Studies of Protein Kinase C alpha as a Potential Target for Inhibition of Murine Melanoma Metastasis

By

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2004

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

August 26, 2004
Date

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ABSTRACT

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Pharmacological and genetic techniques were used to establish the role of PKCα in the migration and adhesion of murine melanoma cells (B16F10). PKC is a monomeric serine, threonine protein kinase that exists as a family of isoforms. An isoform profile revealed PKCα as both the most abundantly expressed isoform and the only conventional isoform. The pharmacological approach to these studies involved the use of the PKC inhibitor, dequalinium, which previously was shown to inhibit the catalytic activity of highly purified PKC. Using analogues to the parent compound C10-DECA, PKCα is shown to be the major intracellular target of the most potent analogue C14-DECA. At a concentration of 250nM and in combination with long-wave UV light, which causes irreversible binding of the inhibitor, inactivation of intracellular PKCα is correlated to inhibition of migration. C14-DECA is also shown to inhibit the metastasis of B16F10 cells in C57BL/6 mice. In a genetic approach, B16F10 cells were stably transfected to overexpress a kinase-defective PKCα. In migration assays these cells show reduced migration on both collagen IV and fibronectin. Furthermore, these transfections show reduced phosphorylation of focal adhesion kinase (FAK). The phosphorylation of FAK is likely to be an autocatalytic event that is critical to the formation of focal adhesions. Taken together, these results define PKCα as a mechanistic component in the migration pathway of murine melanoma cells and implicate FAK as a downstream effector of PKCα in this pathway.

ACKNOWLEDGEMENTS

I am grateful to my research and dissertation advisor, Dr. Susan A. Rotenberg for her invaluable support and guidance during my graduate studies. I gratefully acknowledge the members of my committee for their time and their many helpful suggestions. The faculty members in both the Chemistry and Biochemistry Department and the Biology Department at Queens College have encouraged me throughout my many years at Queens College. I thank my many friends who never lost faith in me. Finally and most importantly, I thank my sons, Ryan and Gregory and my husband, Gene for much love and support during my graduate studies.

DEDICATION

This dissertation is dedicated to the memory of Marcos Zapater. When his brief life ended, the world lost a brilliant mind and a most generous person. Oftentimes, I doubted I would complete this dissertation the opportunity to write this dedication pushed me forward.

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Abbreviations Used:

ATP	adenosine triphosphate
BSA	bovine serum albumin
DECA	1,1 - decamethylenebis-4-aminoquinaldinium diiodide
DMSO	dimethylsulfoxide
EDTA	N, N, N', N'-ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis (2-aminoethylether) - N,N,N',N'-tetraacetic acid
PBS	phosphate buffered saline
PKC	protein kinase C
PMSF	phenylmethanesulfonylfluoride
PS	phosphatidylserine
SD	standard deviation
TPA	12-*O*-tetradecanoylphorbol-13-acetate
UV	ultraviolet

INTRODUCTION

Structure-function Relationships of PKC

PKC is a monomeric serine/threonine protein kinase that plays a critical role in cellular signal transduction pathways governing proliferation, adhesion, migration and metastasis (Li et al., 1998, Yoshikawa et al., 2003 and Mostafavi-Pour et al., 2003). Often these phenotypes are dictated by different PKC isoforms of which eleven have been identified in mammalian cells.

The family of PKC isoforms consists of three categories: (1) conventional PKCs α, β1, βII and γ are activated by Ca^{2+} and DAG; (2) novel PKC's δ, ε, η and θ are Ca^{2+}-independent but require DAG for activation; and (3) atypical isoforms ζ and ι are both Ca^{2+}- and DAG-independent isoforms (Newton, 2001). Upon activation, PKC isoforms redistribute from the cytosol to the membrane, a process called translocation. In 1982, workers in the Nishizuka laboratory showed that PKC is the major intracellular ligand for the tumor promoting phorbol ester, 12-O-tetradecanoylphorbol-13-actetate (TPA), a natural product of the plant *croton tigulius* (Castagna et al., 1982). Because of its nanomolar specificity in activating the conservative and novel PKC isoforms, TPA is traditionally used as a tool to implicate PKC activity in signaling pathways. In recent years however, several non-PKC binding proteins of TPA have been identified (Kazanietz, 2002; Yang and Kazanietz, 2003), thus calling into question the interpretation of results obtained with this reagent.

There are four conserved (C) regions of homology among family members. The C1 domain (present in the conventional and novel PKCs) is defined by two zinc-finger motifs in tandem (C1a and C1b domains), each of which contains a conserved pattern of cysteine

and histidine residues that is organized around two Zn^{2+} atoms. Studies have shown that in order to activate the enzyme, DAG must bind in a stereospecific manner at each of the two zinc-finger projections, where it exhibits a significantly higher affinity for the C1b domain (Szallasi et al., 1996). Low nanomolar concentrations of TPA are sufficent to compete with DAG for the C1 domain, resulting in potent activation of conventional and novel PKC isoforms and their subsequent recruitment to the plasma membrane. When incubated longterm (>18h) with TPA, cells downregulate the conventional and novel PKC isoforms via proteolysis. By contrast, the atypical PKC isoforms which possess only one zinc-finger motif are unresponsive to phorbol esters.

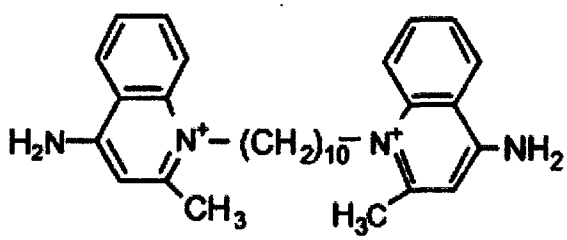
The C2 domain, which contains the Ca^{2+} binding site, is present in conventional PKC isoforms and is missing in the Ca^{2+}-independent PKC isoforms. Evidence from in vitro studies using the isolated C2 domain from PKC indicates that phospholipid (e.g., phosphatidylserine) binds in a Ca^{2+}-dependent manner. Although the stoichiometry of bound Ca^{2+} varies, most C2 domains contain an eight-stranded antiparallel β sheet regions connected by variable loops that define three Ca^{2+} binding sites. Stahelin and Cho (2001) showed that binding of Ca^{2+} resulted in increased membrane association by 37-fold. Using site-directed mutagenesis and monolayer studies, the authors showed that Asp193 and Asp248 play critical roles in conformational changes that lead to inter-domain conformation changes and membrane penetration. Other studies identified asparagine (N189), arginine residues (R216, R249) and a threonine residue (T251) as important in binding of phosphatidylserine to PKC. Site-directed mutagenesis of each residue to alanine showed that the point mutation N189A inhibited Ca^{2+}-stimulated translocation and impaired catalytic activity (Conesa-Zamora et al, 2001). A mechanism by which the C2 domain acts

to modulate membrane binding has been suggested by an X-ray crystal structure. The structural model depicts calcium in a direct interaction with membrane phosphatidylserine, and thereby allows the C2 domain to penetrate the membrane (Verdaguer et al., 1999).

The role of the C2 domain in the membrane targeting of conventional PKC's has been investigated. A prominent example is the interaction of the C2 domain with a cellular protein called RACK (Receptor for Activated C-kinase). RACK proteins bind and therefore sequester PKC to specific locations in the plasma membrane and the cytoskeleton.

In the amino-terminal region of conventional PKC isoform is a sequence called the pseudosubstrate site that is in physical contact with the catalytic domain and upon the binding of activators is released to enable the binding of protein substrates (Mellor and Parker, 1998). A synthetic peptide modeled on a modification of the pseudo substrate sequence in which Ala-25 is replaced with a serine residue, has been employed as a potent peptide substrate in *in vitro* assays (House and Kemp, 1987).

Dequalinium



Dequalinium (DECA)

Scheme 1: Structure of Dequalinium.

1,1[1]- Decamethylenebis-4-aminoquinaldinium diiodide (DECA) was first proposed as an anticarcinoma agent by Chen and coworkers (Weiss et al., 1987).

Because of their abnormal metabolism, cancer cells exhibit high membrane potentials (Pedersen, 1978). Therefore the Chen group explored the possibility that the di-cationic nature and lipophilicity of DECA would enable cells to concentrate this molecule to a greater degree in cancer cells. Indeed, the Chen group found that low micromolar concentrations of DECA was selectively concentrated in the mitrochondria of many cell types and was shown to inhibit aerobic respiration of many cell types (Bernal et al., 1983). Because of its selective uptake by cancer cells, DECA was more effective in slowing tumor growth than other well-established anti-cancer drugs including doxorubicin, 5-fluorouracil, cisplatin and methotrexate (Weiss et al., 1987). The drug inhibited the growth of implanted rat colon carcinoma in rats and human colon carcinoma in nude mice. Interestingly, DECA may not be concentrated in the mitochondria of all types of cancer cells. L1210 leukemia, Lewis lung carcinoma, and notably B16 melanoma cells did not retain rhodamine 123, a mono-cationic dye that is used as a mitochondrial probe for most cells (Bernal et al., 1983). This finding suggests that the DECA di-cation may accumulate in the cytoplasm of B16 cells where it would be available to interact with soluble proteins like PKC.

In 1990, DECA was shown to be a fairly potent inhibitor of PKC in vitro (IC_{50} = 10 μM) (Rotenberg et al., 1990). Analysis of N-terminally truncated mutants of PKCα for inhibition by DECA indicated that the drug interacts primarily with the catalytic domain of PKCα but can also interact with the regulatory domain (Rotenberg et al., 1998). However, potent inhibition of the catalytic fragment of PKCα showed that inhibition of catalytic activity does not require the regulatory domain interaction (Rotenberg et al., 1998).

An interesting property of DECA is that when irradiated with longwave UV light, it binds irreversibly to the target protein. This effect was first demonstrated with

mitochondrial ATPase (Zhuo et al., 1988). This approach was used in the Rotenberg lab to show that DECA interferes with the translocation of PKCα in human breast adenocarcinoma cells (Rotenberg and Sun, 1998). Interference by DECA requires the RACK1 binding domain of PKCα (located in the C2 region of the regulatory domain). This interaction probably impedes the interaction of enzyme with membrane components and occurs in parallel with inactivation of PKCα catalytic activity (Rotenberg and Sun, 1998). In the present work, newly synthesized analogues of DECA will be used to show structure-activity relationships of PKC inhibition in B16 melanoma cells with concomitant effects on the PKC-mediated phenotypes of adhesion and migration (Sullivan et al., 2000).

PKC and Integrins

Migration is critical to the metastasizing cell and draws upon adhesion as a means to engage the substratum for productive locomotion. In order to better understand the role of PKC in the metastasis of melanoma cells, both cell adhesion and cell migration were addressed as phenotypes that are tied to cellular metastasis. A discussion of the relationship between cell adhesion and cell migration follows.

Cell migration requires a coordinated cycle of cytoskeletal-mediated process extensions (filopodia), that form adhesive contacts at the leading edge of the cell while breaking adhesive contacts at the trailing edge. An important study by Palecek et al. showed that cell migration speed can be predicted by cell-substratum adhesion strength and this in turn is determined by three separate factors. These factors include substratum ligand level, cell integrin expression level, and integrin-ligand binding affinity. Altering one of these

factors changes the extent of adhesion and subsequently the rate of migration (Palecek et al., 1997).

To engage the substratum in a productive manner, the motile cell must first form focal adhesions. Focal adhesions provide a link between the extracellular matrix and the actin cytoskeleton that gives the cell sufficient traction to generate a force at the leading edge of the cell. Weak leading edge adhesions do not allow the cell to generate enough traction for motility. However, if the attachments are too strong migration rate is reduced due to an inability of the cell to cycle between the necessary adherent/nonadherent states.

Integrins comprise a family of transmembrane adhesion receptors that play a large part in determining the strength of focal adhesions at the leading edge of the motile cell. Several members of the integrin family also mediate cell-cell adhesion. Integrins are heterodimers composed of one α subunit and one β subunit. To date, eighteen α subunits and eight β subunits have been identified. These subunits pair to form twenty-four known combinations (Petit and Thiery, 2000). While integrins do not contain enzymatic activity per se, their binding interactions with proteins that interact with the integrin's short cytoplasmic tails will convey signaling events. The extracellular binding activity of integrins is regulated from inside the cell (known as inside-out signaling), while the binding of the extracellular matrix elicits signals that are transmitted into the cell (outside-in signaling) (Schlaepfer et al., 1998).

While most integrins bind ligands that are part of the extracellular matrix, certain integrins also bind soluble ligands or adhesion molecules on adjacent cells. Clustering of integrins in the plane of the cell membrane allows for the formation of a cytoskeletal signaling complex that promotes the assembly of actin filaments. The reorganization of

actin filaments into larger stress fibers in turn causes more integrin clustering, thus enhancing the matrix binding and organization of integrins in a positive feedback system (Giancotti and Ruoslahti, 1999).

A number of interactions between PKC and integrins have been reported. A notable example is the interaction between PKCα and β1 integrin, whose extracellular matrix ligands include collagen, fibronectin, and laminin (Ng et al., 1999). Ng et al. showed that the interaction between PKCα and β1 integrin promotes the movement of integrin to the tip of the filopodia in MCF-7 cells that had been engineered to overexpress PKCα. This interaction provides a mechanism by which PKC could influence the rate of migration. Following TPA treatment, a redistribution of PKCα to the cell surface was observed. MCF-7 cells overexpressing PKCα were found to have increased migration on fibronectin, laminin, and collagen. Cells overexpressing constructs that lack the PKCα regulatory domain however did not bind to fibronectin, but binding of PKCα to β1 integrin was unaffected. Also, a kinase-defective PKCα did not support internalization of the ligand-receptor complex. The authors concluded that migration of MCF-7 cells is dependent on recycling of the integrin-ligand complex, which requires PKCα activity (Ng et al. 1999). Parsons et al. mapped the interaction between PKCα and β1 integrin through site-directed mutagenesis of PKC. The β1 integrin cytoplasmic tail binds the V3 region (aa 312-325), which lies in the hinge region between the regulatory and catalytic domains (Parsons et al, 2002). Cell migration in a phorbol ester gradient is enhanced even in the absence of the kinase domain. However the kinase domain is required for haptotactic migration towards β1 integrin. The authors suggest that β1 integrin directs PKCα to the leading edge of the

cell, an idea that fits well with the work of Ng et al mentioned above. Once the β1-integrin/PKC complex has been formed, events such as actin polymerization can occur.

The dependence of migration on the association between PKCα and β1 integrin was also seen in multiple myeloma cells. In this system, vascular endothelial growth factor (VEGF) induced translocation and activation of PKCα in a phosphoinositide-dependent manner. Following VEGF treatment in the presence of bisindolylmaleimide (an inhibitor of conventional PKC isoforms) and a β1 integrin blocking protein, migration of these cells was inhibited (Podar et al, 2002). Interestingly, non-motile, non-transformed human breast epithelial cells (MCF-10A) that had been genetically engineered to over-express PKCα consequently acquired high migration activity and exhibited upregulation of β1 integrin protein (Sun and Rotenberg, 1999).

The linkage between PKC and β1-integrin may involve RACK1. This ubiquitously expressed protein was identified by its ability to bind activated conventional PKC isoforms (Stebbin and Mochly-Rosen, 2001; Schechtman and Mochly-Rosen, 2001). Liliental and Chang used a yeast two-hybrid screen to show that the cytoplasmic domain of β1-integrin interacts with RACK1 (Liliental and Chang, 1998). Full-length RACK was found to bind β1 integrin only after TPA treatment, thereby implicating the involvement of TPA-sensitive PKC isoforms (conventional and novel).

PKC has been implicated in both "inside-out" and "outside-in" signaling pathways in muscle cells, namely through activating interactions with the α4 and α5 integrins, which promote adhesion to fibronectin. When muscle cells that were deficient in α5 integrin were treated with PMA (phorbol myristoyl acetate; same as TPA), the cells showed rapid adhesion to fibronectin. Increased adhesion to fibronectin is due to activation of αβ4

integrin, suggesting an interaction between this integrin and PKC in "outside-in" signaling pathways. Since PKC is required to activate integrins, a positive feedback loop is established, where the external environment elicits PKC-dependent responses from the cell. Activation of PKC leads to activation of integrins that in turn leads to the further establishment of focal adhesion (Distanik and Rando, 1999).

In A375-SM melanoma cells, PKCα activity is associated with ligand engagement of the α5β1 (a fibronectin-binding integrin) but not with ligand engagement of α4β1 (also a fibronectin-binding integrin). Inhibition with bisindolymaleimide, an inhibitor of PKC catalytic activity, reduced α5β1-mediated focal adhesion formation and cell migration but had no effect on α4β1pathways (Mostafavi-Pour et al., 2003).

These studies demonstrate complex interactions between PKCα and a number of integrins that suggest a critical role for PKCα in signaling pathways that govern cell migration and therefore metastasis of cancer cells. Since integrins have no catalytic activity per se, it is their association with adapter, adhesion, and scaffolding proteins that positions PKCα physically for interaction with specific substrate proteins and consequently for activation of one or more signaling pathways that actuate cell movement.

PKC and Focal Adhesion Proteins

In addition to a number of studies linking PKC to integrins, interactions with adapter and scaffolding proteins that are recruited to focal adhesions have also been reported. Focal adhesions are distinct sites where integrins and adaptor proteins physically link the actin cytoskeleton to the substrate in the extracellular matrix.

Focal adhesion kinase (FAK) is recruited to the nascent focal adhesion. A C-terminal focal adhesion targeting (FAT) sequence is necessary and sufficient to localize FAK or a FAT–containing chimeric protein to a focal adhesion. The FAT sequence, found by x-ray crystallography to be a four-helix bundle, is required for binding to the adhesion/adaptor protein paxillin (Hayashi et al., 2002), a known substrate of PKC (DeNichilo and Yamada, 1996). Upon binding to paxillin, FAK auto-phosphorylates on Tyr^{397} and enables binding of src kinase via its SH2 domain to FAK. Src kinase then phosphorylates several focal adhesion components including paxillin and tensin. Src also phosphorylates FAK at Tyr^{925}, which creates a binding site for the complex of Grb and mSOS (a ras-like guanosine 5'-triphosphate exchange factor) (Petit and Thiery, 2000). The above interactions are summarized in the scheme below:

Scheme 2: Formation of Focal Adhesions

FAK-PKC substrate(e.g. paxillin)

↓

FAK autophosphorylation at Y397

↓

Src binds FAK via SH2 region

↓

Src directed phosphorylation of FAK at Y925

↓

Binding of Grb via FAK SH2 region

↓

Focal Adhesion

Although tyrosine phosphorylation predominates in the focal adhesion, a number of studies have given evidence of serine/threonine phosphorylation. For example, when a serine phosphorylation site in paxillin is mutated, localization of paxillin to the focal adhesion and consequent adhesion to fibronectin are prevented in CHO.KI cells (Brown et al., 1998). Since PKC also localizes to focal adhesions (Jaken et al., 1989), it is possible that PKC is responsible for at least part of the observed serine phosphorylation in paxillin. Another phosphorylation target of PKC may lie in vinculin, a cytoskeletal protein that contains a binding site for paxillin and can undergo phosphorylation by PKC *in vitro*. However, evidence for vinculin as a cellular substrate of PKC has not been demonstrated (Weekes et al., 1996).

In the case of filamin, a scaffolding protein present in focal adhesions, both PKCα binding sites and PKCα-dependent phosphorylation has been demonstrated in HeLa cells. Interestingly, other than allowing the cells to adhere to collagen, no other PKC-dependent binding phenomena were observed. Phosphorylation of filamin influences binding to adhesion and/or scaffolding proteins or influences interaction with the actin cytoskeleton (Tigges et al., 2003). Filamin interacts with Rho family GTPases leading to activation of signaling pathways implicated in cell migration (Ohta et al., 1999).

There is general agreement that PKCα plays a role in cell adhesion and migration. However, the precise details of many of the mechanisms remain unknown. Elucidation of these mechanisms is complicated by multiple PKC isoforms that may have over-lapping roles in pathways that control these phenotypes. Further complexity lies in the nature of focal adhesion formation which requires a complex organization of integrins and adhesion

proteins that enable transient interactions with the extracellular matrix. The work presented here on the role of PKCα in metastatic melanoma cells is intended to contribute to the ongoing study of PKC isoforms in adhesion and migration.

PKC and Metastatic Mouse Melanoma Cells

The B16 mouse melanoma cell line has proven to be a useful model system in which to study the role of PKC in metastasis. B16 cell lines have been established that show varying degrees of metastatic potential (F1 low metastatic potential and F10 high metastatic potential). Metastatic potential is judged by the number of lung nodules resulting from intravenous injection of the cells into syngeneic C57 BL6 mice. A correlation between PKC activity and metastatic potential in B16 cells was reported (Gopalakrishna and Barsky, 1988). Higher levels of intracellular PKC have been found in the membrane fraction of B16 cells with higher metastatic potential suggesting that, in highly metastatic cells, PKC undergoes endogenous activation and translocation. When weakly metastatic B16F1 cells are treated with phorbol ester (TPA) for one hour (which promotes translocation of PKC), an increase in the metastastic potential resulted and reached a level comparable to the higher metastatic F10 cells (Gopalakrishna and Barsky, 1988). These studies did not identify specific PKC isoforms in this process. A major focus of this work is to establish PKCα as the key target of DECA and in doing so, clarify its mechanism of action in migration and metastasis of melanoma cells.

Hypothesis

This project was undertaken to test the hypothesis that PKCα drives the metastasis of melanoma cells. A tool to be used in testing this hypothesis is DECA because it has been

shown to cause irreversible inhibition of purified human PKC isoforms when used in combination with long-wave UV light. Since unspecified PKC isoforms have been implicated in the metastasis of murine melanoma cells, studies that utilize a kinase-defective PKCα mutant will establish the specific contribution by the α-isofrom to the metastatic phenotype and demonstrate the use of dequalinium as an anti-metastatic agent.

MATERIALS AND METHODS

Conditions for Cell Culture

B16F10 murine melanoma cells were obtained from Prof. Ian Hart, U.K. Cells were cultured at 37°C and 5% CO_2 in RPMI with 10% fetal calf serum, 1% penicillin/ streptomycin, and fungizone present at 0.125 μmg/ml. Stock cultures of cells were grown to 75% confluence in 10-cm^2 tissue culture dishes. Cells were passaged by rinsing the subconfluent monolayer twice with phosphate buffered saline (PBS). A 0.25% trypsin-0.02% EDTA solution in PBS was applied to the cells for one minute at 37°C, 5% CO_2 for one minute. After the incubation the cells were removed with gentle pipetting and split 1:10 or 1:20 depending on the planned experiment. After 10 passages, a new culture was initiated from frozen cell stocks. All cell culture media, phosphate buffered saline, trypsin, fetal bovine serum was purchased from Gibco-BRL (Gaithersberg, MD).

Treatment of Cells with Dequalinium and UV light

B16F10 melanoma cells were grown in a monolayer to 75-80% confluence on 150mm plates. The cells were washed twice with PBS. Cells were incubated for 1 h at 37°C and 5% CO_2 with the indicated concentration of dequalinium analogue dissolved in dimethyl sulfoxide (DMSO) or 0.05% (v/v) DMSO. The cells were washed twice with PBS and the medium was replaced with serum-free RPMI. The cells were irradiated for 5 minutes at room temperature (on a laboratory bench) with a 365 nm UV lamp (American Ultraviolet Co., Murray Hill, NY) which delivered an intensity of 1200 μW/cm^2. During irradiation, the dish lids were removed and gentle shaking was applied. Following

irradiation, the cells were washed twice with PBS and prepared for specific assays (Sullivan et al. 2000).

Isolation of PKC Activity

Treated cells were harvested by scraping into PBS and centrifuged at 552xg for 5 minutes. The pellets were resuspended in Buffer A: 20 mM Tris pH 7.4, 2 mM EDTA, 2 mM EGTA and 1 mM 2-mercaptoethanol with protease inhibitors (0.25 mM phenylmethanesulfonylfluoride (PMSF), 10 μg/ml soybean trypsin inhibitor, 10μmg/ml leupeptin and 10μg/ml calpain inhibitor II). The cells were lysed by douncing 40X and centrifuged at 552 x g for 10 minutes to remove cell debris. The lysates were diluted 5-fold with buffer A. The lysates were applied to a Bio-Rad disposable column that contained 0.5 ml of DEAE-Sephacel that had been equilibrated in Buffer A. After the sample was applied to the column, the column was washed with 5 ml of Buffer A. PKC was eluted with 1.0 ml 150 mM NaCl and assayed for PKC activity (Rotenberg et al., 1990). Protein concentration was determined with the Bio-Rad protein reagent using bovine serum albumin (BSA) as standard.

Assay of Protein Kinase C Activity

The PKC activity of the eluates was determined by the difference of ^{32}P transferred from [γ-^{32}P]-ATP to a modified pseudosubstrate peptide sequence in the presence and absence of the activators TPA and phosphatidylserine. The reaction medium (0.12ml) consisted of the following: 20 mM Tris pH 7.4, 10 mM $MgCl_2$, 26.6 μM ^{25}SER peptide, and PKC sample (typically 20 μg). Under activating conditions, the reaction mixture included

0.5 mM $CaCl_2$, 83μg phosphatidylserine, and 1.0 μM TPA. For Ca^{2+}-independent PKC activity, 0.5 mM EGTA replaced $CaCl_2$. Each sample was assessed in triplicate with and without activating cofactors. The reaction mixtures were incubated in a 30°C water bath for 5 minutes before the reaction was initiated by the addition of 66 μM [γ-^{32}P]-ATP (in 10μl). The reaction was quenched by transferring 100 μl of the reaction medium to a 2 x 2 cm square of phosphocellulose paper, and the square was immediately immersed in a liter of distilled H_20. After all reactions were terminated, the squares were washed 5 times in 1 liter ddH2O and each square was placed in a vial containing scintillation fluid. The samples were counted in a beta scintillation counter (Searle Analytic) and the results of triplicate values were averaged. The numerical difference in cpm measured for activated and nonactivated conditions was taken as PKC activity (as described in Rotenberg et al., 1990).

Western Blot Analysis

Samples of known protein content were subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently transferred electrophoretically to a PDVF filter (Towbin et al., 1979). To each sample was added an aliquot of 5X sample buffer (50% glycerol v/v, 1% SDS, 0.05 % bromophenol blue, 0.4 M Tris pH 6.8, 2mM 2-mercaptoethanol) diluted to 1X sample buffer, heated for 5 min at 95°C, and subjected to SDS-electrophoresis, followed by transfer to a PDVF membrane. The membrane was placed in a 10-trough manifold (Pharmacia Biotech). Mouse primary antibodies specific for individual PKC isoforms (Transduction Laboratories, Lexington, KY) were applied to each trough and incubated for 2 h with gentle rocking. After removing the antisera and washing the membrane with PBS with 0.1% Tween-20 and 1% BSA, the secondary antimouse IgG

(Santa Cruz Biotechnology, Santa Cruz, CA) was applied for one hour. After washing with PBS (3x5 minutes) the blot was developed by chemiluminescence (Amersham Pharmacia Biotech).

Assay of Adhesion

Adhesion assays were performed in 24-well tissue culture dishes. The bottom surface of each well was coated with matrigel (43.5 μg/cm), fibronectin (2 μg/cm), or collagen IV (2 μg/cm). The dishes were incubated for 1 h at 37°C at 5% CO_2. The wells were washed 3 times with 1x PBS. In order to block any nonspecific binding, 1% BSA in PBS was added to each well and the dish was incubated at room temperature for 30 minutes. Cells were seeded at 1.5x 10^5 cells/well in serum-free RPMI. Following incubation for 30 minutes at 37°C and 5% CO_2, each well was washed three times with PBS and adherent cells were counted immediately. In eight fields chosen at random, cells were counted using a Nikon Diaphot-TMD. Each condition was counted in triplicate and the values were averaged. The results were analyzed statistically using ANOVA available through Sigmastat software (Computer Associates International, Inc. (Islandia, N.Y.). Counts for the fields were entered and the software calculated the standard deviation and P values for each experiment.

Assay of Migration

Migration assays were performed in triplicate in 12-well Costar polycarbonate Boyden chambers (transwells). The distal surface of the microporous membrane was coated with 35μg of Matrigel and incubated at 37°C for one hour. Cells were resuspended in 250 μl of serum-free RPMI and were seeded at a density of 10^5 cells/well. To the lower

chamber was added 800 µl of RPMI containing 0.1% BSA and 1% FBS. The plate was incubated for 3 hours at 37°C and 5% CO_2. Following the incubation, the walls of the upper chamber were carefully wiped using a cotton swab and the cells on the distal surface were stained using the H and E Method (Fisher Diagonstics Leukostat). Each experiment consisted of triplicate wells, in which eight fields per well were chosen at random and counted using a Nikon Diaphot-TMD inverted microscope at 400x magnification. For a single experiment, cell counts were averaged for each of three wells and the values for all three wells were averaged. Each experiment was performed at least three times. Results were analyzed for statistical significance using ANOVA (Sigmastat software).

Transfection and isolation of a kinase-defective mutant of PKCα

A constitutive expression mammalian vector, pEFneo, containing the cDNA of a polyhistidine tagged, kinase-defective mutant of bovine PKCα (Kampfer et al. 1998), was transfected into B16 F10 cells with Lipofectamine Plus reagent (Life Technologies, Inc.). Following their selection with G418 present at 500 µg/ml, stable transfectants were maintained at an antibiotic concentration of 250 µg/ml. To demonstrate the expression of mutant PKCα, cell lysates were prepared, and polyhistidine-tagged protein was isolated by metal chelate affinity chromatography (Pharmacia Biotech). Lysates were prepared in a buffer containing 20 mM Na_2HPO_2, 0.5 mM NaCl, 20mM imidazole and incubated with Ni-charged chelating Sepharose for 30 minutes at room temperature. Polyhistidine-tagged PKCα was eluted with 500 mM imidazole, and the sample volume was reduced to 0.5ml by using a centricon-10 concentrator (Amicon). Protein concentration was determined with the Bio-Rad protein reagent using bovine serum albumin (BSA) as standard.

After adding sample buffer and heating at 95°C for 5 minutes the sample was analysed by Western Blot analysis (22.6 μg of protein/lane) using PKCα-specific antisera (Transduction Labs) and chemiluminescence detection (Amersham). Quantitive analysis of chemiluminescence signals was carried out by two-dimensional scanning densitometry (Molecular Dyanamics).

Cell Fractionation

To prepare cells for fractionation, cells were grown on 150 mm plates to 80% confluence. The cells were washed twice with PBS containing protease inhibitors and then scraped into 5 ml of PBS containing protease inhibitors. The cells were centrifuged for 15 minutes at 1000 x g and resuspended in ice-cold homogenization buffer (20 mM Tris pH 7.4, 250mM sucrose, 2mM EDTA, 10 mM 2-mercaptoethanol). Cells samples were kept on ice while they were lysed by dounce homogenization (40 strokes on ice). The homogenate was centrifuged at 100,000 x g for 1 h. The supernatant was taken as the soluble fraction while the pellet represented the particulate fraction. The pellet was resuspended in 0.5 ml of buffer (50 mM Tris pH 7.4, 5 mM EDTA, 5 mM EGTA, 0.1% TX-100 and 5 mM 2-mercaptoethanol) and incubated on ice for 30 minutes with frequent agitation using a vortex mixer. Both fractions were assayed for protein content and Western blot samples were prepared by adding sample buffer.

PKC Translocation Assay

Cells were grown to 80% confluence and washed twice with PBS. The appropriate concentration of dequalinium analogue was added to serum-free RPMI and the cells were placed at 37°C for 30 minutes. Cells were irradiated with 365 nm long wave UV light.

Following this treatment cells were washed twice with PBS and the medium was replaced with serum-free RPMI containing 1μM TPA or DMSO. After incubation for 1 h at 37°C, 5% CO_2, the cells were washed twice and resuspended in 5 ml PBS containing protease inhibitors. The samples were fractionated as described above. Particulate and soluble fractions were analyzed for protein content and subjected to Western Blot analysis (8.5 μg of protein/lane).

Immunoprecipitation of PKC α treated with 250nM DECA and UV light

Cells were treated with a dequalinium analogue as described above and lysed in immunoprecipitation buffer (IP buffer): 50mM Hepes pH 7.5, 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 10 mM NaPPi, 5 mM EDTA, 1 mM Na_3VO_4, and 1 mM PMSF. To lysates containing equal amounts of protein, 5μg MC5 antibody was added and the samples were rotated overnight at 4°C. Protein A/G agarose (25μml settled volume) was added and incubation was continued for another 3 hours. The immunocomplexes were collected by centrifugation and the pellets were washed three times with IP buffer. The complexes were assayed for PKC activity in presence of activators as described above with the following modification: The duration of ^{32}P transfer proceeded for 30 minutes and PKC activity was taken as the difference between ^{32}P transferred by the immunocomplexes and a control consisting of a lysate treated only with protein A/G-agarose.

Experimental Metastasis

Cells were isolated from ex vivo tumors by pipeting the cells loosened from a metastatic nodule. The cells were placed in complete RPMI medium as described under

"cell culture". Clones usually reached 30% confluence after 5-7 days in culture. At that time cells were passaged and cell stocks were stored at –140°C. C57BL/6 mice were housed in a pathogen-free animal facility under conditions that exposed the mice to a 12-hour light-dark cycle. The animals were injected i.p. with 0.2 ml (2.5 mg/ml) C14-DECA in 0.36% sodium citrate or vehicle only every other day, for one week prior to the tail vein injection of B16F10 cells. Eight animals of comparable age and weight were used in each experimental group. In preparation for tail vein injections, mice were tranquilized with 0.2 mg of acepromazine via i.p. injections. After a 10-20 minute period, it was verified that a deep pain reflex was absent in each animal. B16F10 cells obtained from ex-vivo culture were injected into the tail vein (10^5 cells/ 0.2 ml). Animals were treated IP with vehicle or 5 mg/kg of C14-DECA on the day of the tail vein injection and every other day thereafter for the length of the 2-3 week experiment. At the end of this period, the mice were sacrificed by CO_2 asphyxiation. The lungs were removed and placed in 10% formalin until the metastatic nodules could be counted under a dissecting microscope. The results were analyzed statistically by the Wilcoxon signed rank test. For experiments in which genetically-engineered cells (α6, vector control) were injected, the procedure was identical except that no DECA was injected.

Fluorescence Microscopy

Lab-tech chambered coverslips were coated with 5μg fibronectin for one hour at 37°C. Cells were plated on coverslips at low densities and were allowed to adhere overnight at 37°C and 5% CO_2. Cells were washed with pre-warmed PBS and placed for 10 min in fixative buffer (4% formaldehyde, 0.2% TritonX-100, 60mM PIPES, 25 mM

HEPES, 10 mM EGTA and 3 mM $MgCl_2$ pH 6.1). After washing the cells with PBS, the fixed cells were blocked with 0.5 ml PBS containing 1% BSA for 30 minutes before the addition of 2 units of rhodamine-phalloidin (1.4 units/ml PBS) (Molecular Probes, Inc.). Gel mounting medium (Biomeda Corp., Foster City, CA.) was applied to the coverslips and allowed to dry overnight. The cells were visualized using a Meridian Ultima confocal fluorescence microscope equipped with an argon ion laser and 530/30 and 580/30 band pass filters.

Assay of FAK autophosphorylation

Vector control cells or cells expressing a dominant-negative PKCα were grown on 60mm plates that had been coated with matrigel (43 μg/cm), fibronectin (2 μg/cm) or collagen IV (2 μg/cm). The excess substrate was aspirated and the dish was washed with PBS. The cells were plated at density of 10^5 cells/ml (1 ml/plate) and the cells were allowed to adhere for 30 minutes. After the incubation, the cells were washed twice with PBS containing protease inhibitors and scraped into 1 ml of PBS containing protease inhibitors. The cells were centrifuged for 15 minutes at 1000xg and resuspended in 0.25ml cold homogenization buffer (20 mM Tris, pH 7.4, 5 mM EDTA, 5 mM EGTA, 0.1% TX-100 and 5mM 2-mercaptoethanol). Cells were lysed by dounce homogenization (40 strokes on ice), followed by removal of cell debris and unlysed cells by low speed centrifugation. The resulting lysates were assayed for protein concentration and the samples were applied to SDS-PAGE in sample buffer (40μg/lane). After Western transfer, the membrane was assayed with an antibody directed against the autophosphorylation site of FAK at Tyr^{397} (Upstate Biolabs, Inc.).

RESULTS

I. Evidence that PKCα is an intracellular target of DECA

The goal of these experiments was to establish PKC as an inhibitory target of DECA in B16F10 mouse melanoma cells and to identify a specific PKC isoform as the most likely target.

Isoform Profile and Phosphotransferase Activities of B16F10 Cell Lysates

Levels of expression and catalytic activities of the subclasses of PKC isoforms in B16F10 cells were characterized. To determine the isoform profile of these cells, Western blot analysis was carried out with whole cell lysates (or DECA-Sephacel eluates) and revealed those isoforms that were present. As shown in Figure 1, conventional isoform α, novel isoforms δ, ε, θ, and atypical isoforms λ and ζ were observed.

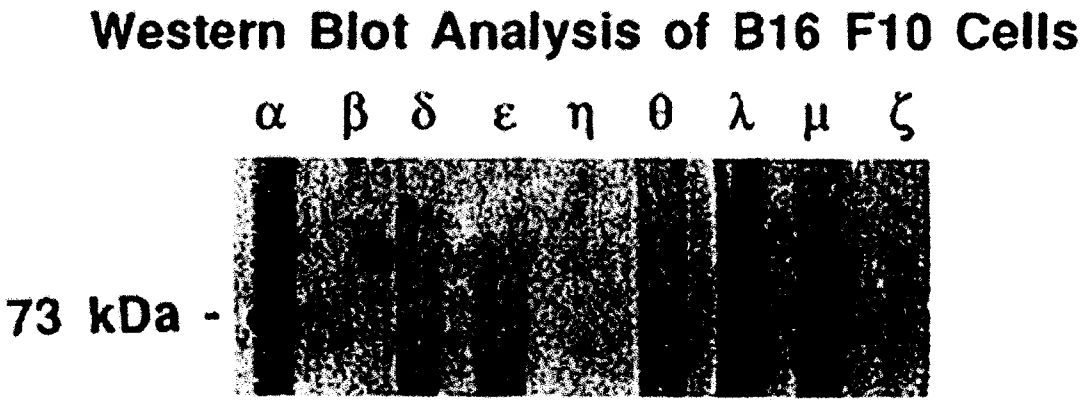


Figure 1. Analysis of PKC isoform expression by Western Blot. Whole cell lysates were prepared from B16F10 cells and analyzed with PKC isoform antisera (46 μg protein/lane).

Of these PKC isoforms the most abundant isoform was PKCα. Characterization of PKC catalytic activity was carried out to establish the extent to which a specific class of PKC (Ca^{2+}-dependent vs. Ca^{2+}-independent) contributed to the total PKC activity. In these

experiments, the intracellular PKC activity of a crude lysate was partially purified by DEAE-Sephacel chromatography and assayed with phosphatidylserine (PS) in the presence and absence of Ca^{2+} and TPA. Chromatography accomplished both partial purification and removal of TX-100 which can interfere with the assay of catalytic activity. For these assays, a peptide substrate was used that corresponds to a modified pseudosubstrate segment in PKC (as described in the "Methods"). This substrate peptide can be utilized by both Ca^{2+}-dependent and Ca^{2+}-independent isoforms. As shown in Figure 2, the results of this experiment indicated that the catalytic activity is 7-fold higher in the presence of Ca^{2+} and TPA than activity measured in the absence of Ca^{2+}.

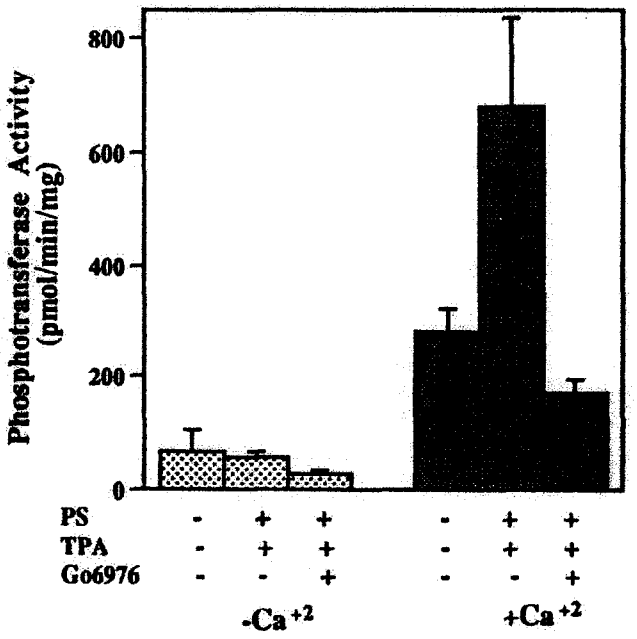


Figure 2. Identification of PKC subclasses present in B16F10 cell lysates partially purified by DECA-Sephacel chromatography. The specified conditions included one or more of the following components: 0.5 mM Ca^{2+}, 83 μg/ml TPA, and 1μm Go6976. Ca^{2+}-independent conditions included 0.5mM EDTA. The error for these experiments was with 10-15%. Error bars, S.D.

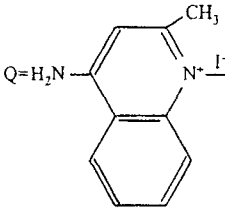
This finding indicates that PKC activity in these cells is primarily due to Ca^{2+}-dependent, TPA-responsive isoforms. In addition, when these lysates were assayed in the presence of Go6976 (a PKCα specific inhibitor), only 25% of the activity remained. As

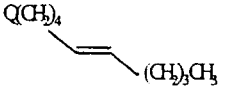
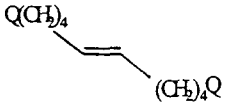
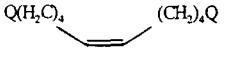
the only detectable Ca^{2+}-dependent isoform present in B16F10 cells (Figure 1), PKCα represents the major contribution to total PKC activity detected in these cells.

Development of DECA analogues

Analogues of the C10-DECA parent compound were developed by Rotenberg and Qin (Qin et al., 2000) with the goal of increasing the potency of the compound as a PKC inhibitor. These analogues differ by the length of the hydrocarbon bridge between the two aminoquinaldinium moieties. The potency of the analogues with recombinant human PKCα in vitro was quantified as the concentration that produces 50% inhibition (IC_{50}). Shown in Table 1 are IC_{50} values for DECA analogues having linkers of C_6 up to C_{16}. The most potent analogues were C14-DECA (IC_{50} = 2.6μM) and C16-DECA (IC_{50} = 2.8μM). To study linker-length dependent effects on the B16F10 cell behavior, the C10-, C12-, and C14-DECA analogues were employed.

Table 1: Linker Distance and Geometry of DECA Analogues Determine Inhibitory Potency with Protein Kinase Cα



Compound		IC50 (μM)[b]	Spacer (Å)[c]	
1a	$Q(CH_2)_6Q$	54 ± 8	6.4	
1b	$Q(CH_2)_8Q$	25 ± 9	8.8	
1c	$Q(CH_2)_{10}Q$	11 ± 5	11.5	parent compound
1d	$Q(CH_2)_{12}Q$	5 ± 2	14.0	
1e	$Q(CH_2)_{14}Q$	2.6 ± 0.2	16.6	
1f	$Q(CH_2)_{16}Q$	2.8 ± 0.2	19.1	
2	$Q(CH_2)_9CH_3$	117 ± 8	---	monomer
3	QCH_3	3590 ± 510	---	monomer
4		137 ± 6	---	monomer
5		12 ± 3	11.1	
6		52 ± 12	10.5	

(Note: All assays performed by Dr. Susan A. Rotenberg) (Qin et al., 2000)

Dequalinium is non-toxic

Proliferation and viability studies were carried out to determine if C10-DECA has adverse effects on the viability of B16F10 cells. A concentration of 2μM C10-DECA with or without UV for 30 min produced no significant effect on the proliferative capacity of these cells for up to 96h (Figure 3).

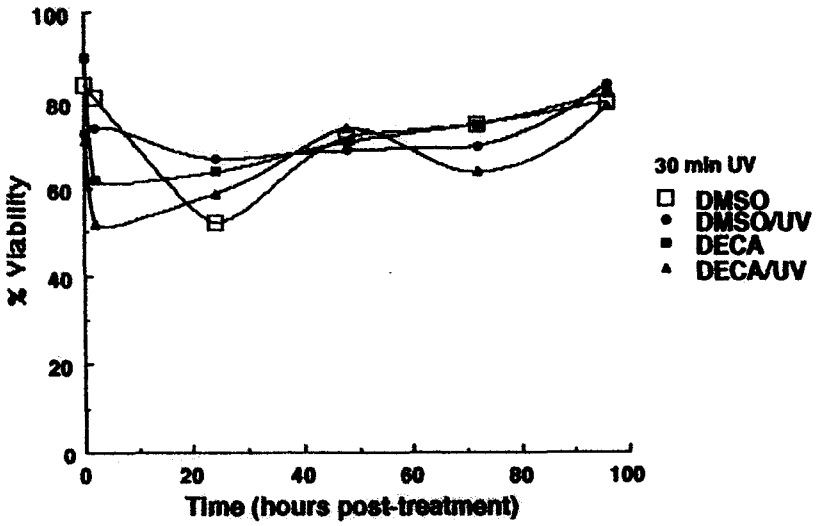


Figure 3. Proliferation of B16F10 cells after C10-DECA UV treatment. B16F10 cells were treated with 2μM C10-DECA or DMSO followed by no treatment or UV irradiation. Cells were replated on to 60-mm tissue culture dishes at 10^4 cells per plate. At the indicated times cells were trypsinized and the number of viable cells was determined by trypan blue exclusion.

Demonstration of irreversible inhibition of PKC activity by DECA when combined with UV.

The C10-, C12- and C14- analogues were used to determine whether there was a corresponding linker-length dependence for inhibiting PKC activity. The cells were treated with 250nM C10-, C12- and C14-DECA followed by 5-min irradiation with 366 nm UV light. Cells were immediately lysed, partially purified by chromatography on DECA-Sephacel, and the eluates were assayed for PKC catalytic activity. As shown in Figure 4, a concentration of 250nM C10-DECA plus UV light was shown to inhibit 20-30% of the Ca^{2+}/PS/TPA-dependent PKC activity, whereas 250nM C14-DECA analogue inhibited 40-60% of the Ca^{2+}/PS/TPA-dependent PKC activity .

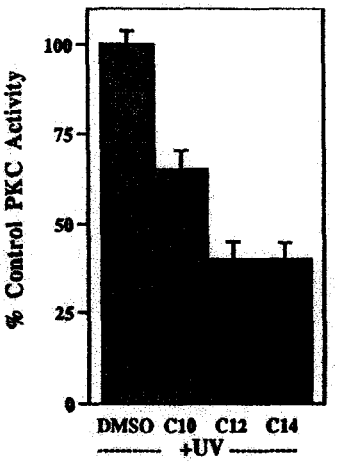


Figure 4. Inactivation of intracellular PKC activity by DECA analogues. PKC activity was partially purified from B16F10 cells that had been treated with 250nM C10-, C12-or C14-DECA following by UV irradiation for 5 min. Intracellular PKC activity was isolated by cell lysis and DEAE-Sephacel Chromatography and assayed as described in the "Methods" section. The error for these experiments was within 10% as indicated. Error bars, S.D.

These analogues showed a pattern of linker-length-dependent inhibition of intracellular PKC activity, in which the C12-DECA analogue showed intermediate or equivalent potency with respect to the C10- and C14- analogues. The C14-DECA analogue at 250nM consistently inhibited intracellular PKC activity by 40-60%, that could not be improved with an analogous experiment using the C16-DECA analogue (not shown). A time course of UV irradiation was performed to determine if the duration of irradiation was a factor. The results indicated that the extent of inhibition is not improved with longer periods of irradiation (not shown) and that maximal inhibition of PKC activity could be achieved with an irradiation time of 5 min. The 5-min irradiation period was therefore adopted in subsequent studies.

Earlier *in vitro* experiments with C10-DECA (Rotenberg and Sun, 1998) demonstrated that long-wave UV light confers irreversibility to DECA-mediated inhibition of PKCα. In this approach, UV light is thought to photolyze the drug thereby making it reactive with the target protein, as shown by others with F1-ATPase (Zhou et al., 1988). In

the present study, UV light was used for the first time with monolayer cell cultures that had been pretreated with DECA. Following irradiation for 5 minutes, the cells were lysed and PKC activity was determined in partially purified samples. Figure 5 shows that the activity levels of cells treated with 250nm C14-DECA/UV is inhibited 50% as compared to control, whereas treatment with 250nM C14 alone does not alter PKC activity levels when compared to controls.

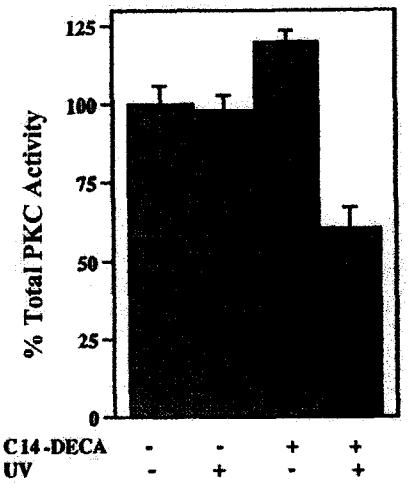


Figure 5. UV light renders C14-DECA-mediated inhibition of PKC irreversible. PKC activity was isolated from B16F10 cells that had been treated with either 250nM C14-DECA or vehicle (0.05% DMSO v/v). Each condition was studied with or without UV light (5 min treatment).

To determine if PKCα is the target of DECA in B16F10 cells, cells were treated with and without the drug, and then prepared as whole cell lysates. An antibody (MC5) that recognizes only conventional isoforms (and therefore would only immunoprecipitate PKCα in the B16F10 cell system) was added to the lysates. The results showed that cells treated with 250nM C14-DECA/UV resulted in immunoprecipitated PKCα activity that was inhibited by 70% when compared to PKCα activity present in immunoprecipitates from cells that had been UV-irradiated but were untreated by the drug (Figure 6).

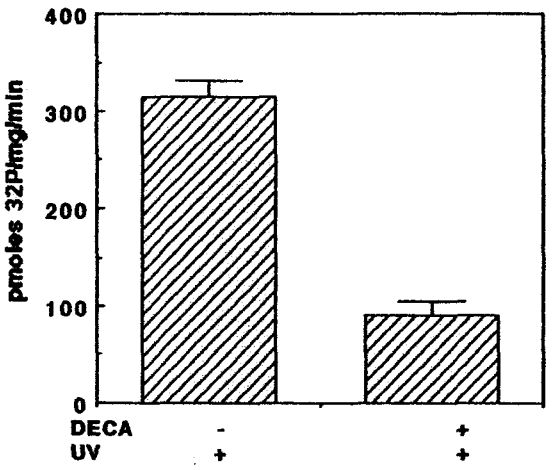


Figure 6. Intracellular PKCα is inhibited by C14-DECA. Cells were treated with 250nM C14-DECA/UV as described in methods. Cell lysates were prepared and PKCα was immunoprecipitated in triplicate with MC5 (0.5 μg/mg cell lysate) and then assayed for catalytic activity. Error within 10%-15%. Error bars, +/-S.D.

That UV irradiation of DECA-treated cells produces irreversible inhibition of PKCα, made it possible to isolate inactive and intact PKC that, unlike use of a reversibly bound (diffusible) inhibitor, survives the lysis and chromatography steps. As a result of UV-induced inactivation, it is therefore likely that PKCα is a target of C14-DECA in B16 F10 cells.

Does DECA impede PKCα translocation in melanoma cells?

It was previously shown that treatment with 10μM C10-DECA/UV inhibits the phorbol ester-induced translocation of PKCα in human breast adenocarcinoma cells by competition with either TPA or RACK (Rotenberg and Sun, 1998). This interaction is in addition to a direct interaction by DECA with the catalytic domain that produces inactivation (Rotenberg et al., 1998.) Experiments were performed to investigate the possibility that DECA inhibits phorbol ester-induced translocation of PKCα in B16F10

melanoma cells in a similar manner. However, concentrations up to 1μM C10-DECA plus UV, produced no inhibition of phorbol ester-induced translocation (data not shown).

As the most potent PKCα inhibitor (Table 1; Figure 4), C14-DECA/UV was tested for an effect on PKCα translocation. B16F10 cells were treated with 250nM, 500nM or 1μM C14-DECA (or DMSO) followed by 5 min UV irradiation. The cells were fractionated into particulate and soluble fractions and analyzed by Western Blot using a PKCα antibody. Although slight translocation was evident at 250nM C14-DECA, translocation of PKCα was stimulated to the highest extent by 1μM C14-DECA in comparison with control particulate fractions (compare lanes 5 and 8).

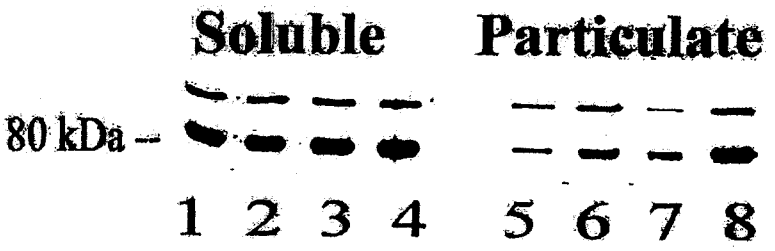


Figure 7. C14-DECA stimulates the translocation of PKCα. B16F10 cells were treated with DMSO (0.05% v/v), UV 5 min (lanes 1 and 5) or C14-DECA/UV in the following concentrations, 250nM (lanes 2 and 6); 500nM (lanes 3 and 7) and 1μM (lanes 4 and 8). Cells were fractionated into soluble fractions (lanes 1-4) and particulate fractions (lanes 5-8) and analyzed by Western Blot with a PKCα-specific antibody (8.5μg/ml of protein).

Because no stimulatory effects by nanomolar concentrations were evident in measurements of cellular phenotypes such as adhesion and migration (to be discussed in the next section), the high degree of stimulation of translocation that was produced with 1μM DECA may have resulted from a lower affinity DECA binding site in PKCα (presumably at the regulatory domain) that does not predominate with DECA concentrations less than 1μM.

C14-DECA inhibits PKC activity in both soluble and particulate compartments.

Treatment of B16F10 cells with 500nM C14-DECA followed by lysis and fractionation, showed that Ca^{2+}-dependent PKC activity was similarly inactivated in both soluble and particulate compartments. As shown in Figure 8, the activity of the soluble enzyme was inhibited by 36% and the activity of the enzyme associated with the particulate fraction was inhibited by 34%. This finding indicates that C14-DECA can inactivate PKC in either compartment, and implies that sites on PKC that are in contact with the membrane do not impede access by DECA to its target site(s).

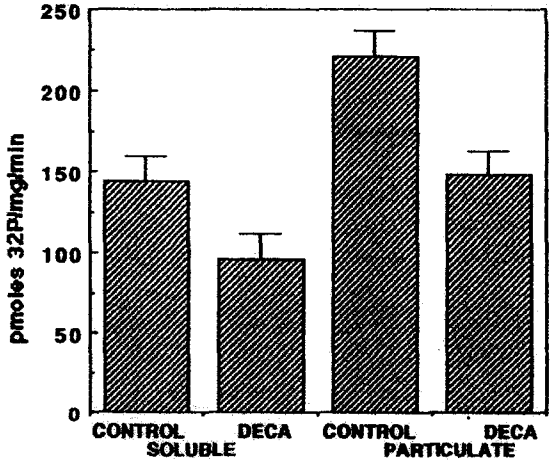


Figure 8. Equivalent inactivation of intracellular PKC activity by 250nM C14-DECA/UV in soluble and particulate compartments. PKC was partially purified from cells that had been treated with DECA/UV or (0.05%, v/v) DMSO/UV and the catalytic activity was assayed in triplicate. The results are representative of two experiments. Error bars,+/- S.D.

Many studies have indicated that PKC isoforms can play diverse and even opposing roles in the cell (Harrington et al., 1997, Tang et al., 1997, and Foey and Brennan, 2004). PKCα has been linked to cell adhesion and movement in several cell types (Zielger et al., 2002 and Anilkumar et al., 2003). In the foregoing experiments, several lines of evidence identify PKCα as the critical target of DECA in B16F10 mouse melanoma cells. In these cells, the isoform profile revealed that PKCα had the highest abundance while much lower

levels were detected for the novel isoforms ε and θ and atypical isoforms ζ and λ (Figure 1). Furthermore, 75% of the total PKC activity in B16F10 was found to be Ca^{2+}-dependent (Figure 2) and could be inhibited by a PKCα specific inhibitor Go6976. The antibody MC5, which recognizes the hinge region of conventional isoforms, was used to immunoprecipitate the enzyme from cells treated with C14-DECA/UV. When isolated from cells, PKC activity measured in a partially-purified preparation or as an immunoprecipitated enzyme consistently showed 40-70% inhibition of Ca^{2+}-dependent activity as compared with cells treated with UV only (Figures 4-6). Since Ca^{2+}-dependent PKC activity makes the largest contribution to the total PKC activity, and PKCα is the only Ca^{2+} isoform (Figures 1 and 2), it follows that the α isoform is the major PKC target of DECA in the intracellular environment. Although the focus of these studies was on PKCα, we cannot exclude contributions by other PKC isoforms (Figure 1) to the cellular attributes to be considered in the following section.

II. DECA treatment of B16F10 melanoma cells inhibits phenotypes related to adhesion, migration and metastasis.

The goal of this series of experiments is to establish the role of PKCα in determining the phenotypes related to metastatic behavior of B16F10 cells. As a first step to determine whether the combination of DECA plus UV light offers a useful approach to study the role of PKC in metastatic related phenotypes, B16F10 melanoma cells were treated with 2μM C10-DECA with or without UV light, and assayed for effects on adhesion and migration. An adhesion assay was initially chosen to study the effect of the drug because of the documented role of PKCα in adhesion and metastasis (Gopalakrishna and Barsky,1988; Zhang et al., 2001a ; Zhang at al., 2001b). Cells treated with C10-DECA but

not irradiated showed a 66% decrease in adhesion to Matrigel (Figure 9). However, adhesion measured for cells treated with both 2μM C10-DECA and UV light was inhibited by 74% as compared to the UV control, thus demonstrating that inhibition of adhesion by C10-DECA can be potentiated to some extent by UV light.

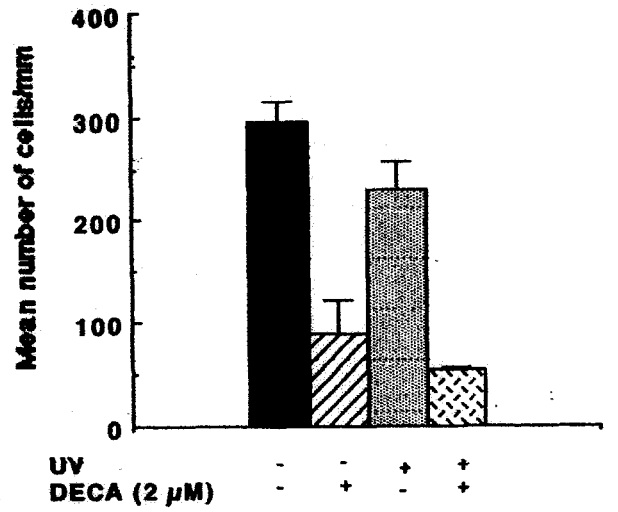


Figure 9. Adhesion of cells treated with C10-DECA and UV light. B16F10 cells were plated on 24-well tissue culture dishes in duplicate. Cells were seeded at 10^4 cells/well for 24 hours. Nonadherent cells were removed by washing each well with PBS three times. Adherent cells were counted as described in the "Methods" section.

To correlate intracellular inhibition of PKC activity with inhibition of adhesion, cells were treated with 0, 25, 50, 100, 250, or 500nM C10-DECA for 1 hour followed by irradiation for 30 min. Cells in each condition were assayed for PKC activity and for adhesion to Matrigel. The results indicated that PKC activity was inhibited by 30% (as in Figure 4), and that adhesion was inhibited by 60%, where both effects were achieved with concentrations of C10-DECA in the nanomolar range (Figure 10).

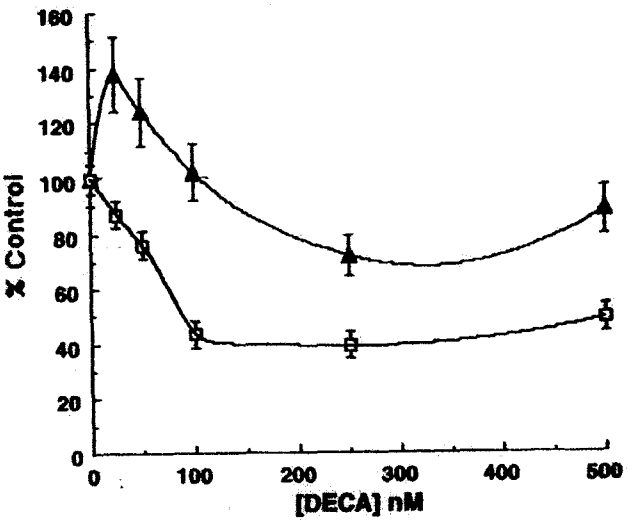


Figure 10. Intracellular PKC activity and adhesion are inhibited in a dose-dependent manner by C10-DECA/UV. B16F10 cells at 50% confluence were treated with increasing nanomolar concentrations of C10-DECA for one hour following by 30 min of irradiation. Half of the total cells in each condition were used to determine intracellular PKC activity ▲. The other half was used for assay of adhesion ❑ as described in the "Methods" section. The error was within 10% -15%. Error bars+/-S.D.

A concentration of 250nM C10-DECA inhibited adhesion to Matrigel by 50%, while a further increase in C10-DECA concentration to 500nM did not enhance inhibition.

Since experimental data presented in Chapter 1 showed that there is a linker-length dependence for inhibition of catalytic activity (Qin et al., 2000), DECA analogues were used to determine whether there was a corresponding linker-length dependence in inhibiting migration in a chemotactic assay. The cells were treated with 250nM C10-, C12- and C14-DECA followed by 5 min UV irradiation. As shown in Figure 11, these analogues showed a pattern of inhibition of migration that paralleled the increasing length of the linker, similar to the linker-length dependence of PKC inhibition (Figure 4). A concentration of 250nM/UV C10-DECA typically inhibited migration by 20-30% of controls. However, 250nM/UV C14-DECA was more potent, as the analogue consistently inhibited 40-60% of control cell migration.

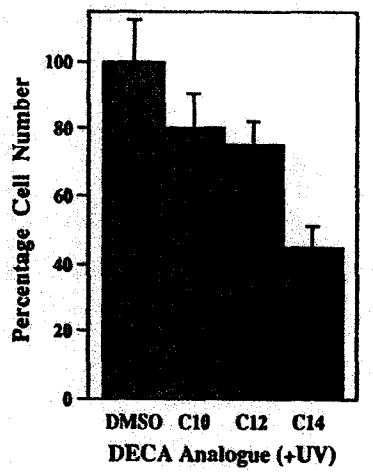


Figure 11. Inhibition of cell migration by DECA analogues. B16F10 cells were treated with the C10-, C12- or C14-DECA at 250nM for 1 hour at 37^{0}C followed by UV irradiation for 5 min. Migration assays were performed as described in the "Methods" section. Each value is the average of triplicate measurements. Error bars indicate +/- S.D. P< 0.001.

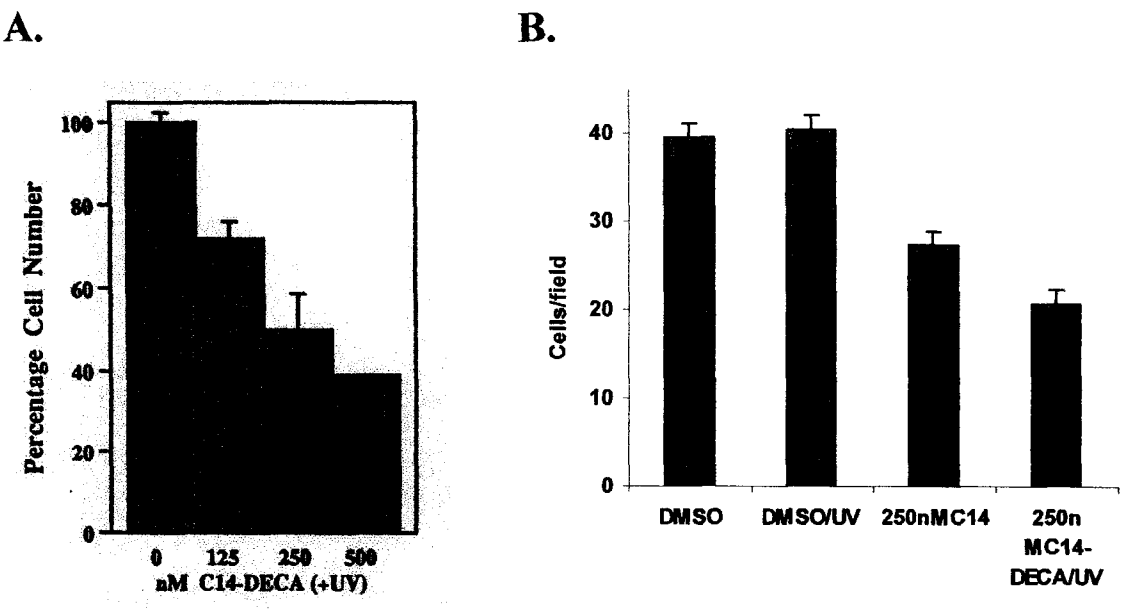


Figure 12. Inhibition of migration by C14-DECA is dose-dependent and potentiated by UV light. A) B16 F10 cells were treated with increasing nanomolar concentrations of C14-DECA for 1 hour at 37°C followed by UV irradiation for 5 minutes. B) Cells were treated with 250 nM C14-DECA or the vehicle (0.05% v/v DMSO) in the presence or absence of UV light (5 min). Migration assays were performed as described in the "Methods" section. Error bars, +/- S.D. P <0.001.

Under the same conditions, C14-DECA also produced inhibition of 40-60% total PKC activity (Figure 4). Inhibition of migration was also dose-dependent with concentrations up to 500nM with the C14 analogue (Figure 12A). Like inhibition of PKC activity (Figure 5)

and inhibition of adhesion (Figure 9), inhibition of migration by C14-DECA was potentiated by UV irradiation (Figure 12B).

Since DECA treatment can produce substantial inhibition of adhesion (Figure 9) and migration (Figure 12B) of B16F10 cells in the absence of UV, it was reasoned that C14-DECA could be used to inhibit metastasis of B16F10 cells in mice as part of an experimental metastasis assay. Because the C14-DECA analogue showed the comparably highest level of inhibition in both adhesion (Figure 9) and migration (Figure 12B) assays, this analogue was chosen for analysis of anti-metastasis activity. Eight C57BL/6 mice of comparable age and weight were injected with 5mg/kg (2.5 mg/ml) C14-DECA in 0.36% sodium citrate (or vehicle only) every other day for one week, as prescribed by a previous toxicology study with C10-DECA (Gamboa-Vujicic, et al., 1993). At the end of this period, 10^5 cells/0.2 ml were injected into the tail vein of each mouse. Intraperitoneal injections of C14-DECA (5mg/kg) were continued every other day for 2-3 weeks after which the mice were sacrificed and their lungs removed. The metastatic nodules were counted and analyzed statistically by the Wilcoxon signed rank test. As shown in Table 2, the animals in the C14-DECA group exhibited an average of 36% fewer tumors than the control group.

Table 2: Inhibition of In Vivo Metastasis by Dequalinium-14

Treatment	Number of Nodules	Mean	%
Sodium citrate	41, 50, 68, 41, 28, 52, 50, 33	45	100
DECA-14	29, 31, 26, 42, 23, 41, 22, 21	29	64

C57BL/6 mice injected i.p. every other day with 4.5mg DECA-14/kg body weight or the vehicle control (0.36% sodium citrate). There were 8 animals/treatment group. (P=0.016).

This result indicated that treatment of B16F10 cells with C14-DECA decreases the incidence of tumors in vivo. In Figure 13, two lung specimens are shown that represent the

control group (left); and the C14-DECA treatment group (right). In this set of specimens, the control number of nodules in the tissue of animals that did not receive the drug was more that twice (50 nodules) than the number of nodules lung tissue from the drug-treated animals (22 nodules), signifying inhibition of nodule formation by 56% .

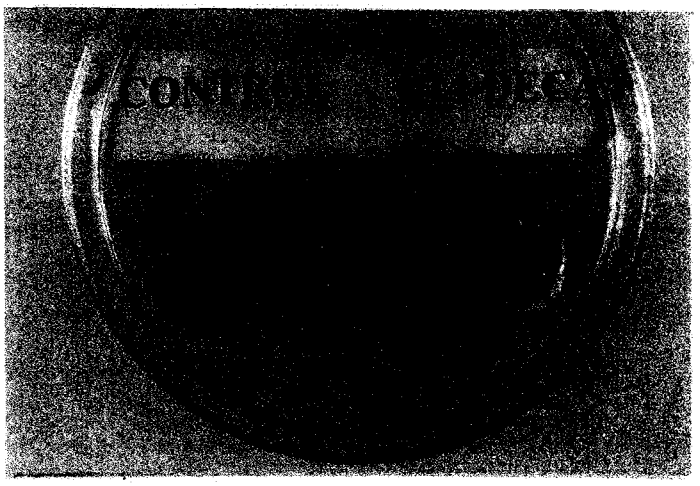


Figure 13. C14-DECA inhibits in vivo metastasis of B16F10 cells. The experiment consisted of two treatment groups(untreated, C14-DECA-treated) each consisting of mice of equivalent weight and age. Animals were injected i.p. with 0.2ml (2.5 mg/ml C14-DECA in 0.36% sodium citrate) or vehicle. Mice were injected every other day for 11 days prior to injection of B16F10 cells. On the eleventh day 10^5 cells were injected into the tail vein of each mouse. After two weeks, the mice were sacrificed and the metastic nodules were counted using a dissecting microscope.

The foregoing experiments were carried out with DECA to establish that the phenotypes of adhesion and migration of B16 cells correlate with PKCα activity. Accordingly, in the presence of DECA, these phenotypes are attenuated due to inhibition of PKC activity. This idea is supported by the linker-length dependence of inhibition by 40-60% of both PKC activity (Qin et al., 2000; Figure 4) and cell migration (Figure 11). The finding that C14-DECA treatment of the whole animal suppresses metastasis of B16F10 cells by up to 56% offers evidence of its potential as an anti-metastatic drug.

III. Construction and analysis of a cell line that stably expresses a kinase-defective PKCα

The previous section demonstrated that DECA/UV effects on cell adhesion and migration could be correlated with inactivation of intracellular PKC activity that, based on several lines of evidence, was presumed to be PKCα activity. The next objective was to demonstrate directly that engineered expression of a kinase-defective PKCα can suppress cell movement by acting as a dominant negative. For this purpose, stable transfectants of B16F10 cells were isolated that overexpressed a polyhistidine-tagged kinase-defective mutant of PKCα protein in which the ATP-binding lysine-368 was replaced by an arginine residue. (This vector was provided by F. Uberall, University of Innsbruck; Kampfer at al., 1998). This mutant PKCα does not phosphorylate cellular substrates and is therefore analogous to native PKCα whose catalytic activity has been inactivated by DECA. Lysates prepared from cells expressing this mutant PKCα (clone α6) or cells that had been stably transfected with a control plasmid, were partially purified by metal chelate affinity chromatography that selectively adsorbs polyhistidine-tagged protein. Western blot demonstrated that following elution with imidazole, eluted polyhistidine-tagged PKCα mutant protein could be detected at a 5-fold higher level as compared to the low signal obtained by the same chromatographic protocol for mock-transfected cells and parental cells (Figure 14).

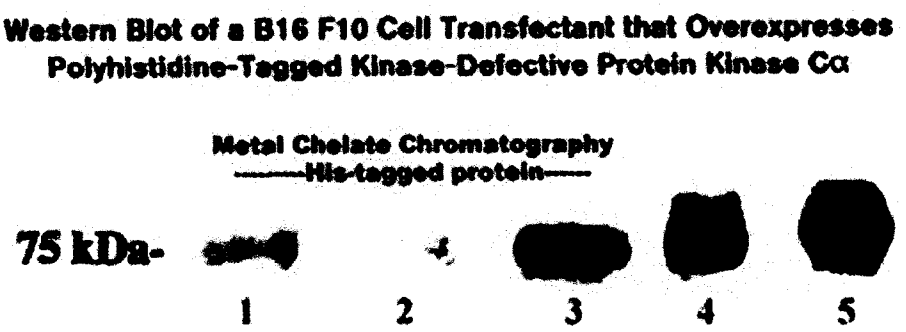


Figure 14. Overexpression of a polyhistidine-tagged kinase defective PKCα mutant by B16F10 cells. Western blot analysis with PKCα-specific antisera was carried out with eluates (23 μg/lane) obtained by metal chelate chromatography (lanes 1-3). Lane 1, B16F10 parental cells; lane 2, mock-transfectant; lane 3, α6 transfectant cells; lane 4, α6 transfectant whole cell lysate and lane 5, recombinant PKCα protein standard (Pan Vera Corp., Madison, WI).

Importantly, in α6 cells, there were no detectable compensatory changes observed in the expression levels of either wildtype PKCα or of other endogenously expressed PKC isoforms (data not shown).

Characterization of B16F10 cells that over-express dominant negative PKCα.

The B16F10 cell line expressing kinase-defective PKCα (α6) was used to specifically define the role of PKCα in migration and adhesion. Against a high background of endogenous PKCα activity, the overexpression of a kinase-defective PKCα mutant would be expected to compromise but not necessarily to eliminate the function of the wildtype PKCα.

Adhesion and migration assays were done using specific extracellular matrix substrates to determine if PKCα activity has a selective role in one or more substrate signaling pathways. Adhesion and migration activities of parental, mock-transfectant, and α6 cells were analyzed over a 3-h period in response to Matrigel (which is composed of

30% collagen IV and 60% laminin) (Figure 15), fibronectin (Figure 16), or collagen IV (Figure 17). With collagen IV, migration of α6 cells was inhibited to the highest extent (48%) (Figure 17), somewhat higher than with Matrigel where the inhibitory effect was 40% (Figure 15). However with fibronectin, there was no significant inhibition of migration (Figure 16). This finding suggests the importance of collagen IV in PKCα-mediated migration. Similar to its effect on migration, expression of kinase-defective PKCα caused substantial inhibition of adhesion, but inhibiton was not affected by the specific substrate used in the assay. Thus, inhibition of adhesion by α6 cells relative to vector control cells (Figures 15-17) was roughly equivalent when cells were plated on Matrigel (66% inhibition), fibronectin (58% inhibition) or collagen IV (67% inhibition).

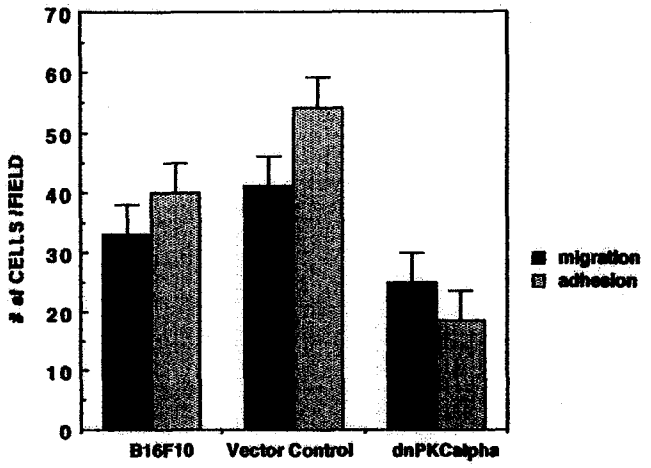


Figure 15. Inhibition of adhesion and migration of B16F10 cells on Matrigel transfected with kinase-defective PKCα. For adhesion, the bottom surface of a 24-well tissue culture dish was coated with 43.5μg/cm of Matrigel for 1h at 37°C. Cells were seeded at 1.5 x 10^5 cells/well and incubated as described in the methods section. For migration, 12-well Costar polycarbonate chambers were used. The bottom microporous surface was coated with 35 μg of Matrigel. Cells seeded at 10^5 cells/well and incubated for 3 h at 37°C as described in the "Methods" section. Error bars, +/-S.D. P <0.001. Results shown are representative of three independent experiments.

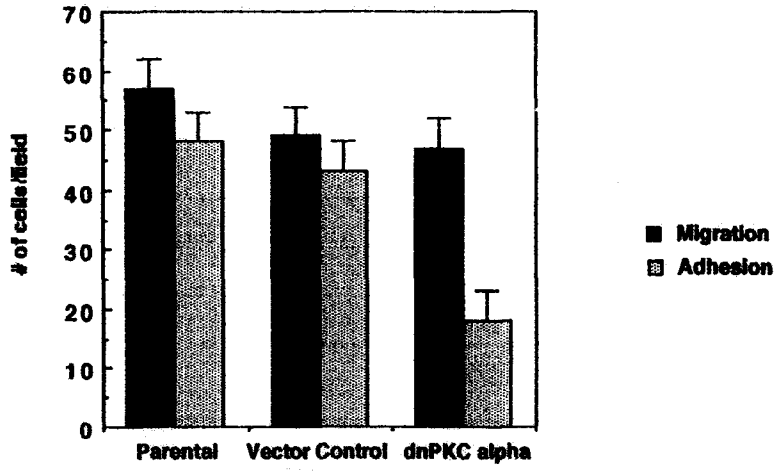


Figure 16. Inhibition of adhesion and migration on fibronectin of B16F10 cells transfected with kinase-defective PKCα. Assays conditions were the same as Figure 15 except that 2μg/cm of fibronectin was used. Error bars,+/-S.D. $P < 0.001$.

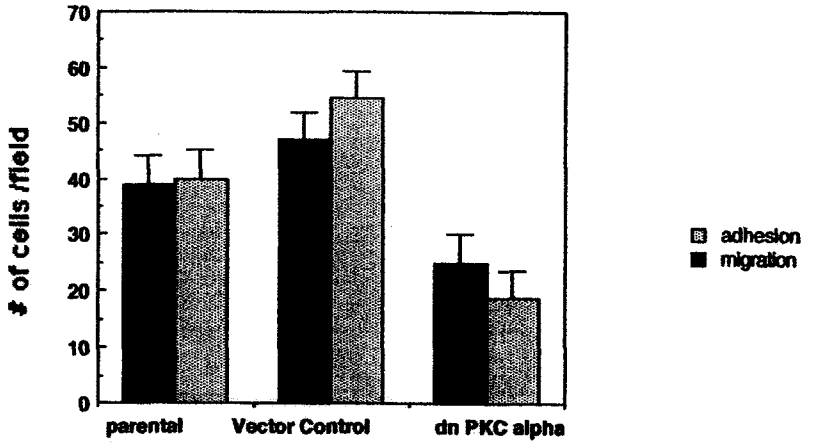


Figure 17. Inhibition of adhesion and migration on collagen IV of B16F10 cells transfected with kinase-defective PKCα. Assay conditions were the same as in Figure 15 and 16 except that 2μg/cm of collagen IV was used. Error bars, +/- S.D. $p<0.001$.

Are the reduced levels of adhesion and migration due to changes in adhesion protein expression levels?

Analysis of lysates of α6 cells for the expression of several adhesion molecules and other proteins identified with cell movement, revealed that there were no detectable changes in their expression levels in α6 cells as compared with mock-transfectant and parental cells.

The proteins tested by Western blot included : β-integrin, focal adhesion kinase, paxillin, VASP, pp120, desmoglein , E-cadherin, γ-and β-catenin (Table 3).

Table 3: Adhesion Proteins Unchanged in Expression in Transfected Cells

Adhesion Protein	B16F10	Control Transfectants	α6 Transfectants
Focal Adhesion Kinase	+	+	+
β_1 Integrin	+	+	+
E Cadherin	+	+	+
γ Catenin	+	+	+
β Catenin	+	+	+
Paxillin	+	+	+
pp120	+	+	+

Inhibition of migration by DECA in cells that express a kinase-defective mutant of PKCα.

To establish a mechanistic link between inhibition of PKCα actvity by DECA and its inhibition of cell movement, the effect of C14-DECA on migration of α6 cells and mock-transfected cells was examined. The premise for this experiment is based on the expectation that kinase-defective PKCα protein expressed by α6 cells will serve as a surrogate target molecule that will compete with native PKCα protein for binding of C14-DECA, thereby decreasing the effective intracellular concentration of C14-DECA. If PKCα is indeed a target of the drug, then C14-DECA would be predicted to be a far less effective inhibitor of migration of α6 cells than with parental or mock-transfected cells. (All three cell lines express an equivalent amount of wildtype PKCα protein.) However, if C14-DECA interacts with another target(s), then the effect by C14-DECA on migration should be undiminished by the kinase-defective mutant. Our findings indicate that treatment of these cells with 500nM C14-DECA produced 50% inhibition of migration in mock-

transfected and parental cells, whereas α6 remained largely unaffected by the drug (Figure 18). This result is consistent with the idea that the PKCα mutant protein in α6 cells served as an effective competitor for the drug. If a target of DECA other than PKCα had been responsible for inhibiting cell migration, then the presence of non-functional PKCα would not have impaired its action. These findings clearly establish that inhibition of migration by C14-DECA is the result of its inhibition of PKCα.

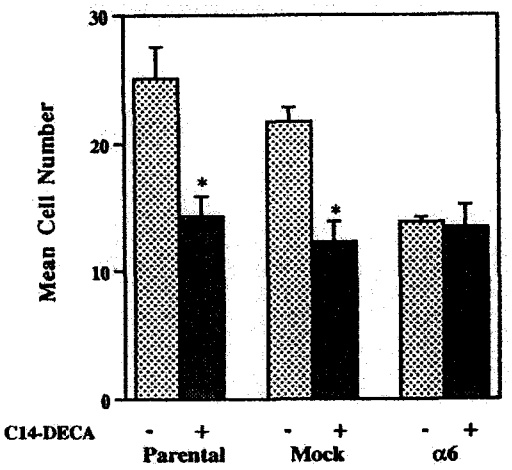


Figure 18. Expression of kinase-defective mutant of PKCα interferes with DECA action in B16F10 cells (parental), mock-transfected cells (mock), and cells expressing a kinase-defective mutant PKCα (α6). Cells were treated with 500nM C14-DECA or DMSO (0.05%,v/v), irradiated with UV for 5 min, and assayed in transwells for migration in response to Matrigel. The results are representative of three independent experiments. The error was within 10% as indicated. Error bars,+/-S.D. $P<0.001$.

The results indicate that inhibition of PKCα leads to inhibition of adhesion and migration, both of which are essential aspects of metastasis. To determine if α6 cells exhibit decreased metastatic potential in mice, experimental metastasis assays were carried out. C57BL/6 mice were injected with either vector control cells or α6 cells. After two weeks, the mice were sacrificed and their lungs removed, as described in the "Methods" section. The results, shown in Table 4 indicate that animals that had been injected with α6 cells produced 30% fewer lung nodules as compared with animals that had been injected

with vector control B16F10 cells. This important result certifies PKCα as a critical determinant of the metastatic phenotype. As a result of overexpression of the kinase-defective PKCα, α6 cells exhibited substantial decreases in migration, adhesion, and metastasis. This stably transfected cell line provided an important means to establish the role of PKCα in the mechanism of adhesion and migration and to verify directly that PKCα serves as the intracellular target of C14-DECA having mechanistic significance.

Table 4: Inhibition of In Vivo Metastasis by B16F10 cells expressing a kinase-defective PKCα

	Number of nodules	mean	%
Vector control cells	30, 56, 51, 5, 59, 63, 44, 41, 58, 20	47.2	100
α6 (kinase-defective)	22, 45, 26, 46, 21, 16, 59, 5, 51, 21, 20 P=0.018	33	70

Nine mice were injected with 10^5 vector control cells. Ten mice were injected with the same number of kinase-defective cells. After two weeks, the mice were sacrificed and their lungs removed. Metastatic nodules were counted twice and the values were averaged.

IV. Possible mechanisms of PKCα in adhesion, migration and metastasis.

Focal adhesion kinase undergoes autophosphorylation on tyrosine residues when cells adhere to extracellular matrix or substrates like fibronectin or collagen. This autophosphorylation event results in increased cell spreading and migration. Cells expressing kinase-defective PKCα and vector control cells were plated on different substrates, specifically collagen, fibronectin, or Matrigel. Cell lysates were prepared and Western blot analyses were carried out with antibodies directed against FAK that had undergone autophosphorylation on Tyr^{397} (Figure 19).

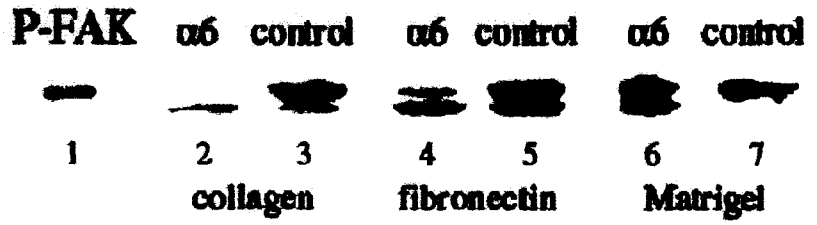


Figure 19. B16F10 cells expressing dominant-negative PKCα display decreased autophosphorylation of Focal Adhesion Kinase. B16F10 cells expressing a dominant-negative PKCα or vector control cells were plated on collagen IV, fibronectin or matrigel and allowed to adhere for 30 minutes. Cell lysates were prepared and analyzed by Western Blot with a phospho-FAK antibody (40μg protein/lane).

The cells expressing kinase-defective enzyme showed decreased phosphorylation on FAK after the cells were plated on collagen and fibronectin as compared to the mock-transfected cells. The most pronounced inhibition of FAK phosphorylation was seen with collagen IV, although α6 cells that had been plated on fibronectin also showed a substantial loss. However, no significant decrease was observed with α6 cells that had been plated on Matrigel. Results obtained with fibronectin and collagen parallel the results obtained for cell adhesion on these individual substrates (Figures 16 and 17) and implicate wildtype PKCα activity in signaling pathways that promote FAK autophosphorylation.

Confocal microscopy studies were carried out to determine if, when plated on fibronectin, α6 cells display differences in actin structure. In these studies, kinase-defective expressing cells (Figure 20A) and mock transfectant cells (Figure 20B) were plated on fibronectin and then stained with rhodamine phalloidin, a reagent that binds to F-actin filaments. These studies revealed a qualitative difference in the appearance of actin filaments present in α6 cells that appear disorganized and exhibit punctate signals throughout the cell (Figure 20B). In contrast, mock-transfectant B16F10 cells exhibit extensive fiber networks (Figure 20A). These findings suggest that expression of kinase-

defective PKCα influences the ability of the cell to form appropriate F-actin filaments (Figure 20).

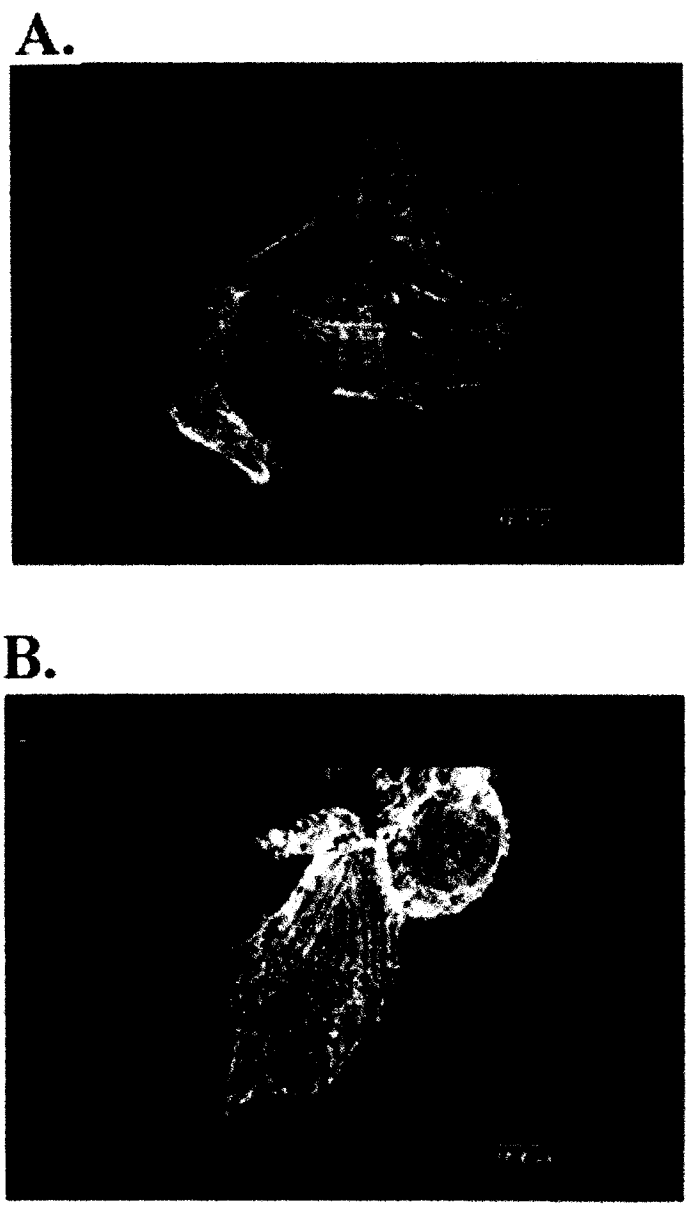


Figure 20. B16F10 cells stably transfected to overexpress a kinase defective PKCα have disorganized actin filaments. Cells were plated onto Lab-tech chambered coverslips that were coated with 5μg of fibronectin. Fixed cells were treated with 2 units of rhodamine-phalloidin (1.4 units/ml PBS). The cells were visualized using a Meridian Ultima confocal fluorescence microscope. (A) Vector control cells, (B) α6 cells.

DISCUSSION

The role of PKCα in the metastatic behavior of B16F10 cells was addressed through the use of pharmacological and molecular techniques. Our major findings are as follows: (1) PKCα is the major intracellular target of C14-DECA in B16F10 cells and can be recovered in the inactivated state from cells irradiated with longwave UV light; (2) inhibition of PKCα correlates with inhibition of adhesion and migration of B16F10 cells; (3) B16F10 cells transfected with a kinase-defective PKCα reproduces the diminished adhesion and migration of C14-DECA-treated cells; (4) PKCα directly or indirectly interacts with focal adhesion kinase; and (5) metastasis of B16F10 cells is inhibited substantially (30%-50%) when they are injected into C57BL/6 mice that are subsequently treated with C14-DECA, or with cells that express kinase-defective PKCα. Discussed below is a consideration of C14-DECA action and PKCα in signaling pathways governing the metastatic phenotype of mouse melanoma.

How does C14-DECA inhibit PKCα activity?

C14-DECA/UV causes the irreversible inhibition of PKCa in B16F10 cells (Figure 5). The mechanism of inactivation involves a direct interaction with the catalytic domain as demonstrated by a deletion analysis by Rotenberg et al. (1998). In that study, inhibition of catalytic activity was not dependent on the presence of the regulatory domain. Previous studies from the Rotenberg lab demonstrated that the parent compound (C10-DECA) can bind to two sites in the regulatory domain in PKC. In the first study, (Rotenberg et al., 1990) C10-DECA displaced [^{3}H]-PDBu (a phorbol ester) from the diacylglycerol binding site of PKC β1, indicating a direct interaction with the phorbol ester binding site in the C1

domain. It is possible that this interaction by DECA accounts for the catalytic activation observed with low doses of C10-DECA with intracellular PKCα (Figure 10), as well as for the induction of translocation of PKCα (Figure 7). We observed that C14-DECA did not inhibit the TPA-induced translocation of PKC in concentrations up to 2μM (data not shown). It was concluded that C14-DECA cannot compete effectively with TPA at the C1 site of the enzyme. However, in the absence of TPA, DECA may bind at the C1 site. In this regard, we found that treatment of melanoma cells with 1μM C14-DECA could induce the translocation of PKCα as analyzed by Western Blot (Figure 7). The reasons for this unexpected finding may lie in its intrinsic lipophilicity which would enable enzyme-bound DECA to increase the affinity of PKCα for the membrane, similar to the mechanism of PKC translocation induced by TPA (Zhang et al, 1995).

A second binding site on the PKCα regulatory domain was demonstrated at the C2 domain, specifically at the RACK site (Rotenberg and Sun, 1998). This interaction was shown to prevent the translocation of the enzyme in human breast cells. Because B16F10 cells express abundant levels of RACK protein (data not shown), DECA (up to 2μM) may not compete well with RACK and therefore would not be expected to inhibit PKCα translocation.

Our results strongly suggest that, C14-DECA primarily influences PKCα through an inhibitory interaction with the catalytic domain. Maximal inhibition of catalytic activity by C14-DECA occurs such that each head group binds at a separate site on the PKC molecule by a distance defined by the optimal linker-length of 16.6 A (Qin et al., 2000). Due to this short distance, it is likely that both sites of contact by the head groups lie in the catalytic domain. Therefore, if DECA binds to the regulatory domain, it does so independently and

would necessarily require a second molecule of DECA. DECA does not compete with ATP or substrate (Rotenberg et al., 1990), nor does it inhibit other protein kinases such as the cAMP-dependent protein kinase (which has 40% sequence homology with the PKC catalytic domain), src, or calmodulin-dependent myosin light chain kinase (Rotenberg, unpublished data). These observations suggest that the interaction of DECA with the PKCα catalytic domain occurs at unknown sites that are apparently unique to PKC. Future investigation will seek to establish the identity of these sites using ^{3}H-DECA in covalent modification of the enzyme with UV light.

FAK is a downstream target of PKCα in the Motility Signaling Pathway

Based on our results and those of others, it is likely that integrin activation is involved in the motility pathway of B16F10 cells. For cells expressing the kinase-defective mutant, both adhesion and migration were inhibited to different degrees when cells were plated on different substrates, and the difference was especially pronounced with collagen IV (Figures 17, 18 and 19). In general, two signaling pathways that involve integrin proteins control such processes of adhesion and migration. Plating the cells on different substrates such as fibronectin, collagen IV or matrigel (which consists of collagen IV and laminin) could initiate different "outside-in" signaling pathways, one of the two types of signaling pathways in which integrins play a role. Molecules inside the cell interact with the cytoplasmic tails of integrin subunits and initiate cascades of signaling events known as "inside-out" signaling. PKCα has been implicated in both "inside-out" and "outside-in" signaling pathways (Disatnick and Rando, 1999). As discussed in the Introduction, binding of β1 integrin and PKC has been mapped to the hinge region of PKCα (Parsons et al 2002).

The interaction between β1 integrin and PKCα has been implicated in migration of multiple myelona cells (Podar, 2002). In our studies with B16F10 cells, expression levels of β1 integrin as assayed by Western Blot analysis are equivalent in parental, vector control and α6 cell lines (Table 4). Therefore the levels of β1 integrin would not be a limiting factor in our cell system. However, the "outside-in" positive feedback loop suggested by Disatnik and Rando (1999) could be in place. Upon integrin binding to substrate, PKC is known to undergo activation in muscle cells (outside-in signaling), whereupon activated PKC can produce "activation" of integrin molecules, that is, integrins acquire a higher affinity for extracellular substrates. As integrin subunits bind to substrates, they cluster in the plane of the cell membrane. Clustering is essential to the formation of focal adhesion complexes. Adhesion proteins are recruited to the focal complex and actin polymerization follows. Hence, the kinase-defective or DECA-inhibited PKCα would be predicted to bind β1 integrin but downstream events that include phosphorylation of PKC substrates leading to actin polymerization would be compromised. Evidence for the latter outcome in the present work was observed by fluorescence microscopy with B16F10 cells plated on slides coated with fibronectin and stained with rhodamine–phalloidin and (Figure 20). When plated on fibronectin, B16F10 cells expressing kinase-defective PKCα showed diminished adhesion (by 60%) and a disorganized, and punctate pattern of actin filaments, as compared with vector control cells. Our results imply that PKCα activity participates in fibronectin-activated outside-in signaling pathways that govern actin structure and consequently adhesion and migration.

An important mechanistic finding of this work was that B16F10 cells expressing kinase-defective PKCα demonstrate a dramatic loss of FAK autophosphorylation (Figure

19). Autophosphorylation of FAK on Tyr^{397} is thought to be a critical event in the formation of focal complexes (Toutant et al., 2002) because it generates a binding site for Src and for Fyn. Upon binding to FAK, Fyn promotes formation of focal adhesions which are required for motility. Both PKCα and FAK have been shown to bind to β1 integrin, suggesting that binding interactions with β1 integrin are essential to this phenotype.

PKCα has been shown to regulate the trafficking of the β1 integrin in breast cancer cells (Ng et al., 1999) which entails a binding interaction with the activated (higher affinity) conformer of the integrin subunit. In addition, PKCα-mediated binding interactions are required for internalization of the integrin-ligand complex (Lawson and Maxfield, 1995). Although inhibition of PKCα-mediated internalization of β1 integrin would be an attractive explanation for how DECA or kinase-defective PKCα acts to inhibit migration, surface expression levels of β1 integrin in both C10-DECA treated cells were unchanged from that of untreated B16F10 cells (S.A. Rotenberg unpublished data). That DECA-mediated inactivation of PKCα does not alter surface expression of β1 integrin reinforces the importance of protein-protein interactions by PKCα in the process of integrin trafficking rather than its catalytic activity.

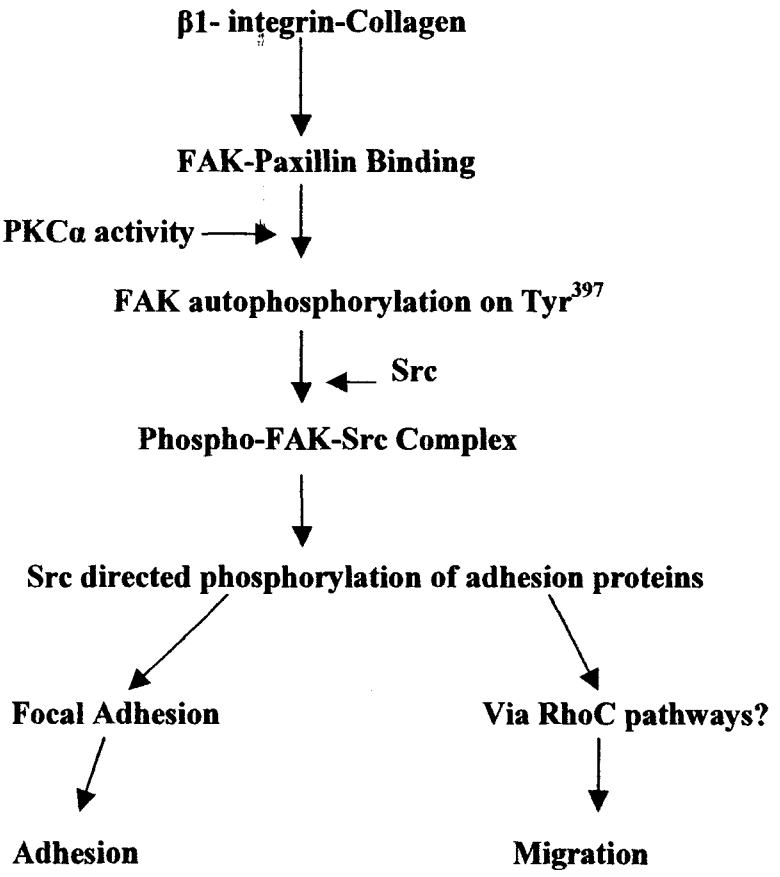
A possible mechanism in B16F10 cells is that PKCα catalytic activity is essential for clustering of the integrin receptor. In this regard, PKC activation was shown to induce clustering of integrin receptors in glioma cells (Besson et al., 2002). Upon integrin engagement, FAK is activated and consequently undergoes autophosphorylation on Tyr^{397}. If PKCα inhibition impedes proper integrin clustering, a fewer number of FAK molecules would be activated leading to a reduced number of stable focal complexes. Evidence for this possibility comes from a study in which the enhanced motility of T-cells due to β1

integrin crosslinking was blocked by prior incubation with calphostin C, a PKC inhibitor (Hauzenberger et al., 1997). In the present study, the autophosphorylation of FAK on Tyr^{397} was greatly diminished when B16F10 cells that express kinase-defective PKCα were plated on collagen IV, and closely correlated with inhibition of adhesion and migration on this substrate.

Interestingly, the binding of the C-terminal focal adhesion targeting sequences (FAT) of FAK to paxillin stimulates autophosphorylation of FAK (Hayashi et al. 2002). Paxillin has been shown to be a PKC substrate (DeNichilo et al., 1996). In our system, PKC could play a role upstream of paxillin-FAK binding. Paxillin is an actin–binding protein that is considered to be an adaptor protein because of its versatile binding capabilities. Since appropriate focal adhesion formation depends on the polymerization of actin, this model could explain the loss of adhesion when PKCα activity is inhibited. The force generated by actin polymerzation is required for migration (Lauffenburger and Horwitz, 1996). Hence placing PKCα upstream of a substrate that binds actin may explain the loss of adhesion and migration observed with inhibition of PKCα activity. These ideas are supported by the loss of actin organization in cells expressing kinase-defective PKCα (Figure 20).

The downstream events in B16F10 cells producing migration are likely to include activation of RhoC pathways, since overexpression of Rho C in melanoma cells induces a more aggressive phenotype (Clark et al., 2000). RhoC is a member of the GTPase family that has been shown to regulate cytoskeleton remodeling (Van Aelst et al., 1997). The mechanism by which adhesion proteins and RhoC produce cell movement is not understood. Taken altogether the results could suggest the following scheme:

Scheme #3: The role of PKCα activity in murine melanoma cell migration and adhesion.



Dequalinium inhibits in vivo metastasis of B16F10 cells.

The activity of PKCα appears to play a mechanistic role in the *in vivo* metastasis of B16F10 cells. When C14-DECA is injected intraperitonally, in vivo metastasis of B16F10 cells is inhibited up to 50% (Figure 13 and Table 2). The mechanism of this inhibition may be explained by mechanisms suggested by our *in vitro* studies. Cancer cells record signals from the extracellular environment and "adjust" the expression of the appropriate adhesion receptors (Varner and Cheresh, 1996). The B16F10 cells used in this study were derived from a normal melanocyte in the deepest layer of the epidermis of the skin. Since collagen

IV is a major component of the extracellular matrix of the dermis, B16F10 cells may be "primed" with adhesion receptors specific for collagen. Interestingly, the *in vitro* study showed that when B16F10 cells overexpressing a kinase-defective PKCα mutant were plated on collagen IV there was a greater decrease in the autophosphorylation of FAK as compared to cells plated on other extracellular matrix components (fibronectin, matrigel). This finding suggests that the mechanism of PKCα action *in vitro* may accurately predict its role in vivo.

Lastly, the expression of invasion-promoting proteases could be playing a role in *in vivo* mechanisms. For example, urokinase (uPA) has been shown to be responsible for invasion through its proteolytic activity. In a recent study, inhibition of uPA reduced the metastasis of a highly invasive breast cancer cell line. The authors further showed that the constitutive activation of AP-1 and NF-kappa B were also inhibited after treatment with the PKC inhibitor bisindolylmaleimide (Sliva et al., 2002), and suggest that cell motility and uPA activity are regulated by the expression of PKC, Ap-1 and NF-kappa B. A similar pathway could be operating in melanoma cells.

CONCLUDING STATEMENT

Taken together, the results in this study have implicated PKCα as a potential therapeutic target in melanoma. These studies were made possible by certain unique qualities of the drug dequalinium, a PKC inhibitor. For example, the drug is presumed to be selectively concentrated in the cytoplasm of B16F10 cells where much of the PKC protein resides. Furthermore, use of long-wave UV light permitted the demonstration that dequalinium irreversibly inhibits intracellular PKCα since inhibition persisted after isolation of the enzyme from cells. Inhibition of experimental metastasis by dequalinium (in the absence of long-wave UV light) is the most promising result in terms of a therapeutic use for the drug.

The development and analysis of a stably-transfected murine melanoma cell line that overexpresses kinase-defective PKCα showed that the defect successfully reproduced phenotypic changes caused by dequalinium with parental cells. Finally, the identification of focal adhesion kinase as a downstream effector of PKCα activity revealed a molecular component by which to analyze PKCα activity, and implicated autophosphorylation of FAK and formation of cytoskeletal actin structure as downstream events of PKCα activity.

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