DMEM containing either 25 mM glucose or 5.5 mM glucose for the designated times. At time of harvest, cells were washed twice with 1 ml ice-cold PBS and lipids were extracted according to the method of Bligh and Dyer (Bligh and Dyer, 1959). One ml and 0.3 ml (in duplicates) of chloroform out of the organic phase were transferred into separate aliquots, dried down, and used for phosphate measurements. DAG

levels were measured with the *Escherichia coli* DAG kinase assay (Preiss et al., 1986). Briefly, the lipids were incubated at room temperature for 30 min in the presence of β-octylglucoside/dioleoyl-phosphatidyl glycerol micelles, 2 mM dithriothreitol, 5 μg of proteins from the DAG kinase membranes, and 2 mM ATP (mixed with [γ-^{32}P]ATP in a final volume of 100 μl. After the lipids were extracted by the method of Bligh and Dyer, the reaction products were separated by TLC in chloroform:acetone:methanol:acetic acid:H_2O (50:20:1.5:10:5), and the radioactivity associated with phosphatidic acid was measured by scrapping and scintillation counting. DAG levels were quantitated by using external standards and were normalized to phosphate.

Adipocyte Differentiation

3T3-L1 adipocytes were differentiated according to previously published methods. Briefly, the adipocyes were plated at a density of 1.0 X 10^6 in either 10 cm dishes and grown to confluence. Three days post confluence, differentiation media consisting of DMEM with10% FBS, 25 mM glucose, 0.5 mM methylisobutylxanthine, 0.25 μM dexamethasone, and 5 μg/ml insulin was added to the cells and incubated for 3 days. At the beginning of differentiation day 4, media was replaced with DMEM containing 10% FBS and 25 mM glucose, and cells were grown for 8 to 12 days. Media was replaced every two days. Cells were used directly for immunofluorescence or transfected via electroporation.

Transfection of 3T3-L1 adipocytes

Fully differentiated 3T3-L1 adipocytes were trypsinized and centrifuged at 800xg. The cell pellet was washed into 10 ml of ice-cold PBS. The cell pellet was then resuspended into a total volume of 500 μl of PBS and DNA was added to 50 μg. Cells were incubated for 10 min on ice prior to electroporation. Cells were electroporated (Gene Pulser) in 0.4 mm cuvettes at 0.15 kV and 960 μFarad. Following electroporation, the adipocytes were rested on ice for 10 minutes and then washed once with DMEM containing 10% FBS and plated

onto 35 mm confocal dishes (MatTeK). Adipocytes were grown for 24-48 hours and then processed for indirect immunofluorescence.

Indirect Immunofluorescence

HEK 293 cells were plated onto MatTek (Ashland, MA) confocal dishes at a density of 2.5-5.0 x 10^{-5} cells/dish and 24 hours later were co-transfected with FLAG-tagged GLUT4 and GFP-cPKCβII using Lipofectamine® 2000 according to manufacturer's recommendations. Twelve hours post transfection, cells were treated either with DMSO (0.01%) or 100 nM PMA for 1 hour and fixed with 3.7% paraformaldehdye/10% methanol for 10 minutes at room temperature. Following fixation, cells were permeabilized at –20°C with 100% methanol for 5 min. Cells were then washed with 1.5%FBS/PBS three times for 5 min each and then blocked with 2.5%FBS/PBS for 1 hour. Primary antibodies were incubated at a dilution of 1:50 or 1:100 in 1.5% FBS/PBS with 0.15% saponin for 2 hours at room temperature or overnight at 4°C. Following incubation with primary antibody, cells were washed three times for 5 min each with 1.5% FBS/PBS. TRITC-conjugated secondary antibody (Molecular Probes) was incubated at a dilution of 1:50 or 1:100 in 1.5% FBS/PBS and 0.15% saponin for 1 hour. Cells were washed a final three times for 5 min each with 1.5% FBS/PBS and visualized immediately with confocal microscopy.

Confocal Microscopy

Cells were plated onto MatTek confocal dishes at a density of 2.5-5.0 x 10^{-5} cells/dish and allowed to adhere for 24 hours. Co-transfection of FLAG-tagged GLUT4 and GFP-cPKCα using Lipofectamine 2000 was carried out according to the manufacturer's instructions. Twelve hours post transfection, cells were treated either with DMSO (0.01%) or 100 nM PMA for 1 hour and fixed with 3.7% paraformaldehdye and 10% methanol for 20 minutes at room temperature. Preparation of samples for immunofluorescence is described above. Cells expressing GFP-fusion proteins alone were viewed immediately following fixation.

All confocal images were taken with an Olympus UltraView Spinning Disk Confocal IX-70 system with an Olympus 60X 1.4 NA lens and Krypton/Argon laser line. Each image is representative of 20 fields over a minimum of three experiments and images represent the equatorial plane of the cell. Green fluorescent protein (GFP) fusion proteins were excited at 488 nm and TRITC was excited at 543. All fluorescent images were captured sequentially and combined within the UltraView software (Perkin-Elmer).

4.3 Results

4.3.1 Hyperglycemia induces an elevation of DAG in HEK 293 cell

To investigate if chronic exposure to hyperglycemia can induce an elevation of DAG in a fibroblastic cell model, HEK 293 cells were incubated in the presence of 10% FBS-DMEM containing either 5.5 mM or 25 mM D-glucose for three weeks. A three weeks exposure was chosen in order to mimic chronic exposure to hyperglycemia that is seen in diabetes. Total cellular lipids were then extracted via the method of Bligh and Dyer and subject to a DAG kinase assay. As seen in figure 4.1A, there was an approximate 75% increase in cellular levels of DAG when cells were exposed to high glucose for 3 weeks when compared to low glucose. These results demonstrate that hyperglycemia can induce an elevation of DAG in fibroblasts.

4.3.2 Hyperglycemia alters the subcellular localization of endogenous cPKCα

We have previously reported that under conditions of sustained activation, cPKC isoenzymes α and βII translocate to a novel, juxtanuclear compartment that functions in the dysregulation of membrane trafficking. Given these previous results, and the strong link between hyperglycemia and activation of the DAG-PKC pathway, we hypothesized that the hyperglycemia-induced elevation of DAG in fibroblasts may also lead to alteration of PKC subcellular localization with an accumulation of PKC at the juxtanuclear compartment. To investigate this hypothesis, cells were either exposed to 5.5 mM or 25 mM glucose for three weeks and then subjected to indirect immunofluorescence (IF) and confocal microscopy to determine the subcellular localization of endogenous cPKCα. After three weeks exposure to low glucose, cPKCα was found in a diffuse cytoplasmic localization in all cells (Fig. 4.1B, panel 1). In contrast, following three weeks exposure to hyperglycemic conditions, cPKCα was found localized to both a diffuse cytoplasmic and a juxtanuclear location (Fig. 4.1B, panel 2). These results suggest that hyperglycemia acting through sustained elevation of DAG may alter the subcellular localization of cPKCα.

4.3.3 Hyperglycemia induces the internalization and sequestration of GLUT4 to a juxtanuclear location in HEK 293 cells

The effects of hyperglycemia on GLUT4 were investigated in the HEK 293 cell model. The HEK 293 cell model was utilized initially due to the clear evidence of DAG elevation with three weeks hyperglycemia and the alteration of PKC subcellular localization. Since these cells do not contain endogenous GLUT4, stable cell lines were generated expressing a FLAG-tagged GLUT4. Overexpression of a FLAG-tagged GLUT4 protein in the presence of low glucose led to a predominant plasma membrane localization of GLUT4 (Fig. 4.2A, panel 1). Interestingly, the localization of GLUT4 to the plasma membrane is unlike what is reported for GLUT4 in 3T3 L1 adipocytes and this may be indicative of a lack of molecular components required to sequester GLUT4. Conversely, another intriguing possibility is that plasma membrane localization is the default localization for GLUT4 and that there are specific conditions that mediate the sequestration of GLUT4 in experimental models. In exploration of these postulations, following 3 weeks of hyperglycemia there was a marked increase in GLUT4 found in a juxtanuclear location (Fig. 4.2B, panel 2). These data suggested that hyperglycemia, on its own, may be partially involved in the compartmentalization of GLUT4.

4.3.4 Hyperglycemia induces the sequestration of GLUT4 at a juxtanuclear compartment in a cPKC-dependent manner

Given the ability of hyperglycemia to induce an elevation of DAG and a translocation of cPKCα to a similar juxtanuclear compartment, it was hypothesized that the effects of hyperglycemia on GLUT4 subcellular localization may be mediated through cPKC. To probe this question, cells that had been exposed to 3 weeks of high glucose, which was shown to be sufficient to induce translocation of cPKC to a juxtanuclear compartment, were incubated for 1 hour in the presence of a 3 μM Gö 6976, a cPKC-selective kinase inhibitor. Incubation with 3 μM Gö 6976 resulted in a cytoplasmic dispersion of the GLUT4-positive

vesicles leading to the loss of concentrated GLUT4 at a juxtanuclear compartment when compared to vehicle control (Fig. 4.2B and C). These data suggest that hyperglycemia sequesters GLUT4 at a juxtanuclear location in HEK 293 cells and that cPKC activity is required for this effect.

4.3.5 cPKCα and GLUT4 colocalize to a juxtanuclear location in HEK 293 cells with PMA

Phorbol esters such as phorbol-12-myristate-13-acetate (PMA) are naturally-occurring tumor promoters as well as specific DAG mimetics. To investigate if the hyperglycemia effect on GLUT4 was through DAG, cells stably expressing FLAG-tagged GLUT4 were transiently transfected with GFP-cPKCα and then treated with 100 nM PMA for one hour. In cells treated with DMSO vehicle alone, GLUT4 was found predominantly at the plasma membrane and GFP-cPKCα was localized to a diffuse cytoplasmic location (Fig. 4.3A). Treatment with PMA lead to the internalization of GLUT4, the translocation of GFP-cPKCα, and the partial colocalizaton of GLUT4 with GFP-cPKCα in a juxtanuclear location (Fig. 4.3B). This PMA induced colocalizaton of GLUT4 and cPKCα was dependent upon kinase activity since incubation with 3 μM Gö 6976 following one hour PMA led to the dispersal of GLUT4 and GFP-cPKCα, and the loss of colocalization (data not shown). These data demonstrate that the DAG-mimicking phorbol ester, PMA, is able to reproduce the effects of hyperglycemia on cPKC and GLUT4 localization demonstrating activation of PKC is sufficient to sequester GLUT4 intracellularly.

4.3.6 cPKC localizes to a juxtanuclear compartment in 3T3 L1 adipocytes and localization is dependent upon kinase activity

To begin to investigate whether long term activated cPKCα exerts a direct effect on GLUT4 trafficking in an adipocyte model, the subcellular location of cPKCα was probed in the 3T3 L1 adipocytes in the presence or absence of phorbol esters. 3T3 L1 adipocytes were

transiently transfected with a GFP-tagged cPKCα via electroporation and treated with PMA to stimulate sustained DAG conditions. In an unstimulated state, (but in the presence of high glucose which is required to maintain the differentiated state of 3T3 adipocytes) cPKCα was found predominantly in a diffuse cytoplasmic location as well as at a juxtanuclear location (Fig. 4.4A, panel 1). Stimulation with PMA for 1 hour led to the translocation of GFP-cPKCα to the plasma membrane and a slight enhancement in the cPKC at the juxtanuclear location (Fig. 4.4A, panel 2). Incubation with 10 μM of the cPKC-selective kinase inhibitor, Gö 6976, for 1 hour following one hour of 200 nM PMA resulted in a total loss of cPKCα accumulation at the juxtanuclear compartment (Fig. 4.4A, panel 3). These results suggest that, similar to HEK 293 fibroblasts, cPKC in 3T3 L1 adipocytes localizes to a juxtanuclear compartment during sustained activation and this occurs in a kinase dependent manner.

4.3.7 cPKCα colocalizes to a subset of GLUT4 positive vesicles in 3T3 L1 adipocytes

To investigate if cPKC and GLUT4 colocalize in 3T3 L1 adipocytes, transiently transfected 3T3 adipocytes were incubated in the presence 200 nm PMA for 1 hour and the subcellular localization of GLUT4 was probed with indirect immunofluorescence for endogenous GLUT4. As shown in Fig. 4.4B (panels 1-3 and inset), GFP-cPKCα was found to colocalize to a subset of GLUT4-positive vesicles at a juxtanuclear location. These results show that in a physiologically-relevant model of adipocytes, cPKC and GLUT4 colocalize, and that PKC may be involved in this sequestration.

4.4 Discussion

These results demonstrate that chronic exposure (three weeks) to hyperglycemia (25 mM) can induce an elevation of DAG as measured by the DAG kinase assay, and that this leads to an alteration in the subcellular localization of cPKC and GLUT4 to a juxtanuclear location in HEK 293 cells. These effects of hyperglycemia on GLUT4 localization in HEK 293 cells were shown to be cPKC dependent and could be fully recapitulated with phorbol esters. Further, the activity-dependent colocalization of cPKCα to a subset of GLUT4-positive vesicles in 3T3 L1 adipocytes suggests that this interaction may occur within a physiological context. The adipocyte cell system represents a relevant model to further investigate the effects of sustained cPKC activity on GLUT4 recycling.

The current understanding of the molecular mechanism(s) regulating GLUT4 compartmentalization in 3T3 L1 adipocytes is incomplete (Bryant et al., 2002). In the absence of insulin, GLUT4 is found concentrated in a membranous compartment concentrated around the trans-Golgi region. It has been proposed that GLUT4 resides in vesicular derivatives of the endosomal system and that GLUT4 is maintained in the intracellular location through a high rate of endocytosis and relatively low rate of exocytosis. Stimulation with insulin triggers a large increase in the exocytic rate and this leads to the rapid redistribution of GLUT4 to the membrane. Confirmation that GLUT4 may be reside in some aspect of the endosomal system arises from biochemical studies that have found 30-40% of GLUT4 associated with recycling markers such as the transferrin receptor. This model; however, is currently in dispute since transferrin recycling is only modestly responsive to insulin signaling (Bryant et al., 2002; Pessin and Saltiel, 2000).

The direct implication of these studies is that hyperglycemia through DAG can mediate a persistent activation of cPKC isoenzymes and that this leads to the dysregulation of membrane recycling i.e. sequestration of GLUT4 at an intracellular location. Overexpression of FLAG-tagged GLUT4 in HEK 293 cells which localized to plasma membrane in the presence of low glucose allowed the specific determination of the effects of hyperglycemia

and the identification of the role of cPKC activity.

The identification of a novel function for cPKC during sustained activation induced by hyperglycemia-derived DAG further illustrates the role of cPKC as a mediator of long term cellular functions. As such, it is important to investigate other disease models where there is an established or proposed elevation of sustained DAG to evaluate the role of membrane recycling dysregulation in the pathological mechanism. Further, these studies serve to underscore the urgent need to elucidate the molecular mechanism(s) behind persistent bioactive DAG.

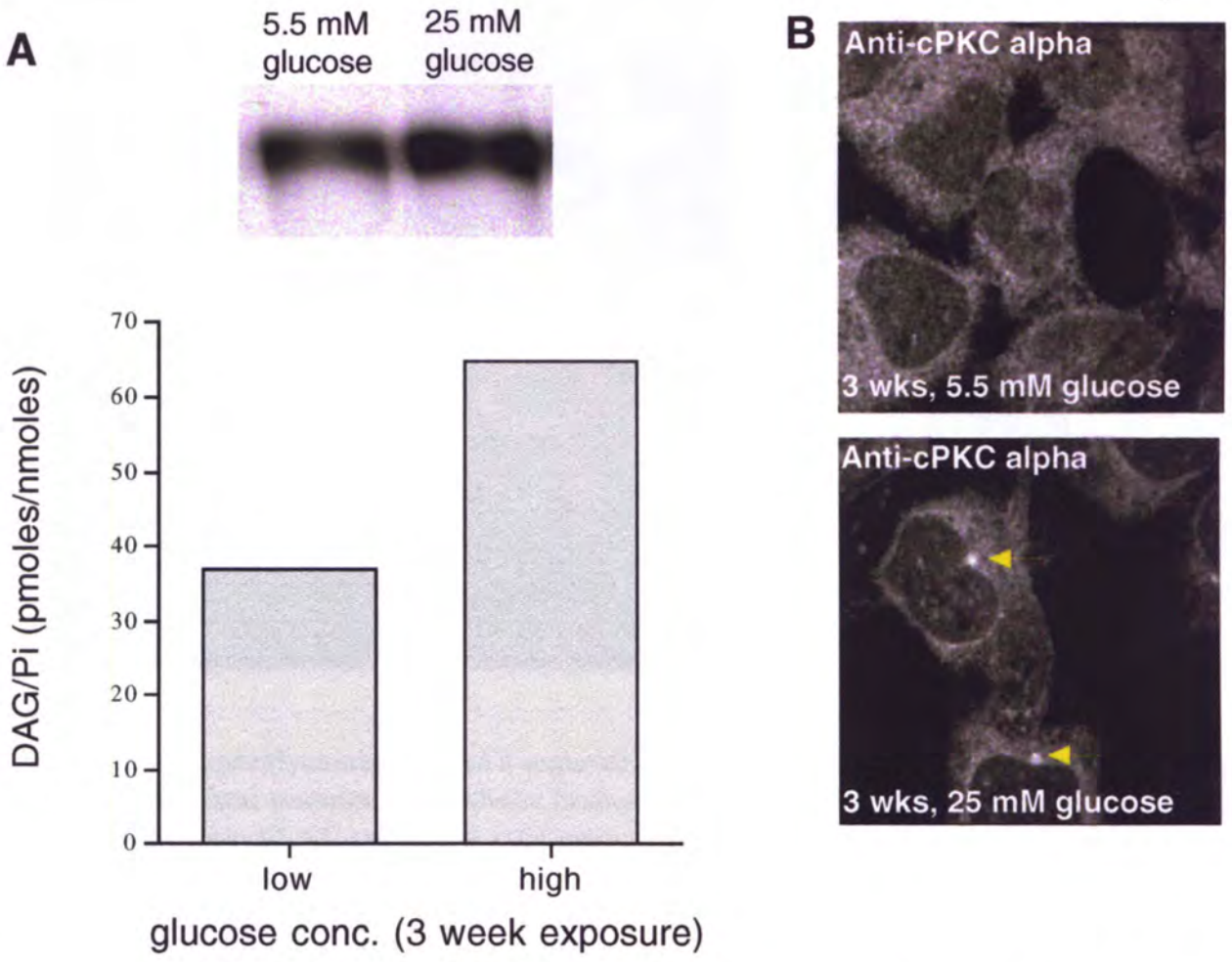


Figure 4.1 Chronic hyperglycemia induces an elevation of DAG and alteration of cPKC subcellular localization. A, HEK 293 cells were exposed to either 5.5 mM or 25 mM D-glucose for three weeks and lipids were extracted via the method of Bligh and Dyer. Total extracted lipids were subjected to a DAG kinase assay and separated via TLC. The 32-P-DAG band was scraped and counted. DAG levels were normalized to phospholipid phosphate to give DAG pmoles/nomles phosphate. **B,** representative confocal images of cells that were exposed to either 5.5 mM or 25 mM D-glucose for three weeks and then processed for immunofluorescence as detailed under "Experimental Procedures". Cells were probed for endogenous cPKCα. Micrographs are single images captured at the equatorial plane of the nucleus and represent a minimum of 20 60X fields.

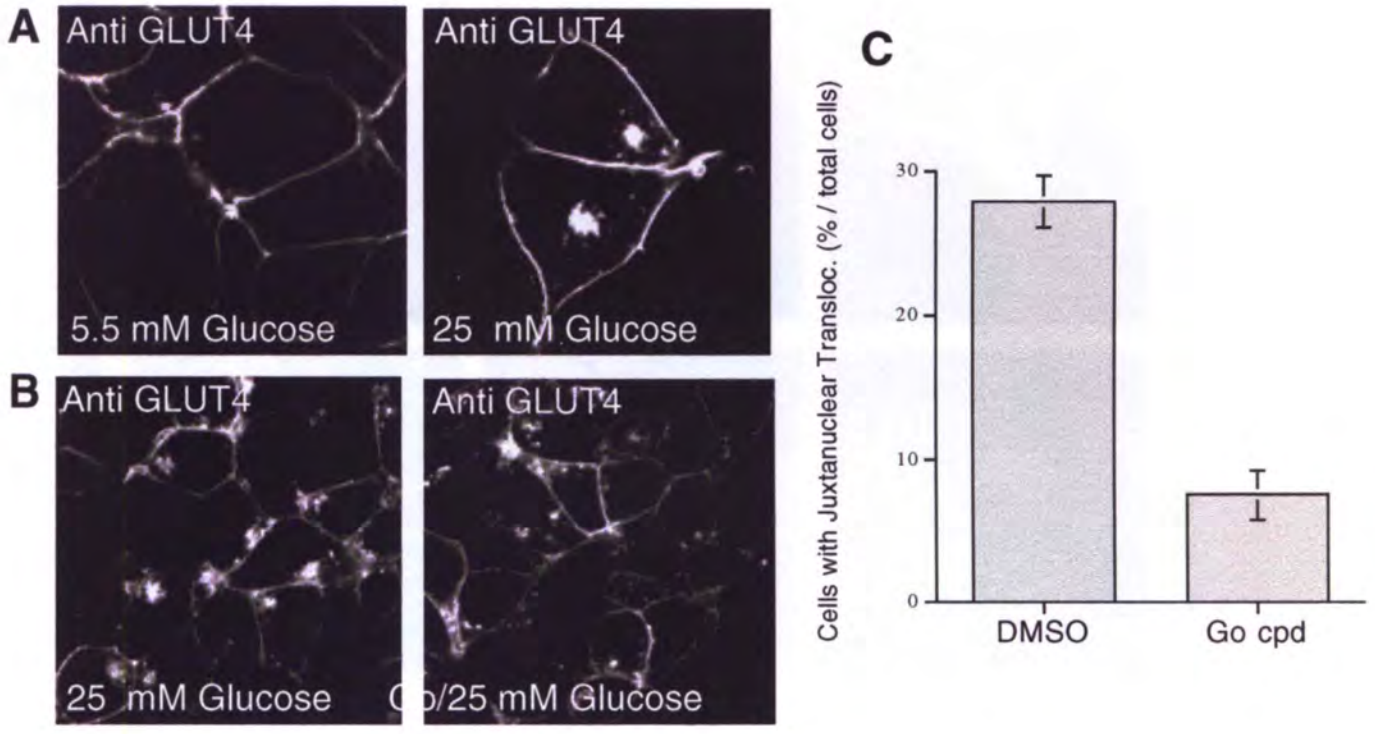


Figure 4.2 Hyperglycemia induces a sequestration of GLUT4 to a juxtanuclear compartment in a cPKC-dependent manner. A, indirect immunofluorescence and confocal images of HEK 293 cells stably expressing FLAG-tagged GLUT4 were maintained in either 5.5 mM or 25 mM D-glucose for three weeks and then processed for indirect immunofluorescence as detailed in "Experimental Procedures". **B,** confocal images of HEK 293 cells stably expressing FLAG-tagged GLUT4 that were cultured in 25 mM glucose DMEM for three weeks and then incubated with either 0.01% DMSO vehicle control or 3 μM Gö 6976 for one hour. **C,** quantitation of cells exhibiting juxtanuclear sequestration of FLAG-GLUT4 induced with hyperglycemia and treated with or without Gö 6976 for one hour. Cells exhibiting uxtanuclear sequestration of GLUT4 were counted in twenty 60X fields over three separate experiments and are represented on the ordinate as a percentage of total cells expressing of GLUT4

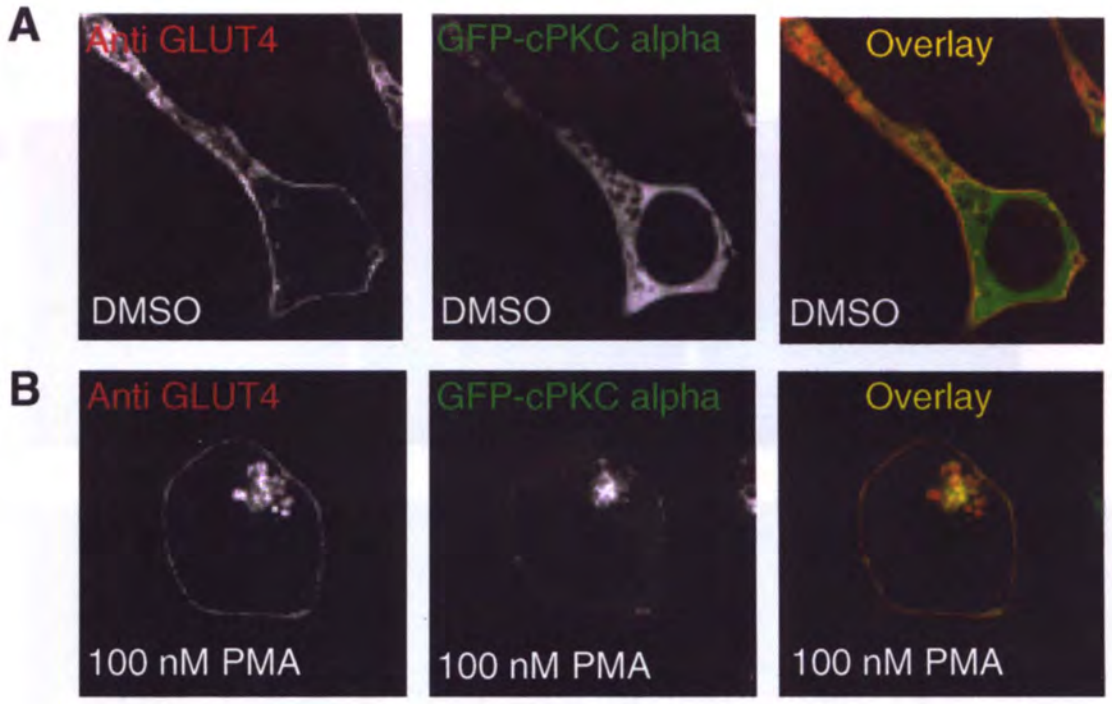


Figure 4.3 PMA induces sequestration of FLAG-tagged GLUT4 and GFP-cPKC alpha in a juxtanuclear compartment of HEK 293 cells. Confocal images of HEK 293 cells stably expressing FLAG-GLUT4 that were transiently transfected with GFP-cPKC alpha. Cells were maintained in 5.5 mM D-glucose at all times. A, cells were treated for one hour with 0.01% DMSO vehicle. B, cells were treated with 100 nM PMA for one hour and processed for indirect immunofluorescence as detailed in "Experimental Procedures".

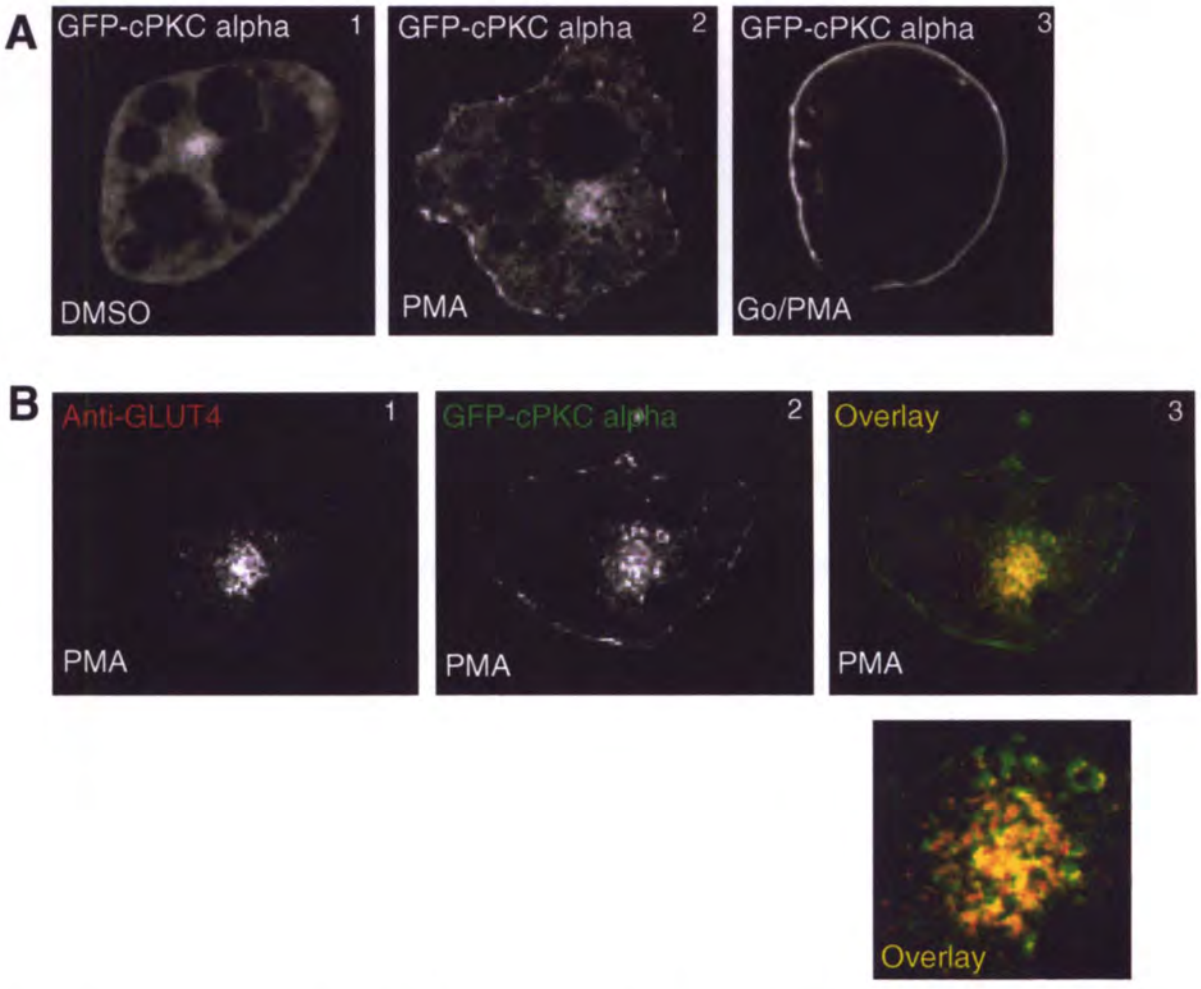


Figure 4.4 cPKC alpha colocalizes with endogenous GLUT4 in 3T3 L1 adipocytes in kinase activity-dependent manner. Representative confocal images of 3T3 L1 adipocytes 48 hours post electroporation with GFP-cPKC alpha. **A,** GFP-cPKC alpha in 3T3 L1 adipocytes treated with 0.01% DMSO control vehicle (panel 1), GFP-cPKC alpha expressing 3T3 L1 adipocytes treated with 200 nM PMA for one hour (panel 2), 3T3 L1 adipocytes expressing GFP-cPKC alpha and treated with 200 nM PMA followed by 10 μM Gö 6976 (panel 3). **B,** confocal imaging of 3T3 L1 adipocytes expressing GFP-cPKC alpha and treated with 200 nM PMA for one hour. Indirect immunofluorescence was performed for endogenous GLUT4.

Chapter 5: Concluding Discussions

5.1 Summary

Protein kinase C (PKC) is subject to spatial and temporal regulation through dynamic fluctuations in lipid second messengers, especially *sn*-1,2-diacylglycerol (DAG). DAG or DAG-mimicking phorbol esters can modulate PKC on several levels: 1) the formation of DAG in the membrane leads to a direct interaction of DAG with the C1 domain of PKC, and the recruitment and anchoring of PKC at the membrane. In the case of the cPKC isoenzymes, recruitment/anchoring involves a cooperative effort between the C1 and the calcium-dependent, acidic phospholipid binding C2 domain; 2) DAG binding to the C1 domain induces maximal activation of PKC in the presence of spatially-defined protein substrates. In this capacity, DAG functions to tightly couple PKC activation to the vicinity of compartment-specific substrates; and 3) metabolic clearance of DAG signal from the plasma membrane leads to the concomitant loss of the PKC from the membrane. "Reverse translocation" or the return of PKC to the cytosol requires kinase activity and autophosphorylation of a conserved serine (660) that acts to counter the C2 domain interactions at the membrane. Thus, the duration of PKC association with membranes is a direct reflection of cellular DAG levels and PKC activity.

During acute signaling events, DAG levels are elevated in the membrane for only a very short period of time (1-2 min). As a consequence, PKC translocation and activation at the membrane is short-lived, but this is of sufficient time to trigger signaling cascades that lead to acute cellular responses (e.g. secretion, glycogenolysis, neurotransmitter release). In contrast to this classical model of PKC activation, there is also significant evidence that DAG levels and PKC activity can be sustained for more prolonged periods. Cell data have indicated that sustained PKC activity is necessary for a variety of normal regulatory processes including cell differentiation, mitogenesis, and cell motility. In addition, PKC has been implicated in several long term pathological processes such as cancer (tumorigenesis, cancer metastasis, multidrug resistance), diabetes, and cardiovascular disease.

These observations have led to the proposal that PKC activity can be divided into two

general categories: 1) those cellular processes that involve a transient activation of PKC as is seen in signal transduction and this is mediated by DAG that is present in the membrane for 1-2 minutes. In this acute model of activation, the plasma membrane is considered the primary target for PKC; and 2) cellular functions that involve a persistent elevation of DAG and PKC activity (hours-days) (Blobe et al., 1996b; Nishizuka, 1995). Currently, little is known regarding the subcellular localization of PKC during exposure to sustained DAG.

The central hypothesis of this study was that DAG produced in the cell through either normal or pathological mechanisms could lead to an alteration of PKC subcellular localization and function. The experimental questions addressed were: 1) where do cPKC isoenzymes locate under conditions of prolonged DAG or phorbol ester exposure; and 2) what is the physiological significance of this localization on cellular functioning. The general experimental approach taken was to use confocal microscopy to monitor the subcellular localization of GFP-tagged PKC isoenzymes in live cells during sustained activation. It was anticipated that this approach would allow the identification of a specific subcellular structure(s) that would not be detectable with other methods eg. subcellular fractionation.

Preliminary studies with DAG-mimicking phorbol esters revealed that short-term activation induced translocation of GFP-cPKCα to the plasma membrane, but that longer exposures led to a localization of cPKCα at a secondary, juxtanuclear compartment. A similar time-dependent accumulation was also evident when endogenous cPKCα was examined with confocal microscopy. These initial results were interpreted to mean that during sustained activation, cPKC may translocate to a juxtanuclear compartment such as the Golgi complex and that this translocation event had a direct consequence on Golgi functioning (e.g. protein secretion).

[*Use of phorbol esters.* Although phorbol esters have been used for more than two decades as DAG-mimetics, there are some data to suggest that phorbol esters may exert some cellular effects unrelated to PKC activation. Therefore, to confirm that PMA was functioning as a true DAG mimetic and to validate the use of PMA in further studies, the

effect of endogenous DAG on PKC subcellular localization was examined using a PC-PLD-coupled agonist, in this case platelet-derived growth factor (PDGF). Overexpression of a FLAG-tagged PDGF-β receptor in HEK 293 cells, cultured in the absence of serum, resulted in a diffuse cytosolic localization of GFP-cPKCα with no specific accumulation of cPKC at either the plasma membrane or juxtanuclear location. On the other hand, the addition of 10% serum or PDGF-BB to the media of cells overexpressing the PDGF-β receptor led to the accumulation of cPKC at both the plasma membrane and juxtanuclear location. Transphosphatidylation assays performed in parallel with the confocal studies confirmed that both serum and PDFF-BB stimulated PLD activity in a time frame that correlated with cPKC translocation. In addition, the translocation of cPKC to the juxtanuclear compartment was found to be sensitive to the lipid phosphate phosphatase inhibitor, propranolol, suggesting that DAG-derived from the actions of PLD was necessary for the localization of cPKC (data not shown). These data suggest that under physiological conditions, sustained DAG can mediate the localization of cPKC to a juxtanuclear compartment and that the effects of endogenous DAG are reproduced with PMA].

5.1.1 Further characterization of sustained PKC activity and the juxtanuclear compartment revealed:

1) PKC translocation was subfamily- and isoenzyme-specific. Translocation was observed for the cPKCα and βII isoenzymes but was not evident with nPKCε or aPKCζ. Specificity for the cPKC subfamily was determined to be due to the presence of the C2 domain. Further, examination of cPKCβI and cPKCβII translocation revealed an isoenzyme specificity that was dependent upon residues within the V5 domain of cPKCβII (note: the V5 region of cPKCα contains residues with chemical properties most similar to cPKCβII). These data suggest that translocation to the juxtanuclear compartment involves a cooperation of the C1, C2 and the V5 variable region of the cPKC isoenzymes, α and βII.

2) Colocalization studies revealed that cPKC does not translocate to the Golgi complex.

Confocal studies revealed that there was no overlap with markers of the cis, medial, or trans subcompartments of the Golgi and cPKC. In addition, the compartment was resistant to dispersal with Golgi disrupting agents such as brefeldin A and nocodazole.

3) cPKC localizes to a subset of recycling endosomes concentrated around the centrosome/ MTOC. The juxtanuclear compartment was centrally defined by markers of the centrosome/ microtubule-organizing complex (e.g. gamma tubulin and pericentrin) and localized to a subset of transferrin-positive recycling endosomes defined by the small GTPase rab11 (for discussion see below).

4) Enrichment with lipid raft components. cPKC translocation to the juxtanuclear compartment coincided with an enrichment of the compartment with plasma membrane lipid raft components, GM1 and caveolin-1. Movement of lipid rafts were detected with bodipy-conjugated GM1, fluorescent cholera toxin, and indirect immunofluorescence for caveolin-1. Electron microscopy studies confirmed the formation of a membranous compartment associated with the appendages of the centrosome and immunogold studies localized cPKCα to these membranes.

5) cPKC activity is required for translocation to the compartment. Kinase inhibitors and kinase-defective mutants of cPKC revealed that cPKC translocates to the plasma membrane in the absence of kinase activity but that subsequent translocation to the juxtanuclear compartment requires an active enzyme.

6) Kinase activity is required for the structural maintenance of the compartment, and the association of cPKC and lipid raft components with the compartment. Application of kinase inhibitors following translocation of cPKC and lipid rafts to the juxtanuclear compartment resulted in the dispersal of these components into the cytoplasm.

7) PLD activity is required for translocation. 1-butanol and the expression of a catalytically deficient PLD (KR-PLD1) had no effect on translocation to the plasma membrane but completely blocked translocation of cPKC to the juxtanuclear compartment.

8) Isoenzyme-specificity of translocation is determined by differential activation of PLD

and involves the carboxy terminus V5 variable region. Examination of cPKCβI and βII revealed that βII preferentially activates PLD upon long term activation with phorbol esters. During this project, it was reported by Exton and colleagues that the interaction of the rat cPKCα with PLD was dependent upon the presence of a single residue (phe663) within the V5 region. These observations and the present study implicate the V5 region in the PKC-PLD interaction and provide a basis for the differential activation of PLD by cPKCβII and isoenzyme-specific translocation to the juxtanuclear compartment.

9) Sustained cPKC activation leads to a dysregulation of membrane recycling. Sustained activation of PKC led to the translocation of cPKCα to a juxtanuclear location and a cPKC-dependent reorganization of transferrin-positive vesicles to a region concentrated around the MTOC. cPKC localized to the subset of reorganized transferrin-positive endosomes. Kinetic studies demonstrated a retention or sequestration of markers of membrane recycling (transferrin) in the cPKC-dependent compartment.

10) Sustained elevation of DAG derived from a chronic exposure to hyperglycemia resulted in an alteration of the subcellular localization of cPKCα to a juxtanuclear location. As has been reported on extensively, we observed a hyperglycemia-induced formation of DAG in fibroblasts as measured with DGK. Hyperglycemia was also observed to alter the subcellular localization of endogenous cPKCα to a juxtanuclear location.

11) Hyperglycemia leads to the sequestration of GLUT4 in a juxtanuclear compartment of HEK 293 cells and this occurs in a cPKC-dependent manner. Although GLUT4 is sequestered at a juxtanuclear location in insulin-sensitive tissues, overexpression of GLUT4 in HEK 293 cells resulted in a plasma membrane localization. Chronic exposure of HEK 293 cells to hyperglycemic conditions led to the internalization and sequestration of GLUT4 to a juxtanuclear compartment and this could be blocked with cPKC-selective kinase inhibitors. The plasma membrane localization of GLUT4 in HEK 293 cells allowed the determination of the effects of hyperglycemia-DAG on GLUT4 in the absence of other variables.

12) Colocalization of cPKCα and βII with a subset of GLUT4 in 3T3 L1 adipocytes suggests a possible novel role for cPKC in insulin resistance. Indirect immunofluorescence studies revealed that cPKC localizes to a subset of GLUT4 vesicles in a kinase activity-dependent manner. The colocalizaton of cPKC to a subset of GLUT4 was lost upon the addition of kinase inhibitors suggesting that colocalizaton requires an active process. A definitive role for cPKC in GLUT4 insulin resistance awaits further investigation.

5.1.2 *The pericentrion*

The rab11 versus cPKC-dependent compartment. During sustained activation, cPKC translocates to a subset of recycling endosomes concentrated in a pericentriolar region and defined by the small GTPase, rab11. Although rab11 and cPKC overlap/colocalize, functional studies revealed that the cPKC-dependent compartment is distinct from the rab11 compartment on several levels (Fig 5.1):

a) rab11 is present at the MTOC/centrosome independent of PKC activation. cPKC requires sustained activation to translocate to this site.

b) rab11 was present at the MTOC location independent of temperature. cPKC and Bodipy-GM1/AF-CTx-B required physiological temperature for localization at this site.

c) in the absence of cPKC activation, the rab11-defined compartment is absent of components of lipid rafts (GM1, caveolin-1). Sustained activation of cPKC leads to an enrichment of GM1 and caveolin-1, and the association of these components with the cPKC-defined compartment was found to be dependent upon cPKC activity.

d) The MTOC/centrosome-associated recycling compartment is sensitive to microtubule disrupting agents e.g. nocodazole. The cPKC-dependent compartment was observed to be resistant to disruption upon nocodazole treatment. *This is a critical point as it shows that the cPKC compartment is not dependent on microtubules and thus can not be easily identified with the MTOC which is strictly dependent on microtubules.*

e) Finally, cPKC translocate to the pericentriolar region even in cells that lack rab11 (data not shown). Thus, the cPKC-dependent compartment cannot be equated with the rab11-defined compartment.

Overall, these studies show that cPKC translocates to a compartment structurally related to the rab11 subcompartment of recycling endosomes but with significant distinguishing features. The cPKC compartment may consist of vesicles and membrane structures that translocate (and may associate with) the MTOC, but can be distinguished structurally (by EM) and functionally. In order to distinguish the cPKC-compartment from other recycling compartments and I propose to name it the *pericentrion.*

5.2 Implications and significance

5.2.1 Novel site of PKC translocation. These studies define a novel site of translocation for cPKC. Previous studies have localized PKC to the Golgi, cytoskeleton, mitochondria, endoplasmic reticulum, nuclear envelope and nucleus but this is the first time that PKC has been localized to a subset of recycling endosomes. Moreover, these studies identify a novel site of translocation for PKC during long term activation which has not previously been described.

5.2.2 Novel compartment. The cPKC-dependent juxtanuclear compartment appears to be a specialized component of recycling endosomes that is strictly PKC and temperature dependent. It is also a (novel) site for intracellular translocation of components of membrane rafts.

5.2.3 Novel function. The DAG-PKC relationships is one of the most ubiquitous and highly conserved signaling mechanisms in biology. Not surprisingly PKC activity has been linked to a large number of cellular functions. The data described here identifies a novel function for cPKC isoenzymes.

5.2.4 cPKC isoenzyme-specific functions: differential activation of PLD. It has been recognized for some time now that cPKC isoenzymes can regulate PLD activity and that there is isoenzyme-specific differences between cPKCα and βII versus βI. These studies provide insight into the cPKCβ isoenzyme-specific activation of PLD and further implicate a critical role for the carboxy terminus of cPKC in the activation of PLD.

5.2.5 PLD regulation of PKC. Most of the studies that have investigated the role of DAG-derived from PC-PLD activity have relied upon either *in vitro* systems or subcellular fractionation for monitoring the translocation of PKC (i.e. activation) to membranes. To date, these studies have produced conflicting results. In the present study visualization of the subcellular trafficking of GFP-tagged cPKC isoenzymes led to the identification of a novel PLD-mediated regulation of PKC through translocation to the pericentrion.

5.3 Speculations

5.3.1 Alteration of membrane composition

The enrichment of an intracellular compartment with lipid and protein components of the plasma membrane (which coincides with cPKC translocation to the pericentrion) may lead to an imbalance in membrane composition with a variety of potential consequences for the cell.

A) Effect of sequestration of membrane proteins

Transferrin. The sequestration of membrane proteins could have profound effects on cellular metabolism. Loss of transferrin-transferrin receptor from the plasma membrane may lead to a deficiency in iron uptake into the cell with direct consequences on iron (Fe)-requiring enzymatic reactions. Previous studies have shown that loss of Fe uptake can dramatically block cell proliferation (Kwok and Richardson, 2002). The sequestration of transferrin receptors away from the plasma membrane at the pericentrion may facilitate the entry into senescence which would be required in order for cell differentiation to occur. In support of this postulation, the PKC-dependent loss of transferrin receptors from the plasma membrane has been identified as a key event in the cellular differentiation of K562 cells (Schonhorn et al., 1995).

Monoamine transporter. There is a large amount of literature that implicates PKC in the internalization and down-regulation of the dopamine transporter (DAT) and other neuronal receptors and transporters from the plasma membrane of neuronal cells (Apparsundaram et al., 1998; Corey et al., 1994; Melikian and Buckley, 1999). It is interesting to speculate if under certain neurological conditions there is an elevation of DAG and a sustained activation of PKC that could lead to the formation of the pericentrion. PKC could then play a role in the pathology of these diseases through the sequestration of DAT (or other transporter/ receptors) in the pericentrion. To that end, aberrant phospholipase (e.g. PLC, PLD, PLA_2) activity has been detected in a variety of neurological diseases.

B) Effects on signal transduction. Signal transduction requires a fine balance between

being able to detect and respond to a stimulus and the ability to rapidly extinguish a signal in order to avoid becoming overly stimulated. The mechanisms that have evolved to protect the cell against uncontrolled stimulation involves either the direct desensitization of the receptor system (e.g. phosphorylation) and/or the removal of the ligand-receptor protein from the membrane surface (down regulation through endocytosis). Thus, a strong interdependent relationship exists between membrane recycling and signal transduction. Any dysregulation of the afferent or efferent branches of the membrane recycling pathway (mediated under conditions of sustained activation of cPKC) could have a direct impact on cell signaling with the resultant inability of a cell to respond appropriately to a particular stimulus.

On the other hand, the sequestration of membrane proteins in a specialized recycling compartment may act to specifically modulate the spatio-temporal properties of an initial stimulus. That is, the formation of an internal cPKC-dependent membranous compartment may facilitate or enhance signaling by providing an alternate platform from which membrane signaling can continue. Data in support of this hypothesis comes from studies that have shown that some receptors are still active when endocytosed from the plasma membrane, and more recently from molecular studies with a dominant negative form of dynamin which revealed that endocytosis is actually required for the full signaling potential of some receptor systems. Of course, this could also lead to a pathological response in that the sequestration of stimulated components in the juxtanuclear compartment may help to elude the normal cellular processes of down-regulation.

5.3.2 Role for PKC in cholera toxin pathology/lipid raft reorganization

To this day, V cholerae still represents a formidable health concern. Cholera toxin, which is the enterotoxin of V. cholerae, acts by binding specifically to the ganglioside-GM1 in the plasma membrane of jejunal epithelial cells and this leads to the internalization of CT and the pathological loss of electrolytes and water. Data from the present study suggests that

the internal accumulation of cholera toxin involves a cPKC-dependent reorganization of lipid rafts to an juxtanuclear location. The development of isoenzyme-specific inhibitors could lead to alternative therapy for cholera toxin-induced disease.

5.3.3 Isoenzyme-specific down regulation. Historically, phorbol esters have been used in the study of cellular PKC in two ways: 1) as a cellular probe in which to dissect signaling pathways that involve DAG and PKC; and 2) to promote down regulation of DAG/PMA-sensitive PKC isoenzymes in an effort to establish or discount PKC-specific functions. The phorbol ester-induced down regulation of PKC typically requires micromolar concentrations of PMA and more than 16 hours of exposure. Unfortunately, the down regulatory effects of phorbol esters are inconsistent among the DAG/PMA-sensitive PKC isoenzymes and this has not been well-appreciated in the literature due to poor controls and over-concluded data.

Data from the work of Blobe et al. and the present study suggest that long term exposure of PKC to PMA may lead to an isoenzyme-specific sequestration of PKC (Blobe et al., 1996a). Sequestration could possibly result in a differential susceptibility of PKC to down regulatory mechanisms resulting in an imbalance of PKC activity and the predominant cellular function of unopposed kinase activity. It is conceivable that a mechanism such as this could account for the tumor promoting effects associated with phorbol esters.

In support of the hypothesis that sequestration at the pericentrion may lead to differential down regulation of PKC, there is evidence that the mechanism of PMA-induced down-regulation specifically targets PKC isoenzymes associated with the plasma membrane. As a consequence, any PKC that is sequestered away from the plasma membrane during phorbol stimulation may be able to elude significant degradation. Preliminary investigation into this hypothesis revealed that at 24 and 48 hours of 100 nM PMA exposure, there appeared to be a loss of PKC from the plasma membrane but no apparent loss of PKC from the juxtanuclear compartment (data not shown).

5.4 Future Directions

5.4.1 PKC and Diabetes

A) *cPKC potentiates insulin resistance through GLUT4 sequestration?* Examine the hypothesis that cPKC translocation to a subset of GLUT4-positive vesicles correlates with GLUT4 insensitivity to an insulin-stimulated translocation and glucose uptake.

B) *Localization of PKC in tissues from diabetic animal models and diabetic patients.* Although the 3T3 L1 adipocyte cell model is a thought to represent a good system in which to study insulin regulated glucose uptake, there are limitations and disadvantages to this model. In order to extend the observations described herein, the subcellular localization of PKC isoenzymes should be examined *in vivo*. Various tissues of animal models such as streptozocin-treated rats should be examined. In addition, the human tissues from diabetic patients (if attainable) should also be examined for the subcellular localization of cPKC in a physiological context of hyperglycemia.

C) *Elucidation of the mechanism(s) of hyperglycemia-induced DAG formation.* It is important to elucidate the molecular mechanism(s) behind the source of sustained PKC activation in diabetes. A therapeutic approach of this type would be even more direct than an isoenzyme-specific inhibition of cPKC.

5.4.2 Molecular/Mechanistic issues

A) *Identification of PKC substrates at the pericentrion.* A requirement for kinase activity for the maintenance of this compartment suggests that there is specific target for PKC at the pericentrion. This could be explored with immunoprecipitation of cPKCβII versus cPKCβI and differential 2-D gel electrophoresis with band cut-out and mass spectrometry. Much care should be exercised in the isolation of pericentrion since it must be done at 37°C. To overcome potential proteolysis of PKC that may arise from performing purification at 37°C, cPKC constructs could be generated with a modified hinge region that is insensitive to calpain I and II-mediated degradation.

B) *Role of PLD.* The studies described herein have shown that PLD is necessary for cPKC translocation to the pericentrion but they did not address the issue of whether PLD is sufficient for translocation. Use of the RNA Interference represents one of the most direct methods to investigate this question. Importantly, a cell line should be chosen that has been well-characterized for PLD isoenzyme expression and that exhibits pericentrion translocation. (e.g. HeLa).

C) *Identification/development of pericentrion-specific inhibitors.* The identification of pericentrion-specific inhibitors should facilitate the study of PKC functions exclusive to the pericentrion compartment. Possible hints may come from the work of Pagano, Maxfield or Hoestra who have shown chain length and saturation characteristics of lipid moieties that are important for sorting to various recycling compartments.

5.5 Concluding remarks

These studies have extended our understanding of PKC biology at the subcellular level and have further served to distinguish the functional role of PKC as an acutely activated signaling enzyme from the role of PKC as a mediator of sustained DAG signals.

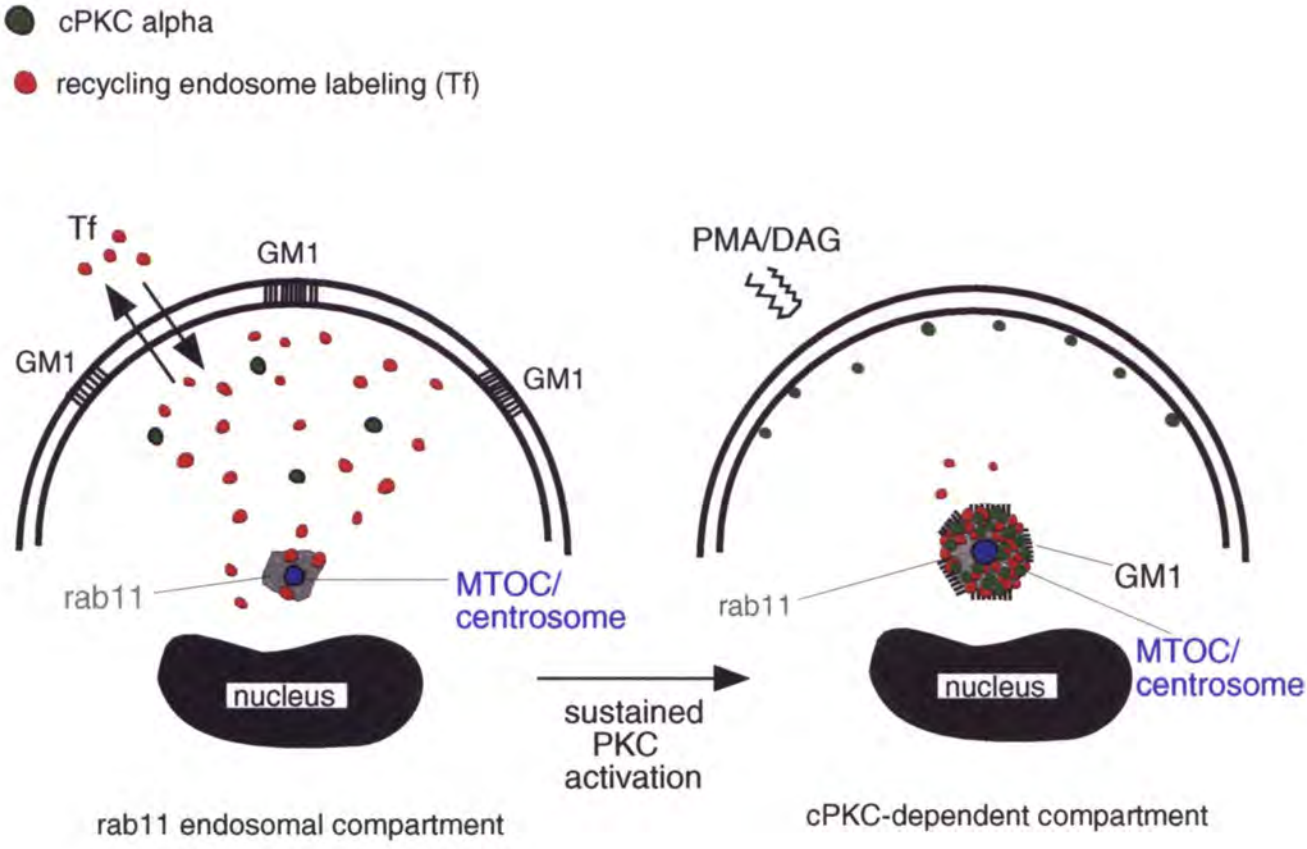


Figure 5.1 rab11 subcompartment versus cPKC-dependent compartment. In the absence of sustained PKC activation, the recycling endosomes (as defined by steady state labeling with fluorescent transferrin) can be found in a diffuse cytoplasmic location with a slight concentration in the juxtanuclear region. The rab11 subcompartment of recycling endosomes is located at the MTOC/centrosome and this localization is independent of PKC activity but dependent on microtubules. At this stage, the predominance of lipid rafts (e.g. GM1, caveolin-1) are located at the plasma membrane. A sustained elevation of DAG or exposure to DAG-mimicking phorbol esters leads to the translocation of cPKC to the plasma membrane and to a juxtanuclear compartment that overlaps the rab11-defined subcompartment. Coincident with cPKC translocation is a reorganization of recycling endosomes to the pericentriolar region and an enrichment of this site with lipid raft components. Unlike rab11, the association of cPKC and lipid rafts at the MTOC/centrosome is dependent upon kinase activity but independent of microtubules. Moreover, translocation and formation of this compartment coincides with the sequestration/retention of recycling components and this is also activity-dependent. Given the distinct properties of the cPKC-dependent from the rab11 subcompartment, I proposed to name the cPKC compartment the *pericentrion*.

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