Journal of Cellular Biochemistry 101:517–528 (2007)

Proliferation of Human Breast Cancer Cells and Anti-Cancer Action of Doxorubicin and Vinblastine Are Independent of PKC-α

Daniel SD Liu,[1] Chad E. Krebs,[1] and Shi J. Liu[1,2]*

[1]Department of Pharmaceutical Sciences, University of Arkansas for Medical Sciences, 4301 West Markham St., Little Rock, Arkansas 72205
[2]Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, 4301 West Markham St., Little Rock, Arkansas 72205

Abstract Protein kinase C (PKC) has been considered for a potential target of anticancer chemotherapy. PKC-α has been associated with growth and metastasis of some cancer cells. However, the role of PKC-α in human breast cancer cell proliferation and anticancer chemotherapy remains unclear. In this study, we examined whether alterations of PKC-α by phorbol esters and PKC inhibitors could affect proliferation of human breast cancer MCF-7 cells and the cytotoxic effect of chemotherapeutic agents. Exposure for 24 h to doxorubicin (DOX) and vinblastine (VIN) caused a concentration-dependent reduction in proliferation of MCF-7 cells. However, these two anticancer drugs altered cellular morphology and growth pattern in distinct manners. Phorbol 12,13-dibutyrate (PDBu, 100 nM), which enhanced activities of PKC-α, increased cancer cell proliferation and attenuated VIN (1 μM)-induced cytotoxicity. These effects were not affected in the presence of 10 nM staurosporine. Phorbol myristate acetate (PMA, 100 nM) that completely depleted PKC-α also enhanced cancer cell proliferation and attenuated VIN-induced cytotoxicity. Three potent PKC inhibitors, staurosporine (10 nM), chelerythrine (5 μM) and bisindolylmaleimide-I (100 nM), had no significant effect on MCF-7 cell proliferation; staurosporine and chelerythrine, but not bisindolylmaleimide-I, attenuated VIN-induced cytotoxicity. Moreover, neither phorbol esters nor PKC inhibitors had an effect on cytotoxic effects of DOX (1 μM) on MCF-7 cell proliferation. Thus, these data suggest that MCF-7 cell proliferation or the anti-cancer action of DOX and VIN on breast cancer cells is independent of PKC-α. J. Cell. Biochem. 101: 517–528, 2007.

Key words: protein kinase C; doxorubicin; vinblastine; cytotoxicity; MCF-7 breast cell; proliferation

Protein kinase C (PKC), a family of serine/threonine kinases, is well known to play an important role in regulating many cellular functions, including cell proliferation, differentiation, apoptosis and survival [Clemens et al., 1992; Hug and Sarre, 2005], and in pathological development such as tumor promotion [Blobe et al., 1994; Tanaka et al., 2003]. Therefore, PKCs have been considered as potential targets for anticancer chemotherapy [O'Brian et al., 2001]. Expression and activation of different PKC isozymes in various cell types have shown to induce diverse cellular responses [Mandil et al., 2001; Hug and Sarre, 2005]. Among PKC isozymes, protein expression of PKC-α, a member of conventional PKCs (cPKC), is found to distribute ubiquitously in a variety of tissues [Nishizuka, 1995; Hug and Sarre, 2005] and cells [Hug and Sarre, 2005]. PKC-α was also found to be activated in melanoma cells under conditions permissive of metastasis [La Porta and Comolli, 1997] and increased in prostatic carcinomas [Cornford et al., 1999]. Over-expression of PKC-α in MCF-7 cells enhanced proliferation and displayed neoplastic phenotype [Ways et al., 1995]. Studies using antisense oligonucleotides showed that suppression of

Grant sponsor: Arkansas Cancer Research Center Partners in Research program and National Heart, Lung, and Blood Institute.

Daniel SD Liu's present address is California Institute of Technology, Pasadena, CA 91176, USA.

*Correspondence to: Shi J. Liu, PhD, Department of Pharmaceutical Sciences, University of Arkansas for Medical Sciences, 4301 West Markham St. MS 522-3, Little Rock, AR 72205. E-mail: sliu@uams.edu

Received 31 August 2006; Accepted 16 October 2006

DOI 10.1002/jcb.21202

PKC-α expression decreased melanoma metastasis [Dennis et al., 1998] and gastric carcinogenesis [Jiang et al., 2004], and inhibited neoplasm of human lung carcinoma cells [Wang et al., 1999]. These studies suggest a correlation between PKC-α and the growth and metastasis of cancer cells. However, it is not clear whether PKC-α plays a vital role in human breast cancer cells and whether it is a potential target of anticancer therapy for breast cancer.

Doxorubicin (DOX) and vinblastine (VIN), two effective anticancer drugs, have been widely used in combination in chemotherapy for treatment of various cancers including advanced breast cancer and Hodgkin's and non-Hodgkin's lymphomas [Minotti et al., 2004]. It is well known that the anticancer action of DOX leads to DNA damage in cancer cells, probably mediated by multiple mechanisms including intercalation into DNA and inhibition of topoisomerase II [Minotti et al., 2004]. VIN is known to bind to tubulin and alter spindle microtubules, thereby inhibiting mitosis during metaphase [Jordan et al., 1991]. Recent studies on DOX- and VIN-induced apoptosis in normal and tumor cells showed that DOX activated p53 protein, a tumor suppressor, in both normal [Wang et al., 2004] and tumor [Brantley-Finley et al., 2003; Wang et al., 2004] cells, whereas VIN decreased p53 in tumor cells [Brantley-Finley et al., 2003]. Despite different effects on the nucleus, DOX and VIN both activated c-Jun N-terminal Kinase (JNK) in KB-3 carcinoma cells [Brantley-Finley et al., 2003]. However, it is unclear whether JNK or other cytosolic signaling modulators, such as PKCs, which serve as transducers of nuclear signals [Olson et al., 1993; Nishizuka, 1995], plays a key role in the antitumor or cytotoxic effect of these two anticancer drugs. Thus, the present study was to determine the role of PKC-α in proliferation of breast cancer cells and the cytotoxic effect of DOX and VIN on human breast cancer cells, MCF-7 cells.

MATERIALS AND METHODS

Materials

MEMα culture media supplemented with glutamine, insulin, estradiol, penicillin–streptomycin and fetal bovine serum (FBS) and Dulbecco's modified Eagle medium (DMEM) were obtained from Life Technologies (Rockville, MD). CyQuant cell viability assay kit was purchased from Molecular Probes, Inc. (Eugene, OR). Anti-PKC α and ζ antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphorylated PKC-α antibodies (at Ser^{657}, p-PKC-α) were purchased from Upstates Biotechnology (Charlottesville, VA). Pre-casted polyacrylamide gels and nitrocellulose membranes were from Bio-Rad (Richmond, CA). Chemiluminescence detection reagent (Ultra Super-Signal) and X-ray films were obtained from Pierce (Rockford, IL). Bisindolylmaleimide I (Bim-I) was purchased from Calbiochem (San Diego, CA). Vinblastine, doxorubicin, staurosporine, chelerythrine chloride, phorbol 12,13-dibutyrate (PDBu), phorbol 12-myristate 13-acetate (PMA), DMSO, and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture and Drug Treatments

Mid-passage MCF-7 cell line, a human breast cancer cell line, and Chinese hamster ovary (CHO) cell line were generous gifts from Drs. T. Kelly and P. Prather (University of Arkansas for Medical Sciences, Little Rock, AR), respectively. Cells were grown in culture media (MEMα for MCF-7 cells and DMEM for CHO cells) with penicillin (100 U/ml) and streptomycin (100 μg/ml) in 5%CO_2/95% air at 37°C until ~80% confluent, dissociated with 0.25% trypsin and 1 mM EDTA, rinsed with PBS and collected after centrifugation at 300*g* for 3 min at 4°C. Cells (passage 2–5) were resuspended into culture medium, plated into 6- or 24-well microplates and maintained in a serum-containing medium for 24 h, which were then changed to a control serum-free medium overnight to synchronize the cell cycle before experiments of drug treatments.

MCF-7 cells were treated for 24 h with different concentrations of DOX and VIN. PKC inhibitors or phorbol esters were added to culture medium 30 min before and during DOX and VIN treatments. In experiments of pretreatment with 100 nM phorbol esters to deplete PKC [Young et al., 1987; Hug and Sarre, 2005], cells were incubated with phorbol esters for 24 h followed by a rapid rinse with the control serum-free medium. The cells were then incubated for 24 h in the same control medium but containing DOX or VIN. The culture medium of the time-control group contained DMSO, which final concentration was not greater than 0.01%.

Cell Proliferation and Viability Assay

In the end of experiments, cells were rinsed with PBS to wash away dead cells, then placed into a −70°C freezer for 30 min and thawed at the room temperature to induce membrane lysis. Cells were then stained with DNA-binding fluorescent dyes in lysis buffer (CyQuant, Molecular Probes) for 2–3 min. The fluorescent signals from viable cells were quantified with 485 nm (excitation) and 530 nm (emission) and converted to number of cells according to the standard curve. A standard curve was constructed for each experiment by seeding 1,000–50,000 cells/well in the designated wells of the same plate with experimental groups. The intensity of fluorescence signals is a linear function of the known cell numbers with r^2 of >0.99 (see insets of Fig. 1). The cell number in each group in each experiment was determined according to the standard curve.

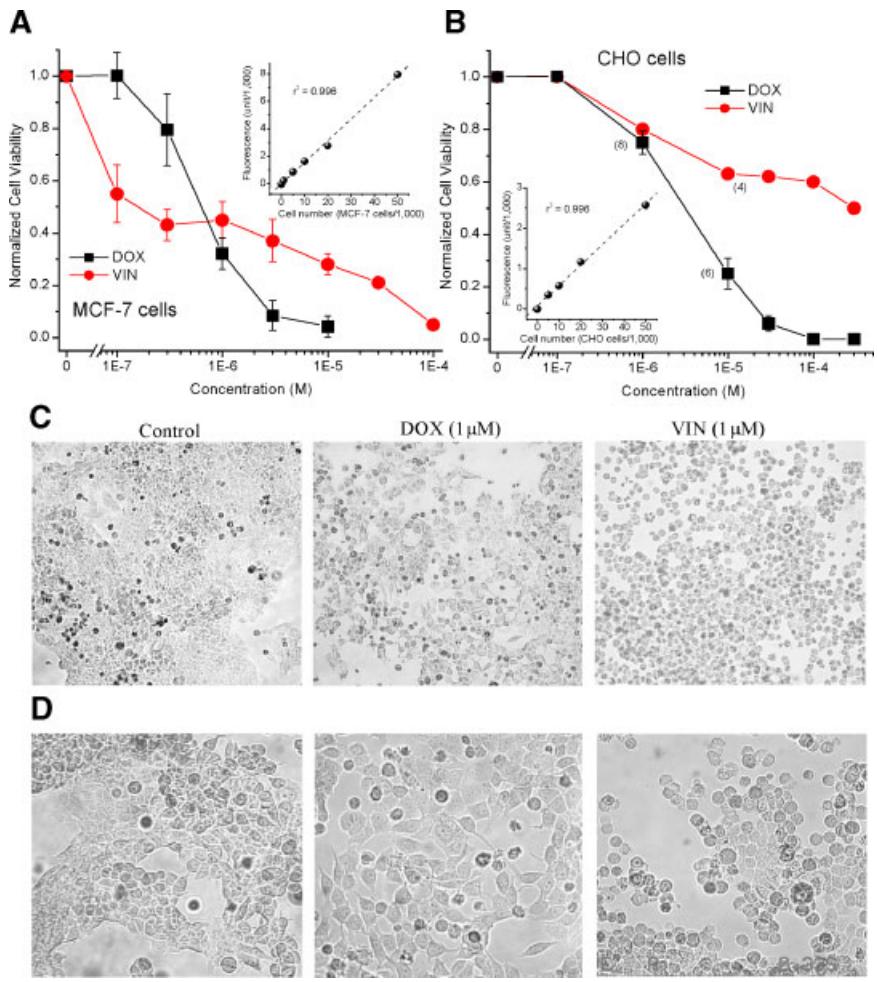

Fig. 1. Effects of of doxorubicin (DOX) and vinblastine (VIN) on growth of MCF-7 and CHO cells, and the morphology of MCF-7 cells. **A**, **B**: Concentration-response curves of growth inhibition induced by DOX (filled squares) and VIN (filled circles) in MCF-7 and CHO cells, respectively. Cells were incubated for 24 h in the absence (time-control with 0.01% DMSO) and presence of various concentrations of DOX or VIN. Number of viable cell in drug-treated groups was normalized to that in each time-control and plotted as a function of concentrations of anticancer drugs. Insets in A and B: Examples of a standard curve constructed in each experiment for determining viable cell number of MCF-cells and CHO cells, respectively. **C**: The growth pattern and morphology of MCF-7 cells in control (left), 1 μM DOX (**middle panel**) and 1 μM VIN (**right panel**) were examined under a microscope (×100 in B) and (×200 in **D**). Photographs are representative fields of more than three independent experiments. Data presented are mean ± SEM obtained from 3 and 6–14 independent experiments for DOX and VIN, respectively, in A. Number in parentheses in B indicates the number of independent experiments, and data for the rest of concentrations were obtained from 2 and 1 experiments for DOX and VIN, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Western Blot Analysis of Phosphorylation and Translocation of PKC-α

Preparation of MCF-7cell lysate and Western blot analyses were carried out as described previously [Liu and McHowat, 1998] with slight modifications. Briefly, cells were rinsed twice with ice-cold PBS and collected after 800*g* at 4°C for 1 min. Cell pellets were resuspended in 0.5 ml ice-cold lysis buffer containing 20 mM HEPES (pH 7.6), 250 mM sucrose, 2 mM dithiothreitol, 2 mM EDTA, 2 mM EGTA, 10 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin and 10 μg/ml aprotinin. After brief sonication on ice, total cell lysate (supernatants) were collected after centrifugation at 10,000*g* at 4°C for 10 min, quantified with the Bradford assay (Bio-Rad) using BSA as standards, and frozen at −70°C before use. In some experiments, cytosolic (the supernatant) and particulate (the pellet) proteins were obtained by centrifugation of total cell lysates at 100,000*g* for 1 h at 4°C. The particulate fraction was further resuspended in a lysis buffer containing 0.5% Triton X-100. Both fractions were quantified in the same manner as described above.

Twenty micrograms of each protein sample was subjected to SDS–PAGE (15% separating gels for total cell lysates or 4%–15% gradient gels for phosphorylation of subcellular fractions), and transferred to nitrocellulose membranes. Proteins on membranes were reversibly stained with 0.1% Ponceau-S Red or SyproRuby (S-11791, Molecular Probes, Carlsbad, CA) to confirm equal protein loading and transfer. Membranes were then blocked with Tris-buffer solution containing 0.05% (v/v) Tween-20 (TBST) and 5% (w/v) nonfat milk for 1 h at room temperature, incubated with antibodies against phosphorylated PKC-α (p-PKC-α, Upstates, 1:1,000) for 3 h at room temperature or anti-total PKC-α and ζ antibodies (1:1000, Santa Cruz) for 1 h at room temperature after stripping membranes. After washes in TBST, membranes were incubated for 1 h at room temperature with horseradish peroxidase-linked secondary antibodies (1:30,000). Immunoblots were detected using an enhanced chemiluminesence method (SuperSignal, Pierce) and quantified by densitometric analysis (Multi-Analyst, Bio-Rad).

Cell Morphological Assay

Before protein extraction, cells grown in 24-well plates were placed on a stage of an inverted microscope (Nikon TE300, Irving, TX). The morphology of cells was examined via 10× or 20× objectives. The video images were acquired by a charge-coupled device camera and stored in an IBM compatible personal computer for later analysis.

Data Analysis and Statistics

All experiments with cell viability assay were performed triplicately. Relative cell viability (or cell number) was determined by dividing the cell number in each treated group by each time-control (which value was 1). The phosphorylation level of PKC-α in each sample was determined by the ratio of the intensity of p-PKC to that of the total PKC in the same lane (or sample). Normalized data obtained from each experiment were then pooled and subject to statistic analysis. All data are expressed as mean ± SEM. Statistical significance ($P < 0.05$) of multiple treatments was determined by ANOVA with a post hoc test of Student–Newman–Keuls Method, while two groups were compared by paired *t*-test in some experiments.

RESULTS

Cytotoxic Effects of DOX and VIN on Proliferation and Morphology of MCF-7 Cells

MCF-7 cells were treated for 24 h with different concentrations of DOX or VIN to yield a concentration response curve. Figure 1A shows that treatment of DOX or VIN resulted in a concentration-dependent reduction in proliferation of MCF-7 cells. While DOX had an apparent IC_{50} of 0.6 μM, VIN showed a different pattern of growth inhibition on MCF-7 cells. In contrast to cancer cells, DOX induced cytotoxicity in non-cancer CHO cells with a higher IC_{50} of 3.2 μM, while 300 μM VIN caused only ~50% reduction of cell viability (Fig. 1B). The results suggest that the antagonizing effect on breast cancer cell proliferation of these two anticancer drugs is much stronger than on CHO cells. This could result from the difference in multiple drug resistance or mechanisms for the associated cytotoxicity. Figure 1C and D illustrate the growth pattern of MCF-7 cells in control, and in the presence of 1 μM DOX or 1 μM VIN for 24 h. With magnification 100× non-

drug treated control cells tightly formed clusters (left panels in Fig. 1C). The growth pattern was disrupted after treatment with DOX (middle panels) and VIN (right panels). Figure 1D shows morphological changes induced by these two chemotherapeutic agents with magnification 200×. DOX treatment enlarged the size of cells and nucleus, and cells became flat and in spindle shape with one or more fine process or pseudopodium (middle panel in Fig. 1D). In contrast, the majority of VIN-treated cells became rounded and smaller, when compared with time-control cells. These results suggest that DOX and VIN not only reduced cell growth but also disrupted the aggregation of MCF-7 cells in different fashions.

Effects of PDBu on MCF-7 Cell Proliferation and Cytotoxicity Induced by DOX and VIN

We used two protocols to examine the effect of phorbol esters on the cytotoxic effect of DOX and VIN. First, cells were pretreated for 24 h with 100 nM phorbol 12,13-dibutyrate (PDBu) to activate PKC, followed by its removal with and without DOX or VIN treatment (pretreatment or p-PDBu). Figure 2A shows that p-PDBu alone induced a ~50% increase in proliferation of MCF-7 cells, whereas 100 nM 4α-PDBu, an inactive form of PDBu, had no effect. Results also show that pretreatment with PDBu, not 4α-PDBu, abolished VIN (1 μM)-induced cytotoxicity (Fig. 2A), confirming the specific action of PDBu on cell proliferation and the VIN action. Similarly, Figure 2B shows that p-PDBu suppressed the growth inhibition induced by a high concentration of VIN (10 μM). Figure 2C summarizes p-PDBu attenuation on VIN-induced concentration-dependent growth inhibition by increasing the IC_{50} to 30 μM. In contrast to VIN, the cytotoxic effect of DOX was not affected by p-PDBu. Figure 2D shows that p-PDBu enhanced cell proliferation (top, right panel), when compared with the time-control (top, left panel). Following p-PDBu, DOX (1 μM) still reduced viable cells that were enlarged and flat (bottom, left panel) as observed in DOX alone (Fig. 1C and D). By contrast, the VIN-induced changes in growth and morphology were not observed with p-PDBu (bottom, right panel). These results suggest that p-PDBu blocks the cytotoxic effect of VIN but not DOX.

In some experiments, cells were treated with 100 nM PDBu 30 min before and during 24-h

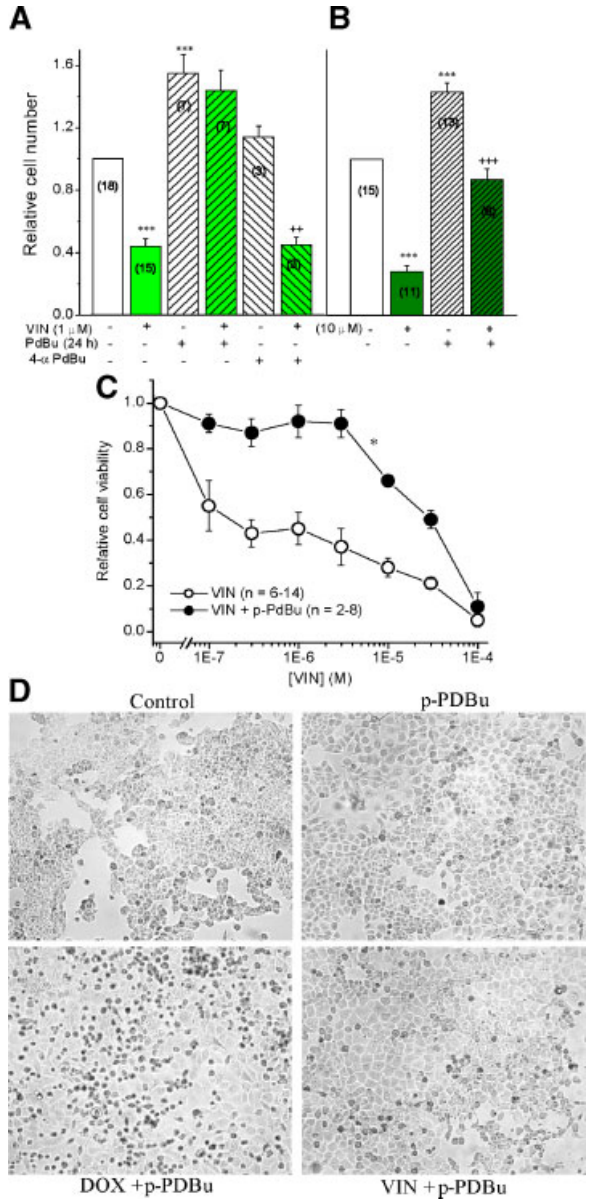

Fig. 2. Effect of phorbol esters on cancer cell growth and VIN-induced growth inhibition. MCF-7 cells were pretreated for 24 h with 100 nM phorbol 12,13-dibutyrate (p-PDBu) or 4α-PDBu followed by 24-h treatment with 1 μM (**A**) or 10 μM VIN (**B**). **C**: Effect of p- PDBu on the concentration-dependent curve of VIN-induced cytotoxicity. Cell viability in treatment with VIN plus p-PDBu was normalized to that with p-PDBu alone (filled circles) and superimposed with that in the presence of VIN alone (open circles). **D**: Morphological examination (×100) of cell growth in DOX plus p-PDBu (bottom, **left panel**) and VIN plus p-PDBU (bottom, **right panel**), compared with the time-control (top, **left panel**) and p-PDBu alone (top, **right panel**). Data presented are mean ± SEM; number in parentheses indicates the number of independent experiments. $^{***}P < 0.001$ versus the time-control; $^{++}P < 0.01$ versus phorbol ester alone. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

treatment (indicated as PDBu) without or with 1 μM VIN. Under these conditions, cell growth was increased by 34 ± 3% (n = 3) and 23 ± 4% (n = 3) in the presence of PDBu alone and co-treatment with VIN, respectively, when compared to the time-control. The growth inhibition induced by co-treatment with 10 μM VIN and 100 nM PDBu was also attenuated (24 ± 11%, n = 9 vs. 72% by 10 μM VIN alone). In other experiments, cells were treated with PDBu for 24 h followed by a refreshment of PDBu without or with 1 μM VIN and incubated for additional 24 h. After 48-h exposure to PDBu, cell proliferation was increased by 87 ± 11% (n = 4). The 24-h co-treatment with PDBu and VIN increased cell proliferation by 70 ± 11% (n = 4). Moreover, in the presence of 10 nM staurosporine, PDBu and PDBu plus VIN still increased cell proliferation by 59 ± 23% and 47 ± 23% (n = 3), respectively. Therefore, regardless how cells were treated with PDBu, chronic exposure to PDBu enhances breast cancer cell growth and abolishes the cytotoxic effect of VIN on cancer cells. These effects were not affected by staurosporine, a potent PKC inhibitor.

Effects of PKC-α Depletion on MCF-7 Cell Proliferation and Actions of DOX and VIN

Chronic treatment of phorbols has been shown to down-regulate cPKCs, and PMA is more potent to inhibit PKC-α than PDBu [Saraiva et al., 2004]. Thus PMA was used to examine the effect of depletion or inhibition of PKC-α in MCF-7 cell on cell growth and the cytotoxic effect of DOX and VIN. Figure 3A

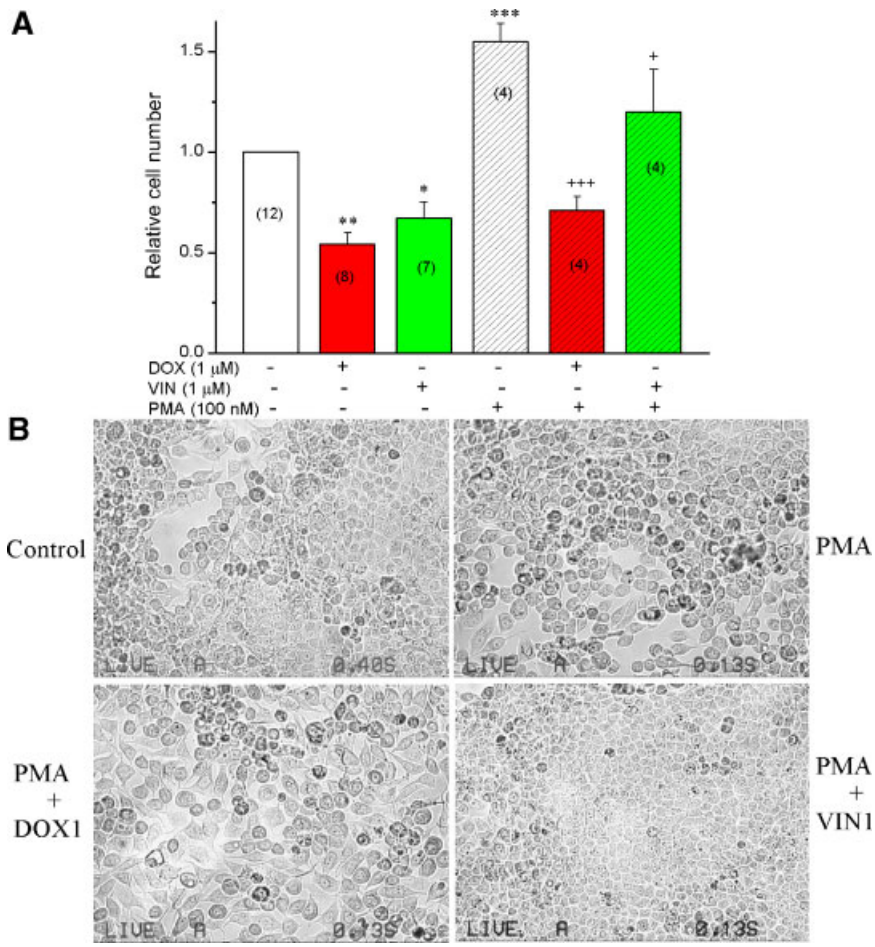

Fig. 3. Effect of PKC-α depletion on cytotoxic effects of DOX and VIN. **A**: Cells were pretreated for 24 h with 100 nM PMA, which depleted PKC-α, followed by 24 h treatment with 1 μM DOX or 1 μM VIN. **B**: Morphology of MCF-7 cells under these experimental conditions examined under a microscope (magnifications 200×). Data presented are mean ± SEM; number in parentheses indicates the number of independent experiments. *, ** and $^{***}P < 0.05$, 0.01 and 0.001 versus the time-control, respectively; $^{+}$ and $^{+++}P < 0.05$ and 0.001 versus PMA alone, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

shows that chronic exposure to 100 nM PMA alone enhanced growth of MCF-7 cells by 55%, similar to the effect of p-PDBu shown in Figure 2A. Similarly, PMA attenuated the VIN-induced growth inhibition but had no effecton the action of DOX. Figure 3B shows the effect of PMA on morphological pattern of MCF-7 cell growth that was also similar to the effect of p-PDBu shown in Figure 2. The evidence for PMA-induced depletion of PKC-α is given in Figure 5A. Thus, the results suggest that PKC-α does not play a vital role to MCF-7 cell growth and the cytotoxic effect of DOX and VIN.

Effects of PKC Inhibitors on Cancer Cell Proliferation and Cytotoxic Effects of DOX and VIN

The effect of inhibition of PKC on cytotoxic actions of VIN and DOX was examined using different PKC inhibitors. First, cells were treated with 10 nM staurosporine, a potent, non-specific PKC inhibitor [Toullec et al., 1991], 30 min before and during 24-h DOX or VIN treatment. Figure 4A shows staurosporine alone had no effect on MCF-7 cell growth or on the cytotoxicity induced by 1 μM DOX. In contrast, growth inhibition induced by 1 μM VIN was attenuated (i.e., $34 \pm 5\%$ vs. $50 \pm 7\%$ inhibition in the absence of staurosporine, $P < 0.05$). Figure 4B shows that survival cells retained aggregation to a high degree in the presence of VIN plus staurosporine. The cytotoxic effect of VIN was further examined using more selective PKC inhibitors, chelerythrine chloride (CC) and bisindolylmaleimide I (Bim-I). CC (5 μM) alone had no effect on MCF-7 cell proliferation but completely blocked the cytotoxicity induced by 1 μM VIN (Fig. 4A). The growth inhibition by 10 μM VIN was also attenuated by CC (from 72% to 60%). In contrast, neither MCF-7 cell proliferation nor the cytotoxicity induced by DOX and VIN was affected by 100 nM Bim-I, a widely-used cPKC selective inhibitor [Martiny-Baron et al., 1993] (Fig. 3C). Thus, these results also suggest that cPKC does not play a vital role in MCF-7 cell proliferation or cytotoxic effects of these two anticancer drugs. The differential effect of PKC inhibitors on the cytotoxic effect of VIN suggests possible involvement of other PKC isozymes or protein kinases.

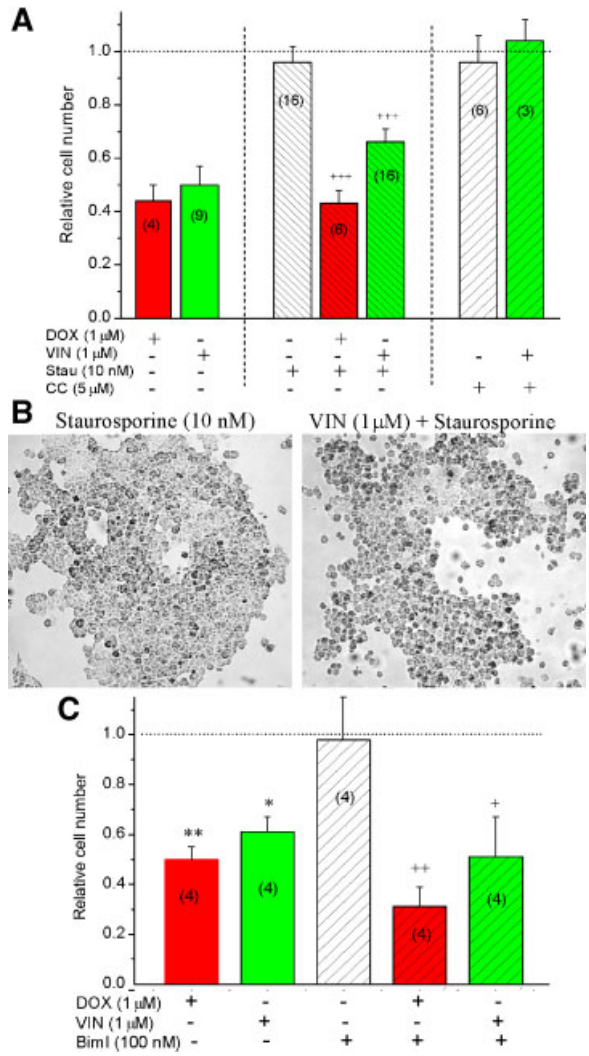

Fig. 4. Effect of PKC inhibitors on cancer cell growth and the cytotoxic effect of DOX and VIN. **A**: Cells were treated for 30 min with 10 nM staurosporine (Stau) or 5 μM chelerythrine chloride (CC) followed by 24-h co-treatment with 1 μM DOX or 1 μM VIN. **B**: Effect of Stau on the growth pattern of MCF-7 cells in the absence (**left panel**) and presence of 1 μM VIN (**right panel**, magnifications 100×). Photographs are representative fields of more than three independent experiments. **C**: Similarly, cells were treated for 30 min with 100 nM bisindolylmaleimide I (Bim-I) followed by 24-h cotreatment with 1 μM DOX or 1 μM VIN. Data presented are mean ± SEM; number in parentheses indicates the number of independent experiments. *, ** and ***$P < 0.05$, 0.01 and 0.001 versus the time-control, respectively; $^{+}$ and $^{++}P < 0.05$ and 0.01 versus PKC inhibitor alone, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Protein Expression and Activity of PKC-α in Response to DOX and VIN Treatment in the Absence and Presence of PKC Activators and Inhibitors

Figure 5A shows a representative result of phosphorylation of PKC-α in the presence and

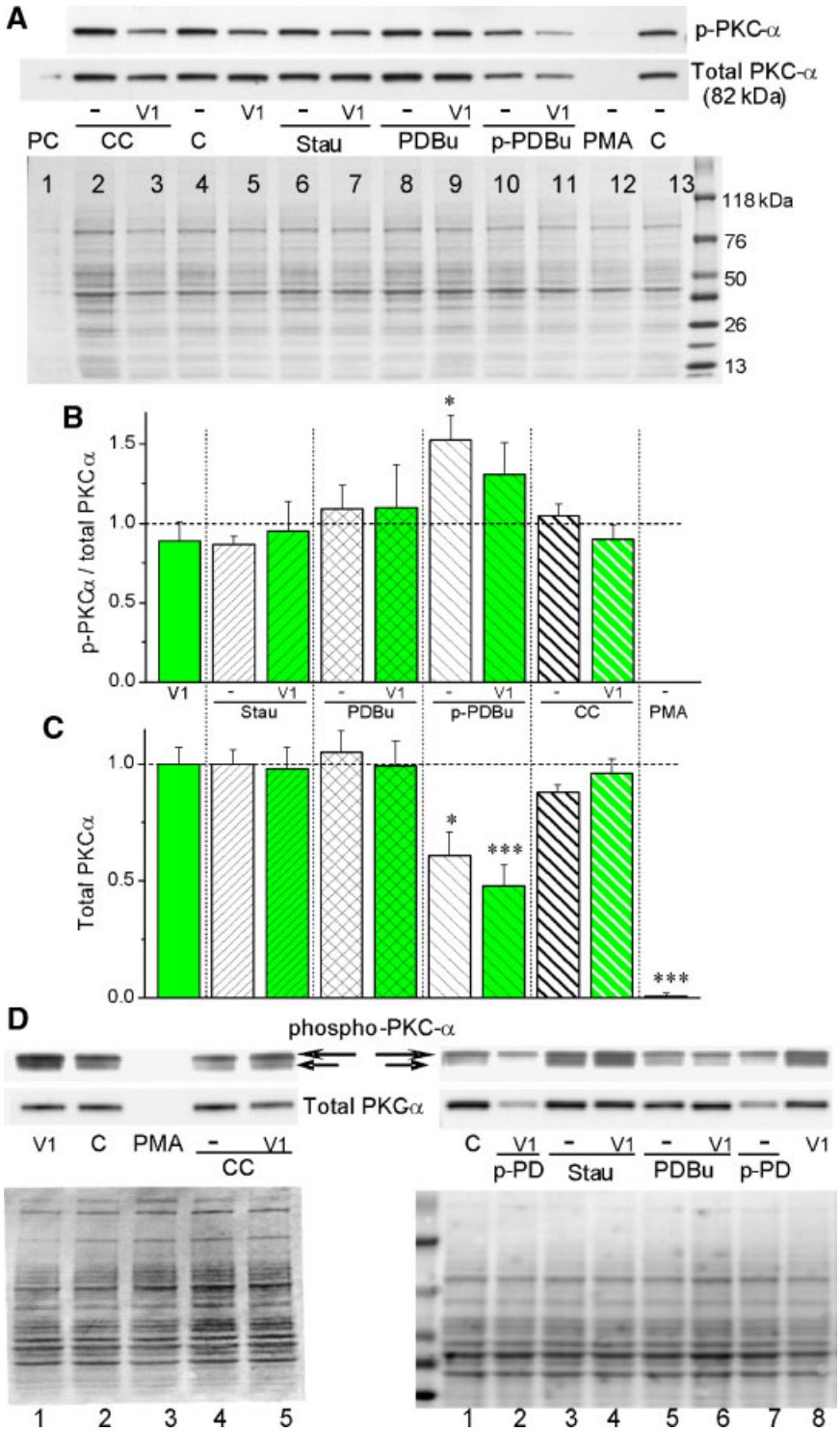

Fig. 5. Effect of PKC activators and inhibitors on the PKC-α activities in MCF-7 cells during VIN treatment. **A**: Phosphorylation of PKC-α in whole cell lysates of each group was detected with anti-phospho-PKC-α antibodies (**top panel**). The respective abundance of PKC-α protein was detected with anti-total PKC-α antibodies (**middle panel**). **Bottom panel**: The membrane was stained with Ponceau-S Red for verification and normalization of protein transferred. Lane 1 (from the left): PKC-α positive control (PC, NIH-3T3 cell lysate); lane 4: time-control; lane 5: 1 μM VIN; lane 13: another control sample; lane 14: protein molecular markers. **B**: Combined data of apparent PKC-α phosphorylation (ratio of p-PKC-α to total PKC-α) in response to each treatment from four independent experiments. **C**: Total PKC-α protein abundance in each group obtained from four independent experiments. **D**, **E**: Dephosphorylation of PKC-α in the cytosolic fraction in response to each treatment was identified as the fast migrating band (lower band with short arrows in top panel) coupled with the phosphorylated form (long arrows) detected with anti-phospho-PKC-α antibodies. The respective total PKC-α abundance in cytosol of each group was shown in the **lower panel**. Note that dephosphorylation was dissolved only in cytosolic fractions (using 4%–15% gradient gel) not in whole cell lysates (using 15% separating gel). Bottom panels: Membranes were stained with SyproRuby for verification and normalization of protein transferred. Data presented are mean ± SEM; * and ***$P < 0.05$ and 0.001 versus the time-control, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

absence of PKC activators or inhibitors during 24-h VIN treatment. Pretreatment with PDBu (not continued exposure to PDBu) reduced abundance of PKC-α but increased phosphorylation of residual PKC-α as expected for PKC-α activation. By contrast, PMA completely depleted PKC-α. This experiment also shows that VIN (1 μM) alone slightly decreased PKC-α phosphorylation, which was also observable in the presence of CC, staurosporine and p-PDBu. Combined data of apparent phosphorylation levels of PKC-α (i.e., p-PKC-α/total PKC-α) are shown in Figure 5B. There was a trend of reduction in phosphorylation of PKC-α induced by VIN and staurosporine alone ($P = 0.051$), and such trend existed in co-treatment with p-PDBu and CC. Figure 5C shows that PKC-α protein abundance was not altered by VIN in the absence and presence of PKC inhibitors or PDBu treatment. Figure 5C also shows that PMA completely depleted PKC-α protein, while p-PDBu reduced PKC-α contents by ~40%. Thus, these results suggest that PKC-α phosphorylation is not associated with phorbol-induced increase in proliferation of breast cancer cells and VIN-induced cytotoxicity.

The activity of PKC-α could also be reduced by an increase in its dephosphorylation [Whelan and Parker, 1998]. VIN (1 μM)-induced increase in the dephosphorylation of cytosolic PKC-α could be observed in Figure 5D and E (indicated by short arrows). By contrast, p-PDBu decreased PKC-α dephosphorylation, consistent with its increase in the PKC-α activity observed in whole cell lysates (Fig. 5A and B). In both p-PDBu and PDBu, VIN-induced increase in PKC-α dephosphorylation was abolished (Fig. 5E). Staurosporine alone also enhanced PKC-α dephosphorylation (Fig. 5E), whereas CC slightly decreased it (Fig. 5D). However, both staurosporine and CC had no effect on the VIN-induced increase in PKC-α dephosphorylation. Taken together, the enhanced PKC-α dephosphorylation, such as by VIN, staurosporine and staurosporine plus VIN, could not account for the inhibitory effect of VIN on MCF-7 cell proliferation.

The PKC-α activity was also assessed by measuring its translocation from cytosol (CP) to membrane or particulate fractions (PP). A representative immunoblotting result of PKC-α protein translocation is shown in Figure 6A. In untreated control cells, there was more PKC-α protein resided in cytosol than in particulate fractions. The distribution of PKC-α protein was not affected by VIN (1 μM) (Fig. 6A). Staurosporine alone had no effect on distribution of PKC-α but slightly enhanced the translocation

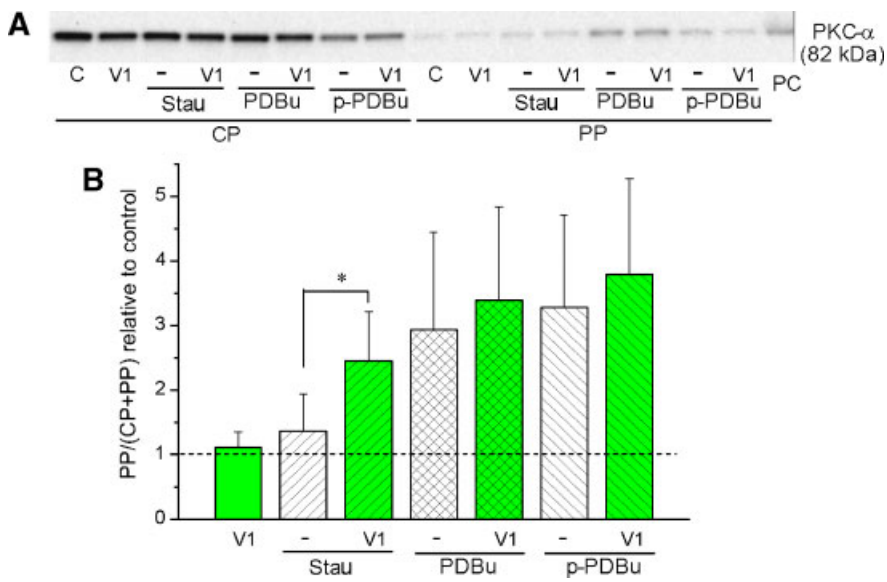

Fig. 6. Translocation of PKC-α in response to PKC activators and inhibitors in the absence and presence of VIN treatment. **A**: Protein expression of PKC-α in cytosolic (CP) and particulate fractions (PP) were detected by anti-(total) PKC-α antibodies 24 h after cells were treated with PKC activators or inhibitors with or without 1 μM VIN. Lane 17: PKCpositive control (PC). **B**: Combined data of PKC-α translocation (ratio of PP to CP + PP) in response to each treatment from four independent experiments. Data presented are mean ± SEM; $^*P < 0.05$ versus PKC inhibitor alone. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

during co-treatment with VIN. CC alone and CC plus VIN had no effect on PKC-α translocation in two experiments (data not shown). By contrast, PDBu and p-PDBu enhanced PKC-α translocation as expected for PKC-α activation, which was not altered in the presence of VIN. Figure 6B summarizes apparent PKC-α translocation (i.e., ratio of PKC-α in PP to the sum of CP and PP) relative to each time-control from four experiments. Results show that in all experimental conditions only a small increase in PKC-α translocation during treatment with VIN plus staurosporine ($P < 0.05$, paired t-test in Fig. 6B). These results suggest that PKC-α translocation is not responsible for VIN-induced cytotoxicity.

DISCUSSION

PKC has been considered as a potential target for anticancer therapy. However, which of PKC isoforms is the main chemotherapeutical target remains unclear. PKC-α was suggested to play an important role in growth of some cancer cell. However, whether proliferation of MCF-7 cells requires PKC-α and whether PKC-α plays a role in chemotherapy of breast cancer also remain unclear. The major findings of this study are (1) proliferation of MCF-7 cells was increased under conditions that PKC-α was activated, inhibited or depleted by different phorbol esters, (2) DOX and VIN suppressed cancer cell proliferation but altered the morphology of MCF-7 cells in different manners, (3) phorbol esters attenuated only VIN-induced cytotoxicity, (4) three PKC inhibitors had no effect on cancer cell proliferation; staurosporine and chelerythrine (but not Bim-I) attenuated VIN-induced cytotoxicity, and (5) neither phorbol esters nor PKC inhibitors had an effect on the DOX-induced cytotoxicity. Thus, these data suggest that PKC-α does not play a vital role in proliferation of MCF-7 cells or the cytotoxic effect of DOX and VIN on MCF-7 cells.

DOX and VIN have been widely used in chemotherapy for various cancers [Minotti et al., 2004] because of their cytotoxic actions on cancer cells. It is known that they act on DNA replication machinery to inhibit cell growth [Jordan et al., 1991; Minotti et al., 2004], which however could not account for differential effects of these drugs on non-cancer cells and breast cancer cells (Fig. 1A,B). In addition to reduction in cancer cell proliferation, these two agents alter morphology of surviving MCF-7 cells in distinct manners, suggestive of different actions other than inhibition on DNA replication. One study using transfected MCF-7 cells showed that overexpression of PKC-α results in loss of cell–cell adhesion and turning the cell shape into a sphere [Williams and Noti, 2001], a change similar to that induced by VIN in the present study. Another study on transformation of non-metastatic MCF-10A showed that cells overexpressing PKC-α became larger and flat with lamellipodia with a slow growth rate [Sun and Rotenberg, 1999], a change somewhat similar to that in MCF-7 cells induced by DOX in the present study. The discrepancy in cell morphology induced by overexpression of PKC-α has been attributed to cell-specific response. Nonetheless, the increase in PKC-α contents does not appear to account for the different effects of DOX and VIN on MCF-7 cells.

If activation of PKC-α such as by PDBu, a cPKC activator, were responsible for cancer cell proliferation, inhibition of PKC-α would suppress MCF-7 cell growth. Phorbol esters have been reported to induce apoptosis in MCF-7 cells overexpressing PKC-α but not in native cells [de Vente et al., 1995]. In the present study we used two phorbol esters, PDBu and PMA, to show their different effects on PKC-α. After 24-h pretreatment, PDBu reduces PKC-α protein contents by 40% but activates the residual PKC-α by increasing phosphorylation and translocation (Figs. 5 and 6). By contrast, PMA completely depletes PKC-α contents (Fig. 5C,D), which is consistent with no Ca-dependent PKC activity observed in PC12 cells [Rasouly et al., 1992]. Note that PMA treatment had no effect on PKC-ζ protein expression in MCF-7 cells (data not shown). Our data are in agreement with findings reported by others who demonstrated that PDBu is less potent to PKC-α than PMA [Saraiva et al., 2004]. Regardless of this difference, both phorbols enhance proliferation of MCF-7 cells and attenuate the VIN-induced cytotoxicity to a comparable degree. In addition, we showed that 10 nM staurosporine, a potent PKC inhibitor with IC50 of 0.7 nM [Rasouly et al., 1992], had no effect on PDBu-induced increase in MCF-7 cell proliferation without or with VIN co-treatment. Thus, these results suggest that MCF-cell proliferation and actions of VIN are PKC-α-independent. Moreover, 100 nM Bim-I, a concentration completely blocking PKC-α (IC50 of 8.4 nM) [Martiny-Baron et al., 1993] has no effect on MCF-7 cell

growth or the VIN-induced cytotoxicity, supporting this suggestion. By contrast, staurosporine and chelerythrine chloride, at a concentration presumably blocking cPKC, have also no effect on cell growth but antagonize the VIN-induced cytotoxicity. We also found that staurosporine, VIN and staurosporine plus VIN decrease PKC-α activities by decreasing its phosphorylation and/or increasing its dephosphorylation. However, under these conditions, these three treatments had different effects on MCF-7 cell proliferation, and staurosporine attenuates VIN-induced growth inhibition. Chelerythrine which had no significant effect on PKC-α activities also attenuates VIN-induced growth inhibition. It has been shown that staurosporine and chelerythrine also inhibit PKC-ζ activity at high at high concentrations [Kochs et al., 1993; Laudanna et al., 1998]. Thus, it is conceivable that PKC isoforms other than PKC-α are involved in the VIN-induced cytotoxicity. Furthermore, neither phorbol esters nor PKC inhibitors have any effect on the DOX-induced cytotoxicity, also suggesting a PKC-α-independent mechanism for the action of DOX. This is in agreement with a previous finding, which reported no effect of selective PKC inhibitors on DOX-induced apoptosis in MTLn3 adenocarcinoma cells [Huigsloot et al., 2003]. Taken together, PKC-α does not appear to play a pivotal role in MCF-7 cell proliferation or in anticancer actions of VIN and DOX.

Isozymes of the PKC family exert various actions on cellular proliferation through a complex network of signal transduction [Hug and Sarre, 2005]. Cellular response to PKC modulators is determined by reactions within each microdomain, such as interactions with substrates and targets during and after alterations in PKC. In addition, one PKC isozyme could be a target of other PKC isozymes [Goode et al., 1995]. The cross talk of isozymes could cause an opposite result in different cells in response to alteration in one isozyme. For example, overexpression of PKC-α increases PKC-δ expression in lymphoma cells [Romanova et al., 1998] but decreases it in MCF-7 cells [Ways et al., 1995]. Complexity of PKC signaling in different cells could be the cause of the controversy regarding the role of PKC-α in anticancer therapeutic strategy. Although antisense targeting PKC-α has been suggested to be a promising therapy, PKC-α has been shown to be anti-apoptotic [Whelan and Parker, 1998; Jiang et al., 2004] or pro-apoptotic in various cancer cells in response to stimulation with phorbol esters [Kennedy et al., 1992; Tanaka et al., 2003; Wen-Sheng and Jun-Ming, 2005]. In summary, our results showed lack of involvement of PKC-α in proliferation of MCF-7 breast cancer cells and in cytotoxic actions of two anticancer drugs. Involvements of other PKC isozymes in these conditions cannot be excluded, and further investigations on possible interactions of different PKC isozymes are required to determine the role of PKC in anticancer chemotherapy.

ACKNOWLEDGMENTS

We thank Ms. Meei-Yueh Liu for her expert technical assistance. This work was supported by grants from Arkansas Cancer Research Center Partners in Research program.

REFERENCES

Blobe GC, Obeid LM, Hannun YA. 1994. Regulation of protein kinase C and role in cancer biology. Cancer Metastasis Rev 13:411–431.

Brantley-Finley C, Lyle CS, Du L, Goodwin ME, Hall T, Szwedo D, Kaushal GP, Chambers TC. 2003. The JNK, ERK and p53 pathways play distinct roles in apoptosis mediated by the antitumor agents vinblastine, doxorubicin, and etoposide. Biochem Pharmacol 66:459–469.

Clemens MJ, Trayner I, Menaya J. 1992. The role of protein kinase C isoenzymes in the regulation of cell proliferation and differentiation. J Cell Sci 103:881–887.

Cornford P, Evans J, Dodson A, Parsons K, Woolfenden A, Neoptolemos J, Foster CS. 1999. Protein kinase C isoenzyme patterns characteristically modulated in early prostate cancer. Am J Pathol 154:137–144.

de Vente JE, Kukoly CA, Bryant WO, Posekany KJ, Chen J, Fletcher DJ, Parker PJ, Pettit GJ, Lozano G, Cook PP, Ways DK. 1995. Phorbol esters induce death in MCF-7 breast cancer cells with altered expression of protein kinase C isoforms. Role for p53-independent induction of gadd-45 in initiating death. J Clin Invest 96:1874–1886.

Dennis JU, Dean NM, Bennett CF, Griffith JW, Lang CM, Welch DR. 1998. Human melanoma metastasis is inhibited following ex vivo treatment with an antisense oligonucleotide to protein kinase C-α. Cancer Lett 128: 65–70.

Goode NT, Hajibagheri MAN, Parker PJ. 1995. Protein kinase C (PKC)-induced PKC down-regulation. J Biol Chem 270:2669–2673.

Hug H, Sarre TF. 2005. Protein kinase C isoenzymes: divergence in signal transduction? Biochem J 291:329–343.

Huigsloot M, Tijdens RB, van de Water B. 2003. Inhibition of protein kinase Cα enhances anticancer agent-induced loss of anchorage-independent growth regardless of

protection against apoptosis by Bcl-2. Mol Pharmacol 64:965–973.

Jiang XH, Tu SP, Cui JT, Lin MCM, Xia HHX, Wong WM, Chan AO-O, Yuen MF, Jiang SH, Lam SK, Kung HF, Soh JW, Weinstein IB, Wong BC-Y. 2004. antisense targeting protein kinase C α and β1 inhibits gastric carcinogenesis. Cancer Res 64:5787–5794.

Jordan MA, Thrower D, Wilson L. 1991. Mechanism of inhibition of cell proliferation by Vinca alkaloids. Cancer Res 51:2212–2222.

Kennedy MJ, Prestigiacomo LJ, Tyler G, May WS, Davidson NE. 1992. Differential effects of bryostatin 1 and phorbol ester on human breast cancer cell lines. Cancer Res 52:1278–1283.

Kochs G, Hummel R, Meyer D, Hug H, Marme D, Sarre TF. 1993. Activation and substrate specificity of the human protein kinase C α and zeta isoenzymes. Eur J Biochem 216:597–606.

La Porta CAM, Comolli R. 1997. Activation of protein kinase C-a isoform in murine melanoma cells with high metastatic potential. Clin Exp Metastasis 15:568–579.

Laudanna C, Mochly-Rosen D, Liron T, Constantin G, Butcher EC. 1998. Evidence of z protein kinase C Involvement in polymorphonuclear neutrophil integrin-dependent adhesion and chemotaxis. J Biol Chem 273: 30306–30315.

Liu SJ, McHowat J. 1998. Stimulation of different phospholipase A_2 isoforms by TNF-α and IL-1β in adult rat ventricular myocytes. Am J Physiol 44:H1462–H1472.

Mandil R, Ashkenazi E, Blass M, Kronfeld I, Kazimirsky G, Rosenthal G, Umansky F, Lorenzo PS, Blumberg PM, Brodie C. 2001. Protein kinase Cα and protein kinase Cδ play opposite roles in the proliferation and apoptosis of glioma cells. Cancer Res 61:4612–4619.

Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, Marme D, Schachtele C. 1993. Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. J Biol Chem 268:9194–9197.

Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L. 2004. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol Rev 56:185–229.

Nishizuka Y. 1995. Protein kinase C and lipid signaling for sustained cellular responses. FASEB J 9:484–496.

O'Brian CA, Ward NE, Stewart JR, Chu F. 2001. Prospects for targeting protein kinase C isozymes in the therapy of drug-resistant cancer—An evolving story. Cancer Metastasis Rev 20:95–100.

Olson EN, Burgess R, Staudinger J. 1993. Protein kinase C as a transducer of nuclear signals. Cell Growth Differ 4:699–705.

Rasouly D, Rahamim E, Lester D, Matsuda Y, Lazarovici P. 1992. Staurosporine-induced neurite outgrowth in PC12 cells is independent of protein kinase C inhibition. Mol Pharmacol 42:35–43.

Romanova LY, Alexandrov IA, Nordan RP, Blagosklonny MV, Mushinski JF. 1998. Cross-talk between protein kinase C-α (PKC-α) and -δ (PKC-δ): PKC-a elevates the PKC-d protein level, altering its mRNA transcription and degradation. Biochemistry 37:5558–5565.

Saraiva L, Fresco P, Pinto E, Goncalves J. 2004. Characterization of phorbol esters activity on individual mammalian protein kinase C isoforms, using the yeast phenotypic assay. Eur J Pharmacol 491:101–110.

Sun Xg, Rotenberg SA. 1999. Overexpression of protein kinase ca in MCF-10A human breast cells engenders dramatic alterations in morphology, proliferation, and motility. Cell Growth Differ 10:343–352.

Tanaka Y, Gavrielides MV, Mitsuuchi Y, Fujii T, Kazanietz MG. 2003. Protein kinase C promotes apoptosis in LNCaP prostate cancer cells through activation of p38 MAPK and inhibition of the akt survival pathway. J Biol Chem 278:33753–33762.

Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F. 1991. The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C. J Biol Chem 266:15771–15781.

Wang XY, Repasky E, Liu HT. 1999. Antisense inhibition of protein kinase ca reverses the transformed phenotype in human lung carcinoma cells. Exper Cell Res 250:253–263.

Wang S, Konorev EA, Kotamraju S, Joseph J, Kalivendi S, Kalyanaraman B. 2004. Doxorubicin induces apoptosis in normal and tumor cells via distinctly different mechanisms: intermediacy of H_2O_2- and p53-dependent pathways. J Biol Chem 279:25535–25543.

Ways DK, Kukoly CA, deVente J, Hooker JL, Bryant WO, Posekany KJ, Fletcher DJ, Cook PP, Parker PJ. 1995. MCF-7 breast cancer cells transfected with protein kinase C-α exhibit altered expression of other protein kinase C isoforms and display a more aggressive neoplastic phenotype. J Clin Invest 95:1906–1915.

Wen-Sheng W, Jun-Ming H. 2005. Activation of protein kinase C alpha is required for TPA-triggered ERK (MAPK) signaling and growth inhibition of human hepatoma cell HepG2. J Biomed Sci 12:289–296.

Whelan RDH, Parker PJ. 1998. Loss of protein kinase C function induces an apoptotic response. Oncogene 16: 1939–1944.

Williams CL, Noti JD. 2001. Reduced expression of Wnt-1 and E-cadherin, and diminished beta-catenin stability in MCF-7 breast cancer cells that overexpress protein kinase C-α. Intl J Oncology 19:1227–1233.

Young S, Parker PJ, Ullrich A, Stabel S. 1987. Down-regulation of protein kinase C is due to an increased rate of degradation. Biochem J 244:775–779.