

Photo-Induced Inactivation of Protein Kinase C α by Dequalinium Inhibits Motility of Murine Melanoma Cells

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ABSTRACT

Dequalinium (DECA) is a potent antitumor agent and inhibitor of protein kinase C (PKC). Previously it was shown that PKC α activity in vitro could be irreversibly inhibited when treated with DECA at low micromolar concentrations and irradiated with 366 nm of light. This approach was used to probe the role of intracellular PKC activity in the motility of metastatic murine melanoma B16 F10 cells and as a target for DECA analogs with increasing PKC inhibitory potencies. Pretreatment of a monolayer of B16 F10 cells with 250 nM of a DECA analog in the presence of UV irradiation for 5 min resulted in 1) complete inhibition of cell motility for up to 4 h in a time-lapse motility assay and 40 to 60% inhibition of cell migration in a Boyden chamber, and 2) inhibition by 40 to 60% of intracellular phos-

phatidylserine/Ca²⁺-dependent PKC catalytic activity, signifying inactivation of a conventional PKC isoform. Because PKC α is the only conventional PKC isoform detected in B16 F10 cells, a stably transfected clone expressing a kinase-defective mutant of PKC α was developed that exhibited a substantial loss of adhesion and motility and was refractory to further inhibition by DECA. These findings identify PKC α catalytic activity both as a mechanistic component of cell motility and adhesion and as a critical intracellular target of DECA. These studies further suggest that the combined use of UV with nanomolar concentrations of DECA offers an effective chemotherapeutic approach to inhibit metastatic behavior of melanoma cells.

Protein kinase C (PKC) (EC 2.7.1.37) is a serine/threonine protein kinase that participates in numerous signaling pathways. PKC consists of a family of related isoforms variously expressed by cells. Each isoform can be assigned to one of three subcategories based on the means by which it is activated: the conventional PKC isoforms (α , β , and γ) are activated by Ca²⁺, diacylglycerol, and phosphatidylserine (PS) or by PS plus 12-*O*-tetradecanoylphorbol-13-acetate (TPA; a potent and specific phorbol ester); the novel isoforms (δ , ϵ , η , and θ) are insensitive to Ca²⁺ but can be activated by diacylglycerol, PS, and TPA; and the atypical isoforms (λ / ι and ζ) are insensitive to conventional activators and TPA but can be stimulated by phospholipids such as phosphatidylinositol (Kazanietz et al., 1993). When cells are treated with TPA, certain isoforms (except λ / ι and ζ) translocate to the membrane where they interact with specific substrates to initiate effects on cell behavior.

Recent studies have implicated a critical role for PKC α in cell adhesion and motility, two highly integrated activities that are fundamental to metastatic potential (Palecek et al., 1997). Previous work with mouse melanoma cells (Gopalakrishna and Barsky, 1988) showed that PKC has a mechanistic role in the metastatic activity of B16 cells in a syngeneic C57BL/6 mouse model (Fidler, 1975). In that work, highly metastatic B16 F10 cells exhibited proportionately higher PKC activity in the membrane fraction than did B16 F1 cells which had characteristically low metastatic activity. Furthermore, B16 F1 cells having low metastatic activity were rendered highly metastatic by treatment with TPA. The potent and specific nature of TPA action implicated the TPA-responsive PKC isoforms in the metastatic activity of this melanoma cell model. Other laboratories have since recognized a role for PKC in promoting the adhesion and motility of B16 cells (Dumont et al., 1992; Dumont and Bitonti, 1994; Lewis et al., 1996; La Porta and Comolli, 1997). Intervention in the metastatic activity of B16 melanoma cells was demonstrated with PKC-targeted inhibitors (Dumont et al., 1992; Liu et al., 1992), an example of which was the light-activated inhibitor, calphostin C (Liu et al., 1992).

In the present study, dequalinium (DECA) was examined

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ABBREVIATIONS: PKC, protein kinase C; DECA, 1,1'-decamethylene-bis-4-aminoquinaldinium di-iodide; PS, phosphatidylserine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; DMSO, dimethyl sulfoxide.

as a UV light-activated tool by which to explore a specific role for PKC α in the motility of melanoma cells and as a prototype for the future design of antimetastatic agents. DECA was previously shown to be an antitumor agent (Weiss et al., 1987; Christman et al., 1990) that is selectively accumulated by cancer cells because of the higher electrochemical potentials maintained across mitochondrial and plasma membranes (Chen, 1989). As shown for several cancer cell lines, a dicationic molecule such as DECA can be accumulated up to 25,000-fold relative to its exogenous concentration (Chen, 1989). The drug is taken up by the mitochondria, which serve as storage depots that slowly release the drug into the cytoplasm. Previous studies showed that low micromolar levels of DECA are required to inhibit the motility and invasiveness of human melanoma cells in vitro (Fink-Puches et al., 1993; Helige et al., 1993; Hofmann-Wellenhof et al., 1995). An attractive feature of the B16 F10 cell system, however, is that, unlike most other cancer cells, they do not accumulate DECA in their mitochondria (Bernal et al., 1983), thus concentrating much of the drug in the cytosol where many PKC isoforms are localized.

DECA is a noncompetitive PKC inhibitor exhibiting IC₅₀ = 10 μ M (Rotenberg et al., 1990). A fortuitous property of DECA is that it can be rendered chemically reactive by irradiation with UV light (366 nm), producing covalent modification at its enzyme target site (Rotenberg and Sun, 1998). The property of photo-induced covalent modification by DECA was first demonstrated with the mitochondrial F1 ATPase, which underwent covalent modification with coincident inactivation of activity (Zhuo and Allison, 1988). Similarly, when PKC is treated with low micromolar concentrations of DECA and UV light in vitro, there is a dose-dependent, loss of catalytic activity that cannot be reversed by dilution (Rotenberg and Sun, 1998).

In the present work photo-induced inactivation is demonstrated with intracellular PKC in cells that have been treated with the drug and irradiated directly with long-wave UV light. A novel aspect is that UV-induced inhibition of both PKC activity and cell motility can be observed with nanomolar concentrations of DECA or DECA analogs recently developed by this laboratory (Qin et al., 2000). These studies implicate PKC α as a critical component in the motility of metastatic melanoma cells and establish it as an important target for antimetastatic agents.

Experimental Procedures

Materials. Cell culture media, phosphate-buffered saline, and growth serum were purchased from Gibco-BRL (Gaithersburg, MD). Matrigel was acquired from BD Biosciences (Bedford, MA). PS was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL), TPA was purchased from LC Services (Woburn, MA), and Go6976 was acquired from CalBiochem (San Diego, CA). Nitrocellulose membranes were obtained from Amersham Pharmacia Biotech (Piscataway, NJ), [γ -³²P]ATP (3000 Ci/mmol) was from NEN-DuPont (Wilmington, DE), and protein dye reagent was from Bio-Rad (Hercules, CA). The ²⁵SER peptide (RFARKGSLRQKNV) was synthesized by N. Pileggi (Protein Core Facility, Columbia University, New York). All curve-fitting and graphical representations were prepared with CA-Cricket Graph III software purchased from Computer Associates International, Inc. (Icelandia, NY).

Cell Culture. Murine melanoma B16 F10 cells were cultured in 10-cm² dishes to 75 to 80% confluence in RPMI medium containing

10% fetal bovine serum, 1% penicillin/streptomycin, and 0.125 μ g/ml fungizone.

Irradiation of Cells with DECA. DECA analogs were synthesized as the di-iodide salts by an established method (Taylor, 1951; Rotenberg et al., 1990; Qin et al., 2000). A confluent monolayer of cells was washed twice with phosphate-buffered saline, and the indicated concentration of DECA analog was added to the cells in serum-free medium. Cells were treated with or without the drug for 1 h at 37°C, 5% CO₂, and then washed with phosphate-buffered saline. In phosphate-buffered saline and with the lids off, cells were irradiated with long-wave UV light for 5 min (1200 μ W/cm²). Irradiation was carried out with a long-wave UV lamp (American Ultraviolet Co., Murray Hill, NJ) while the plate of cells was subjected to gentle shaking at room temperature. After this treatment, the medium was removed and the cells were washed twice with PBS. At this point, cells were collected for subsequent assay of adhesion or migration (see below), or lysed for PKC isolation.

To isolate PKC, cells were harvested by scraping, pelleted, and lysed in homogenization buffer containing 0.1% Triton X-100. The sample was applied to a 0.5-ml column of DEAE-Sephacel, the column was washed with buffer A (20 mM Tris, pH 7.5, 2 mM EGTA, 2 mM EDTA, 1 mM 2-mercaptoethanol, 0.25 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, and 10 μ g/ml soybean trypsin inhibitor), and PKC activity was recovered by elution with 150 mM NaCl in buffer A, as previously described (Rotenberg et al., 1990).

PKC Assay. PKC catalytic activity was judged by a standard assay in which ³²P was transferred from [γ -³²P]ATP to a peptide substrate, as described elsewhere (Rotenberg et al., 1990). Triplicate measurements of substrate phosphorylation were conducted in the absence and presence of 1 μ M TPA, 83 μ g/ml PS, and 0.5 mM Ca²⁺. The difference in substrate phosphorylation in the two conditions was taken as PKC activity. The peptide substrate used in these studies was the synthetically modified pseudosubstrate peptide (²⁵Ser peptide) (House and Kemp, 1987).

Transfection and Isolation of a Kinase-Defective Mutant of PKC α . A constitutive expression mammalian vector, pEFneo, containing the cDNA of a polyhistidine-tagged, kinase-defective mutant of bovine PKC α (Kampfer et al., 1998), was transfected into cells with Lipofectamine Plus reagent (Life Technologies, Inc., Gaithersburg, MD). Stable clones were isolated by selection with G418 present at 500 μ g/ml, and transfectants were maintained at an antibiotic concentration of 250 μ g/ml. To demonstrate the expression of mutant PKC α , lysates were prepared, and polyhistidine-tagged protein was isolated by metal chelate affinity chromatography (Pharmacia Biotech). Quantitative analysis of chemiluminescent signals on Western blots was carried out by two-dimensional scanning densitometry (Molecular Dynamics, Sunnyvale, CA).

Western Blot. Cell lysates or eluates of metal chelate chromatography were subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently transferred electrophoretically to a nitrocellulose filter (Towbin et al., 1979). Immunochemical assay of PKC isoforms was carried out in a 10-trough manifold (Pharmacia Biotech) with mouse primary antisera that were PKC isoform specific (Transduction Laboratories, Lexington, KY) and secondary anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). The blot was developed by the chemiluminescence (Amersham Pharmacia Biotech).

Wound Closure Assay. To measure cell motility by digital image capture, an Olympus IMT-2 inverted phase contrast microscope (Olympus Microscopes, London, UK), fitted with a Fujitsu TC2-336P charge-coupled device camera (EOS Electronics AV Ltd., Barry, Wales) and surrounded by a Perspex temperature-regulated jacket that was adjusted to 37°C, was used. Cells were plated onto 3-cm² Petri dishes such that a densely confluent monolayer was achieved within 24 or 48 h. After the cells were treated with DECA and UV, a "scratch wound" was created by drawing a sterile micropipette tip along the monolayer with a ruler as a guide. Detached cells were removed by washing twice in growth medium before adding 2 ml of

fresh medium. The dish was placed in a specially constructed two-piece circular aluminum housing that had a glass lid and an epicentric hole in the base through which the cells were observed with the 10 \times objective. The chamber was gassed with 10% CO₂ in humidified air and was placed onto the stage of the microscope. A regular wound edge was chosen and digital images were collected every 30 min for 8 h onto a Power Mac 7100 computer by use of Adobe Premier software. The percentage of the capture window (determined as 726 \times 544 μ m) that had been filled in by the movement of cells (normalized to the original wound edge) was determined with Optilab software and plotted against time.

Migration Assay. A 12-mm Costar Transwell (Boyden chamber) having a 12- μ m pore size was used to measure cell movement across a porous polycarbonate membrane (haptotaxis). The bottom surface was coated with 35 μ g of Matrigel (BD Biosciences) for 1 h at 37°C, 5% CO₂. Cells were seeded into the upper chamber of the Transwell at 10⁵ cells per well and incubated at 37°C, 5% CO₂. After the cells were incubated for 3 h, the upper chamber was carefully wiped with a cotton swab to remove cells that remained on the upper membrane surface. Those cells that had migrated to the lower membrane surface were fixed and stained by the Hema 3 staining system (Fisher Scientific, Pittsburgh, PA). Eight fields of adherent cells were randomly counted in each well with a Nikon Diaphot-TMD inverted microscope at 400 \times magnification, and the results were numerically averaged. Each condition was conducted in triplicate and statistical significance was determined by ANOVA with SigmaStat software.

Adhesion Assay. Twenty-four-well tissue culture plates were coated with 200 μ l of collagen IV (2 μ g/cm²), fibronectin (2 μ g/cm²), or Matrigel (43.5 μ g/cm²) and incubated at 37°C, 5% CO₂ for 1 h, followed by washing with PBS. Immediately before use, the coated wells were overlaid with 1% bovine serum albumin for 30 min, washed five times with phosphate-buffered saline, and dried for 30 min at room temperature in the tissue culture hood. Cells were applied to individual wells at 1.5 \times 10⁵ per well and incubated for 1 h at 37°C, 5% CO₂. Nonadherent cells were removed by aspiration and three additional washes with phosphate-buffered saline. Adherent cells were counted visually with a Nikon Diaphot-TMD inverted microscope at 400 \times magnification. In each well, cells were counted in eight randomly chosen fields and numerically averaged. Each experimental group consisted of triplicate measurements.

Results

UV plus DECA Inhibits Motility of B16 F10 Cells. In initial studies we examined C10-DECA (X = 10 in Fig. 1) and UV treatment for their effects on the motility phenotype of B16 F10 mouse melanoma cells. These cells were previously found to be highly metastatic in a mouse model (Gopalakrishna and Barsky, 1988). To examine the kinetics of cell motility, a wound closure assay was developed. Briefly, the wound closure assay consisted of a plate of cells that was "wounded" with a sterile pipet tip to introduce a cell-free zone across a densely confluent cell field. The movement of cells to fill in the cell-free zone was monitored by time-lapse image

capture, and subsequently analyzed by computer software, as described under *Experimental Procedures*.

When B16 F10 cells were subjected to the combined treatment of C10-DECA/UV, inhibition of motility was observed with drug concentrations in the nanomolar range. Shown in Fig. 2A are the results of testing the effects of 5-min UV alone, 250 nM C10-DECA alone, or their combined use, compared with the vehicle control [dimethyl sulfoxide (DMSO), 0.1%, v/v]. Cells treated with UV only or C10-DECA only were indistinguishable from the vehicle control, whereas cells treated with UV plus C10-DECA were nonmotile for a period of 3 to 4 h. Four hours posttreatment, these cells recovered to some extent, achieving a maximum rate of motility during the next 10 h that was 35% lower than the control behavior. In a related experiment, shown in Fig. 2B, inhibition of motility was found to be dose dependent. The motility of B16 F10 cells that had been UV irradiated for 5 min in the presence of 0, 125, 250, or 500 nM C10-DECA exhibited a dose-dependent decrease in the average rate of motility (calculated from the slopes of two or more individual experiments). In Fig. 2C, the average rate was replotted against the corresponding C10-DECA concentration, producing a linear curve with an IC₅₀ of 450 to 500 nM.

It is noted here that fluorescence-activated cell sorting analysis with antisera directed against specific integrins revealed no changes in cell surface expression levels of β 1, α v, α 4, α 5, or α 6 integrins (data not shown). In addition, it was observed that neither viability nor the proliferative capacity of B16 F10 cells were affected for up to 96 h after 250 nM C10-DECA/UV treatment (not shown).

DECA consists of two aminoquinaldine moieties linked by a 10-carbon alkylene bridge (C10-DECA; Fig. 1). Recently, several analogs of DECA bearing longer alkyl linkers of 12, 14, and 16 carbons were synthesized and tested as PKC α inhibitors in vitro (Qin et al., 2000). Inhibition was found to improve with the longer linkers and was maximized with a linker having 14 carbons (C14-DECA).

Inhibition of cell migration (haptotaxis) by 250 nM DECA analogs (C10-, C12-, and C14-DECA; Fig. 1) was analyzed during the initial 3-h post-treatment by the use of Boyden chambers. Cells that had been treated with a DECA analog plus UV were applied to the upper surface of the membrane, whereupon they moved chemotactically through the membrane and adhered to Matrigel, which had been coated onto the under surface of the membrane. After the initial 3 h, the number of cells migrating to this lower surface was measured. As shown in Fig. 3A, the DECA analogs produced inhibition of cell migration and exhibited a patterned response with analogs having longer linkers. Although 250 nM C10-DECA inhibited migration by 20 to 30% of controls (similar to the effect on motility rate shown in Fig. 2C), inhibition by 250 nM C14-DECA was more potent, producing 40 to 60% of the control. Additional experiments indicated that inhibition by C14-DECA was dose dependent in the range of 0 to 500 nM, as shown in Fig. 3B.

UV plus DECA Analogs Inactivate Conventional PKC Activity. Previous work with DECA showed that the combined use of C10-DECA and UV irradiation induced irreversible PKC inhibition in vitro (Rotenberg and Sun, 1998). For the present study, we addressed the possibility that nanomolar concentrations of DECA analogs caused inactivation of intracellular PKC under the same conditions that

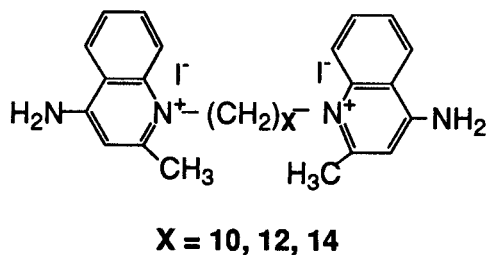


Fig. 1. Chemical structure of dequalinium analogs.

produce inhibition of cell motility. B16 F10 cells were treated with 250 nM C10-, C12-, or C14-DECA and irradiated with UV for 5 min. The results (Fig. 4A) demonstrated that 250

nM C10-DECA produced 35% inhibition of TPA/PS/Ca²⁺-dependent PKC activity. This finding is consistent with a 33% loss of cell motility (Fig. 2C) and 20 to 30% inhibition of

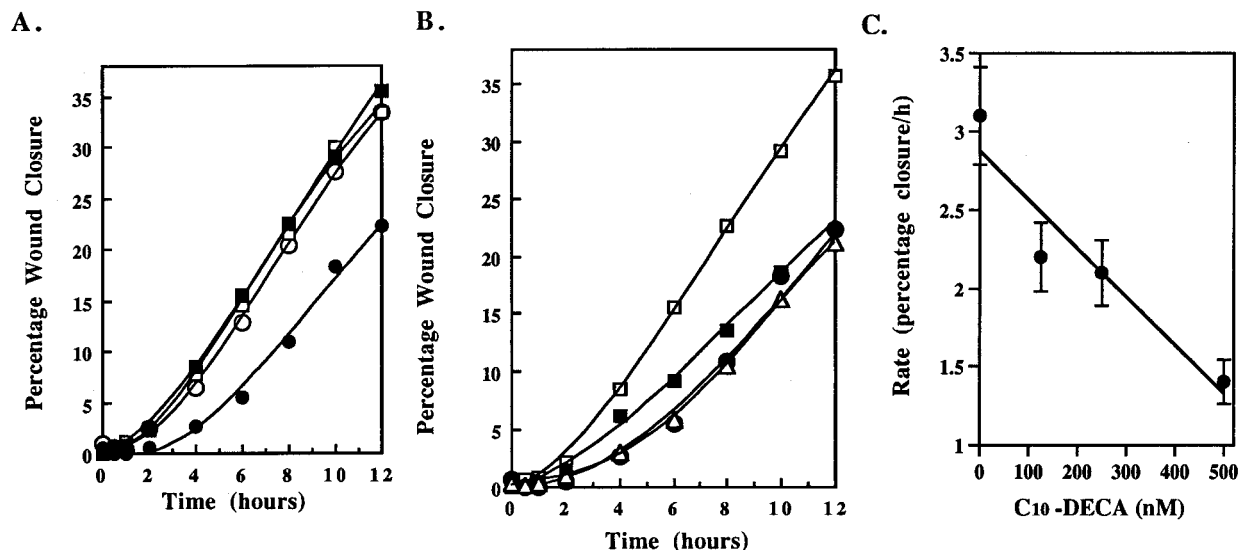


Fig. 2. Inhibition of cell motility by C10-DECA/UV treatment. A, time-dependent movement of B16 F10 cells was monitored for DMSO-treated control cells (0.1%, v/v) without (□) and with (■) 5 min of UV irradiation, cells pretreated for 1 h with 250 nM C10-DECA only (○), and cells subjected to the combined treatment of 5 min of UV plus C10-DECA (●). B, time-dependent cell movement recorded for cells pretreated for 1 h with the specified concentration of C10-DECA, followed by UV irradiation for 5 min. The following C10-DECA concentrations were used: vehicle control (DMSO, 0.1%, v/v; □), 125 nM (■), 250 nM (●), and 500 nM (△). Each time-dependent curve in A and B is the average of two independent experiments for which the error was within 10%. C, replot of the slopes from B against the corresponding DECA concentration.

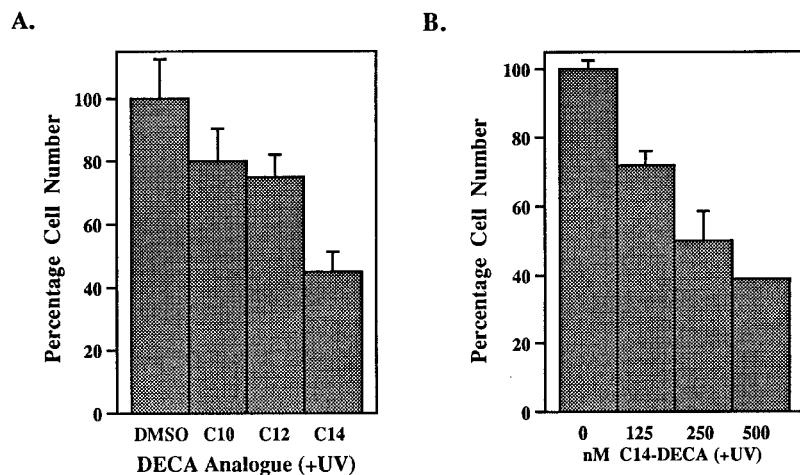


Fig. 3. Inhibition of cell migration by DECA analogs. A, cells were treated with the C10-, C12-, or C14-DECA analog at 250 nM for 1 h, followed by UV irradiation for 5 min. Cells were assayed for migration in Transwells, as described under *Experimental Procedures*. Each value is the average of triplicate measurements and the S.D. was within 15%. B, concentration-dependent inhibition of cell migration by C14-DECA. Cells were treated with the indicated concentration of this analog, as described in A. The results are representative of three independent experiments. For both A and B, ANOVA showed that $P < .001$.

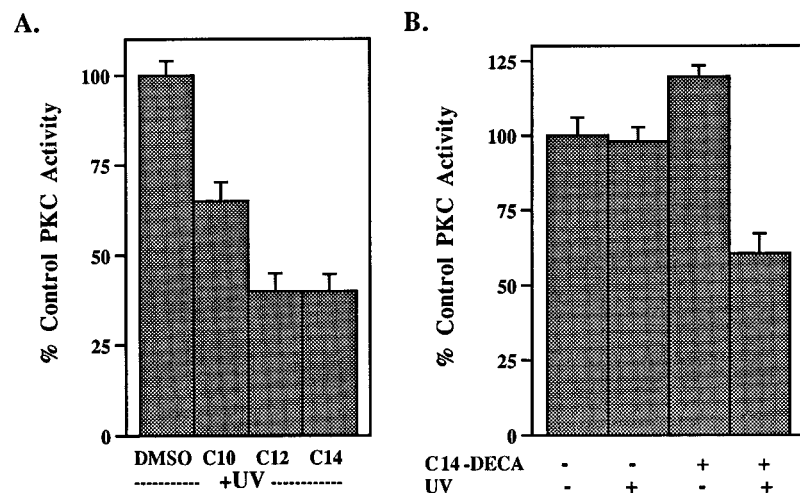


Fig. 4. Inactivation of intracellular PKC activity by DECA analogs. PKC activity was partially purified from cells that had been treated with a DECA analog and UV irradiation and then assayed for catalytic activity. The average of triplicate activity measurements was typically within 10 to 15% error. Each experiment is representative of two or more independent experiments. A, inhibition of intracellular PKC activity was analyzed after treatment of cells with UV plus 250 nM C10-, C12-, or C14-DECA, or DMSO (0.05%, v/v). B, inhibition of PKC activity by C14-DECA is potentiated with UV light. The control TPA/PS/Ca²⁺-dependent kinase activity (165 pmol/min/mg) isolated from vehicle-treated cells (0.05% DMSO) was compared with 5 min of UV irradiation only, 250 nM C14-DECA only, or the combination of these treatments. +, included in treatment; -, not included in treatment. The percentage control PKC activity remaining after each treatment is shown. Error bars, \pm S.D.

haptotaxis (Fig. 3A) produced by C10-DECA under identical conditions. When Figs. 3A and 4A are compared, it can be seen that the C14-DECA analog showed greater potency than C10-DECA in inhibition of both motility and PKC activity. However, the C12-DECA analog, which occasionally produced an intermediate effect in inhibiting these activities, exhibited less predictable activity. Nonetheless, inactivation of PKC activity by 250 nM C14-DECA plus UV typically produced 40 to 60% inhibition, whereas UV light alone or 250 nM C14-DECA alone had no inhibitory effect (Fig. 4B). Thus, an improvement in inhibitory potency was consistently observed when comparing C14-DECA and C10-DECA for inhibition of both PKC activity and migration.

Pilot experiments (not shown) indicated that inactivation of PKC activity was maximal with 5-min irradiation, because

longer periods of irradiation (up to 30 min) neither improved nor diminished the extent of PKC inhibition.

B16 F10 cells possess a complex profile of PKC isoforms (Fig. 5A). When isoform-specific antisera were used, the PKC α isoform (80 kDa) was found to be present in greatest abundance and was the only detectable conventional isoform in these cells. (The β -isoform is absent and the γ -isoform is generally found in neural tissue.) By contrast, the Ca²⁺-independent isoforms (or novel isoforms), PKC- ϵ and - θ , were detected at significantly lower levels. It was noted that a band at ~150 kDa was immunoreactive with antisera recognizing PKC δ (74–79 kDa), but its significance remains unknown. Other experiments indicated that the PKC catalytic activity that was measured in Fig. 4 can be largely attributed to the activity of a conventional isoform, which, in B16 F10 cells, is limited to PKC α . This conclusion follows from experiments (shown in Fig. 5B) in which lysates that had been partially purified by DEAE-Sephacel chromatography, were assayed for activity with no activators, TPA/PS, or TPA/PS/Ca²⁺. The results showed that only TPA/PS/Ca²⁺-dependent activity was detectable and that the level of Ca²⁺-independent activity stimulated by TPA/PS alone was negligible. Furthermore, the addition of 1 μ M Go6976, a specific inhibitor of PKC α , produced substantial (75%) inhibition of TPA/PS/Ca²⁺-stimulated activity. These observations, coupled with the relatively low expression levels of Ca²⁺-independent isoforms (ϵ , η , θ), imply that the measurable catalytic activity was that of PKC α , the only detectable conventional isoform. For these reasons, PKC α was chosen as the focus for subsequent study. It is emphasized, however, that the possibility that other PKC isoforms may serve as targets of DECA cannot be excluded.

Overexpression of a Kinase-Defective PKC α Mutant Decreases Motility and Adhesion of B16 Cells. Because DECA/UV effects on motility could be correlated with inactivation of intracellular PKC α activity, the next objective was to demonstrate that PKC α has a critical role in cell motility. For this purpose, stable transfectants of B16 F10 cells were prepared that overexpressed a polyhistidine-tagged kinase-defective mutant of PKC α protein in which the ATP-binding lysine-368 was replaced by an arginine residue (as described by Uberall et al., 1997). This mutant PKC α does not phosphorylate cellular substrates and is therefore analogous to native PKC α whose catalytic activity had been inactivated by DECA. Lysates prepared from mutant PKC α -expressing cells (clone α 6) or cells stably transfected with a control plasmid were partially purified by metal chelate affinity chromatography that selectively adsorbs polyhistidine-tagged protein.

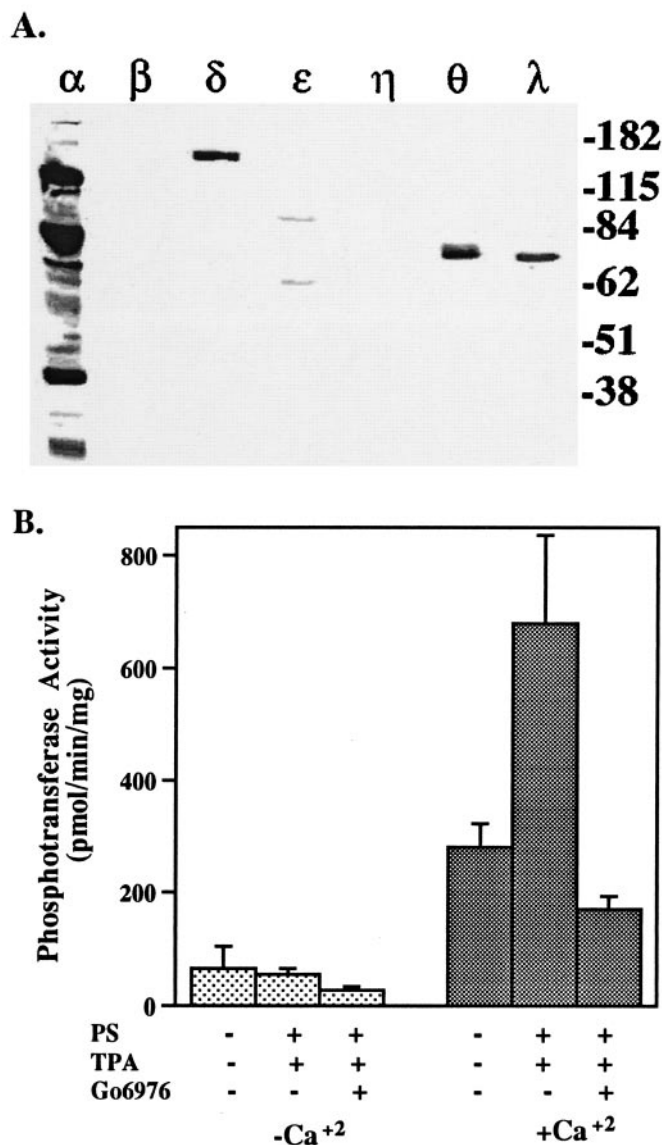


Fig. 5. Analysis of PKC isoform expression and activity in B16 F10 cells. A, whole cell lysates were prepared from B16 F10 cells and analyzed with PKC isoform-specific antisera (46 μ g protein/lane). B, phosphotransferase activities of lysates that had been partially purified by DEAE-Sephacel chromatography. The specified conditions included one or more of the following components: 0.5 mM Ca²⁺, 83 μ g/ml PS, 1 μ M TPA, and 1 μ M Go6976. Ca²⁺-independent conditions included 0.5 mM EDTA. +, included in treatment; -, not included in treatment.

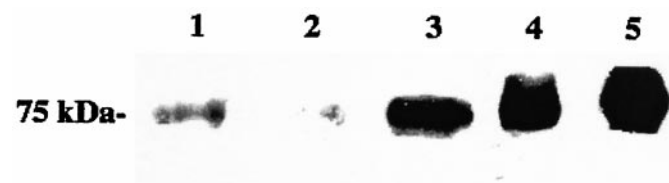


Fig. 6. Overexpression of a polyhistidine-tagged kinase-defective PKC α mutant by B16 F10 cells. Western blot analysis with PKC α -specific antisera was carried out with eluates (23 μ g/lane) obtained by metal chelate chromatography (lanes 1–3), and compared with a whole cell lysate (lane 4). Lane 1, B16 F10 parental cells; lane 2, mock-transfectants; lane 3, α 6 transfectants; and lane 5, recombinant PKC α protein standard (PanVera Corp., Madison WI).

Western blot demonstrated that the eluted polyhistidine-tagged PKC α mutant was expressed at a 5-fold higher level compared to the low signal obtained by the same chromatographic protocol for mock-transfected cells and parental cells (Fig. 6). Importantly, there were no detectable compensatory changes observed in the expression levels of either wild-type PKC α or other PKC isoforms expressed by α 6 cells (data not shown).

Against a high background of endogenous PKC α activity (Fig. 5A), the overexpression of a kinase-defective PKC α mutant in B16 F10 cells would be expected to compromise but not to eliminate the function of the wild-type PKC α . To test whether clone α 6 exhibited a slower rate of motility, a wound closure experiment was conducted. As shown in Fig. 7, α 6 cells exhibited an average motility rate (1% wound closure/h) that was typically 50 to 60% slower than that for mock-transfected cells (2% wound closure/h). In addition, Table 1 displays the haptotactic migration behavior of parental, mock-transfected, and α 6 cells. It was found that α 6 cells also exhibited a decrease in haptotaxis by 60% over a 3-h period in response to collagen IV or Matrigel (which is composed of 30% collagen IV and 60% laminin). In response to fibronectin, however, the migration behavior of α 6 cells was not significantly different from controls.

When plated on single substrates such as fibronectin or collagen IV, α 6 cells displayed diminished adhesion to each of these substrates (Table 1), compared with Matrigel. Effects on adhesion by kinase-defective PKC α expression were most pronounced with either collagen IV or fibronectin, to which adhesion was reduced by 63 and 58%, respectively. By contrast, adhesion by α 6 cells to Matrigel was inhibited by only 24%. These results suggest that wild-type PKC α activity mediates adhesion of B16 cells with collagen IV and fibronectin. Analysis of whole cell lysates for the expression of several adhesion molecules and other proteins identified with cell motility revealed that there were no detectable changes in

their expression levels in α 6 cells compared with mock-transfected and parental cells. The proteins tested by Western blot (not shown) included β 1-integrin, focal adhesion kinase, paxillin, VASP, pp120, dynamin, desmoglein, E-cadherin, and α -, β -, and γ -catenin.

Inhibition of Migration by DECA in Cells That Express a Kinase-Defective Mutant of PKC α . To establish a mechanistic link between inhibition of PKC α activity by DECA and its inhibition of cell motility, we examined the effect of C14-DECA on the motility of α 6 cells and mock-transfected cells. The premise for this experiment is based on the expectation that the kinase-defective PKC α protein expressed by α 6 cells will compete with native PKC α protein for binding of C14-DECA, thereby decreasing the effective intracellular concentration of C14-DECA. As a result, 500 nM C14-DECA (plus UV) would be predicted to be a far less effective inhibitor of PKC α activity in α 6 cells than in parental or mock-transfected cells. (All three cell lines express the same amount of wild-type PKC α protein.) If PKC α is the critical target of DECA that produces inhibition of migration, then the inhibitory action of C14-DECA should be compromised in α 6 cells.

As can be seen in Fig. 8, further inhibition by C14-DECA of migration was completely eliminated in α 6 cells. Treatment of cells with or without 500 nM C14-DECA plus UV revealed that 50% inhibition of migration (in response to Matrigel) had occurred in mock-transfected and parental cells (consistent with Fig. 4B) but that α 6 cells remained largely unaffected by the drug. This finding indicated that the presence of the PKC α mutant protein in α 6 cells served as an effective competitor for the drug. If a target of DECA other than PKC α had been responsible for inhibiting cell migration, then the presence of nonfunctional PKC α would not have impaired its action. These findings link the action of C14-DECA to inhibition of both PKC α activity and cell migration.

Discussion

Cell locomotion is the outcome of concerted events involving cytoskeletal rearrangements and the dynamics of focal adhesion contacts with the extracellular matrix (Palecek et al., 1997). The function of PKC α in these events involves its translocation to membranes where it interacts with cytoskeletal substrates and adhesion proteins, producing changes in cytoskeletal organization and cell movement (Nobes and Hall, 1995; Myat et al., 1997; Chapline et al., 1998). The design of agents that intervene in these PKC-mediated activities may well provide an effective therapeutic approach to halting cellular metastasis.

In the foregoing study, a role for PKC α was examined in murine B16 F10 melanoma cells that had previously been shown to be highly metastatic in a syngeneic mouse model (Gopalakrishna and Barsky, 1988). A causal event in the metastatic activity of these cells was attributed to PKC activation and localization of this isoform to the membrane fraction. Consistent with this idea, our findings demonstrated that both DECA-mediated inactivation of intracellular PKC catalytic activity (attributed to PKC α) and genetically engineered expression of a kinase-defective mutant of PKC α produced significant decreases in cell adhesion and motility (Table 1). Studies in which C14-DECA/UV-treated cells were fractionated into soluble and particulate fractions have

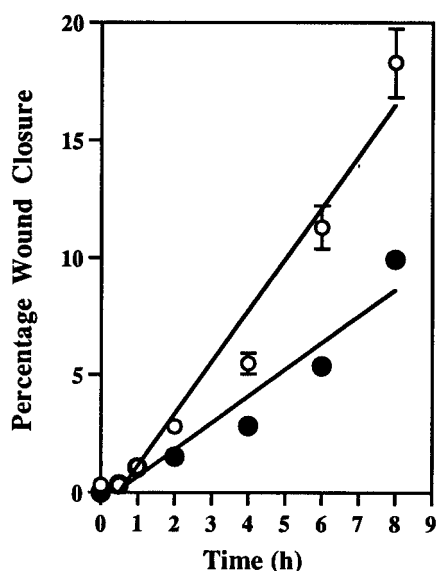


Fig. 7. Motility of cells expressing a kinase-defective mutant of PKC α . Kinetics of motility by cells expressing kinase-defective mutant PKC α (●) compared with mock-transfected cells (○). A wound closure assay was conducted as described under *Experimental Procedures*. The curves represent an average of three independent experiments that was within 8% error. Error bars, \pm S.D.

shown that DECA does not act by displacing PKC α protein from the membrane, but causes equivalent inhibition of PKC activity in both cytosolic and membrane compartments (data not shown). We conclude, therefore, that DECA action with PKC α in B16 F10 cells primarily involves the inactivation of catalytic activity rather than by redistribution of this isoform from membrane to cytosol.

An important outcome of these studies was that the inhibitory actions of DECA were evident in the nanomolar range when coupled with exposure to UV light. The kinetics of cell motility revealed that C10-DECA/UV abolished cell movement for 3 to 4 h post-treatment (Fig. 2), after which cells regained partial motility whose rate was a function of the C10-DECA concentration (Fig. 2C). That this effect could be attributed specifically to inhibition of PKC α rather than of an additional target protein of DECA is supported by the following observations: 1) nanomolar concentrations of DECA plus UV were sufficient to cause inhibition of both intracellular conventional PKC activity and cell movement, and 2) overexpression of a kinase-defective mutant of PKC α led to diminished cell movement (Fig. 7) and eliminated the ability of C14-DECA to inhibit cell migration further (Fig. 8).

Other potential cellular targets of DECA (studied by others without UV light), include the Ca²⁺-activated K⁺-channel (Galanakis et al., 1996), the mitochondrial F1-ATPase, and a calmodulin-dependent phosphodiesterase (Hait, 1987), the inhibition of which require micromolar concentrations of C10-DECA. With regard to the motility phenotype, both the K⁺-channel protein and the F1-ATPase can be ruled out as targets of DECA because of the absence of a patterned response to DECA analogs with different linker lengths (Galanakis et al., 1996; W. S. Allison, personal communication). However, the results obtained with C10- and C14-DECA gave clear evidence of linker-length-dependent action with respect to inhibition of both PKC α activity (Fig. 4A) and migration of B16 F10 cells (Fig. 3A). If a DECA target other than PKC α had been critical to the mechanism of migration of B16 F10 cells, then the expression of a kinase-defective mutant of PKC α would not have impaired the inhibitory action of C14-DECA (Fig. 8).

Other studies from this laboratory have shown that inhibition of highly pure, recombinant PKC α by DECA in vitro entails a direct interaction with the catalytic domain (Rotenberg et al., 1998) in a manner that involves binding of the ring moieties in a *trans*-oid fashion at two distinct sites. The two sites are separated by a distance of approximately 16 to 17 Å (Qin et al., 2000) as defined by C14-DECA, the analog with highest potency. The identity of the two sites remains

unknown because earlier studies demonstrated that inhibition is not competitive with either ATP (Rotenberg et al., 1990) or substrate (S.A. Rotenberg, unpublished observations). Although in vitro assays characterized the effect of C12-DECA as intermediate between C10- and C14-DECA (Qin et al., 2000), the effect of the C12 analog on intracellular PKC α activity was less predictable. It is possible that, compared with the purified recombinant enzyme, the distance between the two sites is a variable with the intracellular PKC α enzyme, which can exist in multiple states of phosphorylation. Binding of DECA across this distance may be achieved best with the C14 analog, thereby accounting for its higher effectiveness and reproducibility with the cellular enzyme. The interaction of DECA with the catalytic domain is distinct from an additional interaction by DECA with the regulatory domain at the binding site for RACK (receptor for activated C-kinase), which consequently inhibits TPA-induced PKC translocation (Rotenberg and Sun, 1998). The interaction by DECA with the catalytic domain is probably an independent binding event, because inhibition of PKC α mutant enzymes that lack the regulatory domain was similar to that of the wild-type enzyme (Rotenberg et al., 1998).

A compelling property of dicationic compounds like DECA is that they are selectively accumulated by cancer cells because of their abnormally high transmembrane potentials (Chen, 1989). It has been estimated that exogenous dicationic drug levels in the nanomolar range can be accumulated by cells to micromolar levels. Thus, in contrast to PKC inhibition in vitro, which requires micromolar concentrations of DECA, inhibition of intracellular PKC activity can proceed with extracellular DECA concentrations in the nanomolar range. An additional factor in the present study is that, unlike many cell types, the mitochondria of B16 F10 cells do not retain the drug for lengthy periods (Bernal et al., 1983), an observation that would predict accumulation of DECA in the cytosol. Our findings that intracellular PKC α activity can be inhibited by exogenous nanomolar doses of DECA are therefore consistent with cytosolic accumulation of the drug to high levels.

Past studies have implicated a role for PKC in adhesion and migration of many cell types with the use of either TPA as a potent stimulus or PKC inhibitors. However, these tools were not selective for specific PKC isoforms. Recombinant methods and immunofluorescence microscopy have established a specific role for PKC α in adhesion and motility of vascular smooth muscle cells, intestinal cells, and human mammary epithelia (Batlle et al., 1998; Haller et al., 1998; Ng et al., 1999; Sun and Rotenberg, 1999). In this regard,

TABLE 1

Migration and adhesion by B16 F10 cells expressing kinase-defective PKC α

Assays of haptotactic migration and adhesion with each substrate were conducted as described under Experimental Procedures. Each experiment is the average of triplicate measurements and the standard deviation is indicated in parentheses. Each experiment is representative of three independent experiments.

Cell	Collagen IV		Fibronectin		Matrigel	
	Cell Number (avg.)	%	Cell Number (avg.)	%	Cell Number (avg.)	%
Migration						
Parental B16 F10	39 (\pm 5)	100	57 (\pm 7)	100	29 (\pm 2)	100
Mock-transfected	47 (\pm 7)	120	49 (\pm 5)	86	27 (\pm 3)	93
α 6 clone	25 (\pm 1)	64	47 (\pm 2)	82	17 (\pm 1)	57
Adhesion						
Parental B16 F10	40 (\pm 2)	100	48 (\pm 5)	100	44 (\pm 3)	100
Mock-transfected	54 (\pm 6)	135	44 (\pm 11)	92	45 (\pm 4)	101
α 6 clone	19 (\pm 2)	47	20 (\pm 1)	42	34 (\pm 3)	76

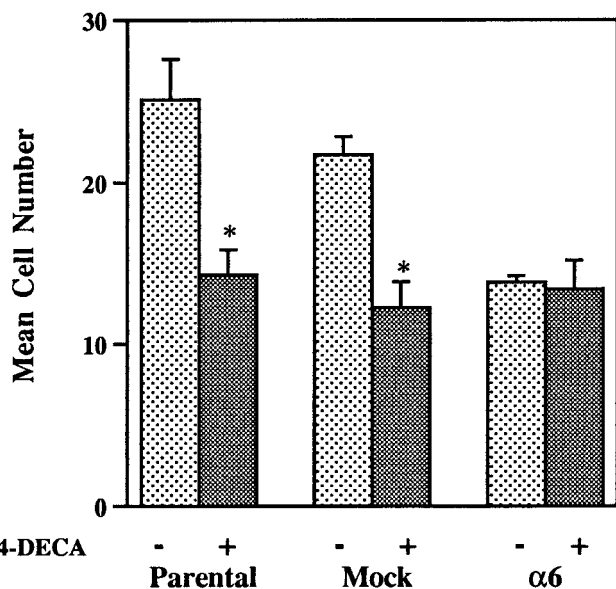


Fig. 8. Expression of a kinase-defective mutant of PKC α interferes with DECA action in B16 F10 cells. Migration assays were conducted with parental B16 F10 cells (Parental), mock-transfected cells (Mock), and cells expressing a kinase-defective mutant PKC α ($\alpha 6$). Cells were treated with 500 nM C14-DECA or DMSO (0.05% v/v), irradiated with UV for 5 min, and assayed for migration behavior in response to Matrigel. The results are representative of three independent experiments. * $P < .001$.

recent findings have linked PKC α catalytic activity with 1) trafficking of integrin $\beta 1$ to the cell surface (Ng et al., 1999) and 2) a signaling component that is upstream of Src kinase and focal adhesion kinase in a motility-signaling pathway between growth factor receptors and integrins (Sieg et al., 2000). Our results (not shown) obtained by fluorescence-activated cell sorting analysis of B16 F10 cells treated with DECA/UV revealed that inhibition of motility (Fig. 2) was not accompanied by changes in cell surface expression levels of certain integrins known to be present in B16 F10 cells ($\beta 1$, α_V , $\alpha 4$, $\alpha 5$, $\alpha 6$). Rather than decrease the trafficking of integrins into the plasma membrane, PKC α inhibition by DECA/UV may act to limit activation of Src kinase¹ and focal adhesion kinase and, consequently, to suppress the function of existent cell surface adhesion proteins. This mechanism is supported by earlier work with human metastatic melanoma cells (Dumont and Bitonti, 1994), which showed that TPA-stimulated PKC activity leads to phosphorylation of $\alpha 3$ and $\beta 1$ integrins with coincident enhancement of adhesion to collagen I and IV.

The foregoing studies reinforce the role of PKC α as a key mechanistic determinant of motile behavior of B16 F10 melanoma cells and as a critical target for the design of anti-metastatic drugs. It is concluded that the PKC α inhibitory and antimigration effects by nanomolar concentrations of DECA are potentiated with UV light, thereby strengthening future clinical use of this drug when coupled with light-mediated technologies.

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¹ Src kinase does not undergo inhibition *in vitro* by concentrations of C10-DECA up to 1 mM (S. A. Rotenberg, unpublished results).

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