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GRANT NUMBER DAMD17-96-1-6022

TITLE: The Role of PKC in Retinoic Acid Regulation of Human Mammary Cancer Cell Proliferation

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REPORT DATE: September 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 1997	Annual (1 Sep 96 - 31 Aug 97)

4. TITLE AND SUBTITLE
The Role of PKC in Retinoic Acid Regulation of Human Mammary Cancer Cell Proliferation

5. FUNDING NUMBERS
DAMD17-96-1-6022

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

The data presented in this first annual report (for award DAMD17-96-1-60220) support our hypothesis for a mechanism of retinoic acid-induced growth arrest of human breast cancer cells. Specifically we believe that retinoic acid induced growth arrest of human breast cancer cells requires protein kinase Cα expression and activity. The inhibition of uncontrolled proliferation following retinoic acid treatment of hormone dependent, T-47D breast cancer cell lines, was consistent with retinoic acid inducing expression of PKCα and concomitant repression of PKCζ expression. The changes in PKCα and PKCζ reflected retinoic acid-induced changes in mRNA. In contrast, retinoic acid had no effect on growth or PKC expression in hormone independent, MDA-MB-231 breast cancer cells. RARα-selective synthetic retinoid, Am580, was equally effective as retinoic acid at growth arrest and the induction of PKCα, but not reduction in PKCζ of T-47D cells. Addition of Gö6976, a selective inhibitor of conventional PKC, prevented the Am580 induced reduction in proliferation. In total, our interpretation is that retinoic acid arrests proliferation of T-47D cells following RARα dependent induction, and activation of PKCα. By manipulating the expression of PKCα, we also have shown that expression of PKCα is sufficient to exert growth inhibitory effects on T-47D cells.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer Retinoic acid, Protein kinase C (PKC), Signal transduction, Vitamin A, Cell cycle, Oncogene expression	35
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Retinoids have anti-tumor effects and a number of clinical trials of retinoids in human breast cancer have been initiated (Band et al., 1984; Costa 1993). Retinoid therapy is based on intrinsic physiological mechanisms of inhibiting cellular proliferation (Gudas 1992). Expression of peptide growth factors, proto-oncogene and transcription factors, such as AP-1, are all potential targets for the molecular mechanisms of retinoid action (Dickens and Colletta, 1993; de Groot et al., 1991; Schule et al., 1991). Although retinoids inhibit proliferation of numerous breast cancer cell lines, responsiveness appears to be limited to estrogen-dependent cells, in which retinoic acid (RA) inhibits estrogen stimulation of proliferation (Liu et al., 1996; Wilcken et al., 1996).

The proliferative effect of estrogen on mammary cells has been linked to the production of mitogenic growth factors and increased expression of surface receptor tyrosine kinases (Daly et al., 1994; Halter et al., 1992). Growth factor binding to receptor tyrosine kinases activates multiple, interactive signaling pathways, most of which involve sequential activation of serine/threonine protein kinases. Two of the targets for receptor tyrosine kinases, phospholipase Cγ (PLCγ), and phosphatidylinositol 3-kinase (PtdIns 3-kinase), produce second messengers that activate multiple isozymes of protein kinase C (PKC)

Twelve distinct PKC isozymes are encoded by a family of at least 11 genes. These isozymes are divided into three groups based on sequence homology and cofactor requirement (Blobe et al., 1994); the conventional PKCs (α, βI, βII and γ), the novel PKCs (δ, ε, μ, η and θ), and the atypical PKCs (ι, λ and ζ). Physiologically conventional and novel PKCs are activated by the concerted action of phospholipid and the second messenger diacylglycerol, hydrolytic product of PLCγ (Blobe et al., 1994). Conventional PKCs also require calcium (Blobe et al., 1994). Atypical PKCs are calcium independent, and are not activated by DAG, but can be activated by the novel phospholipid, PtdIns 3,4,5-P_3, a product of PtdIns 3-kinase (Nakanishi et al., 1993). These observations support a model in which PKC isozymes are differentially activated in response to distinct growth factor or hormonal activation of either PLC or PtdIns 3-kinase.

Retinoic acid (RA) inhibits signaling between receptor tyrosine kinases and the nucleus (Leid et al., 1993; Talmage and Listerud, 1994). The components of these signaling pathways that serve as targets for RA have yet to be identified. At least two signaling pathways stimulated by receptor tyrosine kinase utilize PKC family members as mediators. RA-induced differentiation of embryonal carcinoma and melanoma cells results in growth arrest, and is associated with changes in PKC expression (Rosenbaum and Niles, 1992; Khuri et al., 1996). In our preliminary data (Proposal Illustration 3), RA treatment of T-47D, but not MDA-MB-231 cells resulted in increased PKCα and decreased PKCζ expression. Because of the relationships between hormonal dependent mammary cell proliferation, RA growth regulation and protein kinase C, **we have hypothesized that retinoic acid inhibits the uncontrolled proliferation of mammary carcinoma cells, by increasing the overall activity of**

PKCα, and/or decreasing the overall activity of PKCζ. Two HBC cell lines are used in our study; the hormone-dependent T-47D cell line, originated from a ductal carcinoma and the hormone-independent MDA-MB-231 line, originated from an adenocarcinoma.

BODY FOR ANNUAL REPORT; YEAR 1.

Specific Aims.

Aim 1. Determine if retinoic acid arrest of mammary carcinoma cells is associated with changes in the expression of, activation of, or signaling through the protein kinase C family (Task 1, 2 and 3).
Aim 2. Determine the importance of PKCα and/or PKCζ in mammary cell proliferation and/or differentiation (Task 4).

Task 1. Characterization of cell lines for expression, translocation and activity of PKC isozymes, month 1-10.

Methods

Cell culture

T-47D human breast cancer cells (ATCC# HTB133) were grown on plastic culture dishes in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% fetal bovine serum (UBI, Saranac Lake, NY) and insulin (7.4 μg/ml). MDA-MB-231 human breast cancer cells (ATCC# HTB26) were grown on culture dishes in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum. Cells were treated with 10^{-9} M-10^{-6} M all trans retinoic acid or Am580 dissolved in ethanol (final ethanol concentration was 0.1%), for up to 5 days.

Measurement of relative proliferation

T-47D and MDA-MB-231 cells were plated at 15 x 10^4 cells/60 mm culture dish. Media was changed 24 h later, at which time cells were treated with indicated concentrations of all-trans retinoic acid, Am580, or 500 nM Gö6976 dissolved in DMSO (final concentration of DMSO was 0.05%). Media and experimental treatment were renewed every 72 h. On the days indicated, cells were harvested, and counted with a hemocytometer. Each sample was counted in duplicate, and each condition was done in duplicate or triplicate.

Western blot analysis for PKC isozyme expression

Cell protein lysates were prepared and separated on 10% SDS-PAGE gel as described previously (Cho, et al., 1997). Following electrophoretic transfer to nitrocellulose, membranes were blocked by 5% non fat dry milk in PBS. PKC isozymes were detected by incubating with PKC isozyme specific antibodies(affinity purified polyclonal PKCα and β antibodies; protein G-purified polyclonal γ, δ, ε, ζ antibodies)(Gibco BRL, Inc., Gaithersburg, MD), followed by extensive washing with PBS containing 0.05% Tween 20 (PBST) and subsequent incubation with the

horseradish peroxidase coupled anti-rabbit IgG (1:7500 dilution)(Amersham Corp.). Immunoreactive proteins were visualized by enhanced chemiluminescence.

RNA isolation and northern blotting

Total RNA was isolated from treated cells and northern blots were performed as described by Dorsett et al (1989). In every case, filters were hybridized to ^{32}P-cRNA probes, which were synthesized from either PKCα or PKCζ cDNA cloned into the pSP72 vector (Melton et al., 1984). Blots were washed and exposed to X-ray film at -80°C with intensifying screens.

Results

Proliferation of the hormone dependent, T-47D human breast cancer line was inhibited by retinoids (Figure 1A and 1B). All-trans retinoic acid (RA), and the RARα-selective synthetic retinoid, Am580 were equally effective at arresting growth of these cells. The similar dose response for growth arrest seen between RA and Am580, particularly at concentrations of 10^{-8} M where selectivity is greatest, argue for a primary role for RARα in regulating mammary cell proliferation. In contrast, hormone-independent cell line, MDA-MB-231 was insensitive to 10^{-6} M RA (Figure 1C). Consistent with the ability of Am580 to inhibit T-47D proliferation, the lack of RA responsiveness of MDA-MB-231 cells has been explained partly by the low level of RARα expressed (Sheikh et al., 1994).

Untreated T-47D cells expressed the novel PKC isozymes, PKCδ and PKCε, and the atypical PKCζ (Figure 2A), but not conventional PKCs (PKCα, β or γ; Figure 2A and 2C). When T-47D cells were treated with 10^{-6}M RA, PKCα expression was induced and concomitantly PKCζ expression decreased (PKCδ and PKCε expression was marginally decreased)(Figure 2A). Increased PKCα expression was apparent within 1-2 days of RA treatment(see also Figure 4). By 5 days, PKCα expression was predominant and PKCζ expression was dramatically decreased. Untreated MDA-MB-231 cells also expressed PKCε and PKCδ, and PKCζ, but not PKCα, PKCβ and PKCγ (Figure 2B and 2C). In contrast to T-47D cells, RA treatment of MDA-MB-231 cells had no effect on PKCα or PKCζ expression (PKCδ expression was marginally increased after 5 days)(Figure 2B).

RA induced changes in PKCα and PKCζ expression of T-47D cells resulted from changes in mRNA levels (Figure 3). Again, the induction of PKCα and the reduction in PKCζ expression was observed after 2 days of treatment. No change in either mRNA was seen in MDA-MB-231 cells. Curiously, PKCα mRNA was readily detected in these cells, despite the absence of measurable immunoreactive PKCα. Whether this indicates a defect in translation of this mRNA, or mutations that lead to failure of this protein to be stably expressed is under further investigation.

The changes in PKCα and PKCζ expression following RA treatment coincided with the cessation of proliferation. This, along with the observation that neither changes in PKC expression nor growth arrest occurred in MDA-MB-231 cells, raised the

possibility that these changes in PKC expression were responsible for the anti-proliferative effects of retinoids in T-47D cells. At low retinoid concentrations (10^{-9} M) growth arrest of T-47D cells was delayed (Figure 1A and 1B). If the changes in PKC expression observed were related to the cessation of cell division, we reasoned that the time course of these changes should also be sensitive to the dose of RA. After 5 days, maximal expression of PKCα was seen with RA at concentrations of 10^{-8} - 10^{-6} M (Figure 4A). Slight elevations were typically seen with 10^{-9} M RA only after 5 days. In contrast, PKCζ expression remained unaffected at RA concentrations less than 10^{-6} M. These results indicate that RA is regulating expression of these two PKC isozymes via fundamentally different mechanisms and that decreased PKCζ is unlikely to contribute to growth arrest.

The data presented in Figure 1, and by others (Sheikh et al., 1994; Talmage and Lackey, 1992) indicate that RARα is partially responsible for the anti-proliferative effects of RA on human breast cancer cell lines. To extend the association between expression of PKCα and RARα-induced growth arrest, we examined the concentration and time dependent effect of the RARα selective ligand Am580, on PKC expression (Figure 4B). PKCα was detected after 1 day of treatment with 10^{-8} M Am580. At 2 days, PKCα was seen in cells treated with 10^{-9} M Am580, and maximal expression was seen between 10^{-7} and 10^{-6} M Am580. High levels of PKCα expression were maintained after 5 days at 10^{-9} and 10^{-8} M. The higher concentrations of Am580 resulted in decreased PKCα expression after 5 days, indicating that PKCα expression was down-regulated by these higher concentrations of Am580. Compared to the rapid induction of PKCα by 10^{-9}- 10^{-8} M Am580, no decrease in PKCζ levels were seen after 2 days of treatment with 10^{-8} M Am580. Levels were marginally reduced at 2 days by the higher concentrations (10^{-7} or 10^{-6} M) of Am580. Even after 5 day, PKCζ was still expressed at low concentrations (10^{-9} or 10^{-8} M) of Am580 and intermediate (10^{-7} M) concentrations of RA. At low concentrations, Am580 is a selective agonist of RARα (kd = 8 x 10^{-9} M for RARα, 1.1 x 10^{-6} M for RARβ and γ) (Delescluse et al., 1991). The sensitivity of T-47D proliferation and PKCα expression to low concentrations of Am580 indicate that both of these effects are mediated by RARα. In contrast, the decreased expression of PKCζ after only extended periods of treatment with high concentrations of RA or Am580, is not consistent with RARα mediated regulation of this gene, or with a causal association between the loss of PKCζ expression and growth arrest.

In order to determine if retinoic acid-induced PKCα plays an active role in mediating the anti-proliferative effect of retinoic acid, we assayed proliferation of T-47D cells in the presence of the synthetic indolocarbazole, Gö6976 which is a selective inhibitor of conventional PKCs (Martiny-Baron et al., 1993., Qatsha et al., 1993) Martiny-Baron et al (1993) demonstrated that Gö6976 inhibits conventional PKCs (IC50 of PKCα = 2.3 nM, IC50 of PKCβ = 6.2 nM) but not other PKCs (no inhibition for PKCδ, ε, and zeta). T-47D cells were treated with 10^{-8} M Am580, 500 nM Gö6976, or both for 72hr. Untreated cells underwent 1.5-2 population doublings during this period compared to less than 1 doubling for Am580 treated cells (Figure 5). Addition of Gö6976 prevented the Am580 induced reduction in proliferation. Cells treated with

retinoid and Gö6976 alone or in combination were fully viable; greater than 95% of cells excluded trypan blue. Therefore retinoid arrested proliferation of T-47D cells required the activity of conventional PKCs, presumably the retinoid-induced PKCα.

Task 1d (measurement of cytosolic and membrane protein levels of PKC isozymes) and Task 1e (Assay of PKC activities with partially purified PKC enzyme fraction) were not completed in this first year. Currently we are establishing T-47D and MDA-MB-231 cells stably expressing PKCα, PKCζ, antisense PKCα and antisense PKCζ (see page 9; method section in Task 4). After stable transfection is established, we plan to complete Task 1d and 1e in each cell line including control T-47D and MDA-MB-231 cells.

Task 2, Characterization of cell lines for proto-oncogene expression, month 11-16.

Currently we are establishing T-47D and MDA-MB-231 cells stably expressing PKCα, PKCζ, antisense PKCα and antisense PKCζ (see page 9; method section in specific aim 2). After stable transfection is established, we plan to complete Task 2 in each cell line including control T-47D and MDA-MB-231 cells.

Task 3, Characterization of retinoic acid effect on cell cycle progression, month 1-20.

After establishing T-47D and MDA-MB-231 cells stably expressing PKCα, PKCζ, antisense PKCα and antisense PKCζ (see page 9; method section in specific aim 2), we plan to characterize intensively retinoic acid effect on cell cycle progression in each cell line including control T-47D and MDA-MB-231 cells.

Task 4. Manipulation of individual PKC isozyme gene expression, month 18-36.

Methods

Plasmid

The anti-sense PKCα plasmid (pMV7-As PKCα) was constructed by ligating the 2.44 -kb EcoR I fragment of PKCα cDNA from pβ-actin SP72-PKCα (Cho et al, 1997) into the unique EcoR I site of the pMV7 vector (Talmage and Lackey, 1992). The antisense-orientation was confirmed by BamHI restriction digestion. The sense and antisense PKCζ plasmids (pMV7-PKCζ and pMV7-As PKCζ) were constructed by ligating the 2.2-kb EcoR I and Hind III fragment of antisense and sense PKCζ cDNA from pβ-actin SP72 vector (Cho et al., 1997)into the EcoRI and Hind III site of pMV7 vector (Talmage and Lackey, 1992). The orientation was confirmed by BamH I restriction digestion.

In Stratagene's LacSwitch™ II inducible mammalian expression system, sense or anti-sense PKCα cDNA (pOPRSVI-PKCα; pOPRSVI-As PKCα) were subcloned by

ligating the 2.48-kb Sma I and Kpn I fragment of sense or antisense PKCα cDNA from pβ-actin SP72-PKCα into Sma I and Kpn I multicloning site of pOPRSVI/MCS.

Stable transfection

To stably express PKCα, antisense PKCα, PKCζ or antisense PKCζ, pSVXα encoding bovine PKCα (Borner et al., 1991), pMV7-As PKCα, pMV7-PKCζ or pMV7-As PKCζ was transfected into the NIH3T3 GP + envAm12 packaging cell line (Markowitz et al., 1988) and colonies stably producing recombinant virus were isolated following selection for G418 resistance (250 μg/ml). T-47D and MDA-MB-231 cells were infected with freshly conditioned medium derived from subconfluent NIH 3T3 GP + envAm12 cultures. Following infections, cultures were subjected to G418 (250μg/ml) selection. Resistant cells were maintained in $100\mu g.ml^{-1}$ G418.

For Stratagene's LacSwitch™ II inducible mammalian expression system, both pCMVLac I repressor and pOPRSVI/MCS encoding either PKCα or antisense PKCα were transfected simultaneously into T-47D and MDA-MB-231 cells by lipofectAMINE reagent (Gibco BRL, Inc., Gaithersburg, MD)(Bebok et al., 1996). Following transfection, cells were subjected to hygromycin B (200 μg/ml) and G418 (800 μg/ml) selection over 4 weeks. Expanded clones will be isolated, and examined expression levels of Lac repressor and PKC α by western blotting with polyclonal antiserum to Lac repressor (Stratagene, Inc.) and PKCα antibody (affinity purified polyclonal PKCα antibody; GIBCO BRL, Inc.).

Results

Stable expression of PKCα inhibits the proliferation of T-47D cells. In order to demonstrate further the functional role of PKCα in mediating the proliferative response of T-47D cells to retinoids, we isolated T-47D cells constitutively expressing PKCα following infection with a recombinant retrovirus (pSVXα; Borner et al., 1991). SVXα infected T-47D cells (αT-47D) expressed PKCα in the absence of retinoic acid (Figure 6A). No additional alterations in PKCβ, γ, δ, ε, and ζ were seen in αT-47D cells (Figure 6A), demonstrating that constitutive PKCα expression did not alter basal expression of other PKC isozymes. T-47D and αT-47D cells were treated with 10^{-8} M trans retinoic acid, 50ng/ml TPA or 500 nM Gö6976 for 72hr. Untreated T-47D cells underwent 1.5-2 population doublings compared to less than 1 doubling for αT-47D cells. Constitutive expression of PKCα slowed T-47D proliferation more effectively than 10^{-8} M RA. During the first 3 days of treatment addition of retinoic acid to αT-47D cells did not reduce proliferation further. Treatment of αT-47D cells with the selective inhibitor of cPKCs, Gö6976 increased proliferation to near control levels (~75% of T-47D). Although less pronounced, chronic phorbol ester treatment, which down-regulates multiple PKCs, also increased αT-47D proliferation (Figure 6B).

To further delineate the role of PKCα and/or PKCζ in RA induced growth arrest of human breast cancer cells, we are using the Stratagene's LacSwitch™ II inducible mammalian expression system, in which gene transcription can be reversibly turned on

or off. In the Escherichia coli lactose (lac) operon, the Lac repressor binds as a homotetramer to the lac operator, blocking transcription of the lacZ gene. Physiological or synthetic inducers, such as allolactose or isopropyl β-D-thiogalactopyranoiside (IPTG), respectively, bind to the Lac repressor, causing a conformational change and effectively decreasing the affinity of the repressor for the operator. When the repressor is removed from the operator, transcription of genes from the lac operon resumes. In Stratagene's LacSwitch™ II inducible mammalian expression system, sense/ anti-sense PKCα or PKCζ cDNA was subcloned in Sma I and Kpn I site of pOPRSVI/MCS, in which RSV (Rous sarcoma virus)-LTR promoter drives expression of the gene, and ideal operator sequences for the Lac repressor binding (op) are present in the RSV promoter and in the intron. After establishing T-47D and MDA-MB-231 cells stably expressing PKCα, antisense PKCα, PKCζ or antisense PKCζ mRNA, we plan to investigate the temporal on and off effect of genes (especially PKCα) on cell cycle progression and oncogene expression. Currently, we are in progress of selection with hygromycin (pCMVLac1) and G418 (pOPRSVI/MCS) for double transfection in T-47D and MDA-MB-231 cells.

Discussion

The second messenger regulated PKCs are emerging as important components of retinoid responsiveness. On one level, Maciaszek et al (1994), and Tahayato et al (1993) demonstrated synergistic activation of SIV/HIV-1 LTRs and the RARβ2 promoter respectively by retinoic acid and phorbol esters. Synergistic transactivation represents convergence of diverse signaling pathways on a common target. In the former case, retinoic acid, acting via RARs, was proposed to induce expression of a transcription factor that became functionally active following PKC dependent phosphorylation (Maciaszek et al., 1994). In the latter example, the common molecular target was proposed to be RAR itself, following retinoic acid binding and PKC-dependent phosphorylation (Tahayato et al., 1993).

A second level at which PKC and retinoid action intersect involves changes in PKC isozyme expression, frequently including increased expression of PKCα, that accompany retinoid-induced differentiation and growth arrest (Rosenbaum and Niles, 1992; Khuri et al., 1996; Gruber et al., 1992). In most cases the significance of altered PKC isozyme expression in achieving or maintaining the altered differentiated or proliferative state is unresolved. Here we have demonstrated that retinoids induce PKCα and growth arrest in hormone-dependent but not hormone-independent human breast cancer cells. The synthetic nonglycosidic indolocarbazole, Gö6976, is a specific inhibitor of conventional PKCs with no significant inhibitory activity against novel and atypical PKCs (Martiny-Baron et al., 1993). Gö6976 abrogated the retinoid anti-proliferative effect on T-47D cells. Since PKCα was the only conventional PKC detected in retinoid treated T-47D cells, we conclude that PKCα expression and activity is required for retinoid action. This conclusion was supported by demonstrating that PKCα transfected T-47D cells grew at rates comparable to low dose, retinoid treated

parental cells; a response partially reverted by the PKC inhibitor Gö6976, or by chronic phorbol ester treatment.

Other studies on human breast carcinoma cell lines revealed that the level of PKC expression is altered depending on estrogen receptor status (Borner et al., 1987), and that chronic exposure to phorbol ester results in growth arrest in Go/G1 and acquisition of a more differentiated phenotype (Darbon et al., 1990; Valette et al., 1987; Kennedy et al., 1992). As is frequently the case, it is difficult to determine if these effects of phorbol ester result from activation of conventional and/or novel PKCs, or result from PKC down-regulation following chronic activation, nor is it clear to what extent these responses are mediated by PKCα or other PKC isozymes. Recently Ways et al. (1995) reported a more " aggressive " phenotype resulting from PKCα expression in MCF-7 cells. Whether the difference in the effect of PKCα expression in our study compared to theirs results from the use of different parental cell lines, is secondary to alterations in other PKCs, or is because of a non-kinase dependent effect in MCF-7 cells as suggested by Ways et al. (1995) is not clear.

In F9 embryonal carcinoma cells, retinoic acid-induced changes in conventional PKC expression, including PKCα induction, are related directly to changes in the regulation of cell cycle associated genes and markers of terminal differentiation (Khuri et al., 1996). Expression of PKCα in the absence of retinoic acid resulted in phorbol ester induced expression of the differentiation marker, type IV collagen. This report, and the current study clearly establish PKCα as a key component of retinoid induced biological responses in multiple cell types.

The mechanism(s) by which PKCα inhibits T-47D cell proliferation is not known. Retinoid-induced growth arrest of T-47D cells also involves disrupted mitogenic signaling from receptor tyrosine kinases (Tighe, Cho and Talmage; in prep). It is conceivable that PKCα-dependent phosphorylation of the EGF receptor (and/or similar receptors) desensitizes growth factor signaling (Cochet et al., 1984; Friedman et al., 1984; Saloman, 1981; Lee and Weinstein 1978). Alternatively PKCα might indirectly regulate the expression of a gene whose product is actively involved in removing T-47D cells from the cell cycle.

In addition to inducing PKCα expression, retinoic acid also repressed PKCζ expression in T-47D cells. The functional relevance of this is not clear. Retinoid-dependent inhibition of PKCζ expression followed a time course distinct from PKCα induction and growth arrest, and was significantly reduced only in cells treated with high retinoic acid concentrations. Neither the kinetics of PKCζ repression nor the retinoid concentration dependence paralleled the proliferative effects of retinoids on these cells. This, coupled with the ability of Gö6976 to block retinoid action, argues strongly against the functional importance in decreased PKCζ expression in mediating the retinoid anti-proliferative effects.

The extent to which different RARs regulate distinct versus redundant or overlapping biological responses is unclear. T-47D cells express both RARα and RARγ, and RARβ expression increases following prolonged RA treatment (Liu et al., 1996). Several observations strongly favor RARα as the mediator of RA growth regulation, and PKCα expression in these human mammary carcinoma cells (Sheikh et

al., 1994; Valette et al., 1987; Kennedy et al., 1992). RARα is the major RAR subtype expressed in T-47D cells, but is present at low levels in MDA-MD-231 cells (Sheikh et al., 1994; Fitzgerald et al., 1997). Expression of RARα cDNAs in MDA-MB-231 cells results in increased sensitivity to the growth inhibiting effects of RA (although they remain considerably less responsive than T-47D cells) (Sheikh et al., 1994). The synthetic retinoid, Am580 arrested T-47D proliferation at concentrations ($<10^{-8}$ M) at which it demonstrates greatest selectivity for RARα (Delescluse et al., 1991). At these concentrations Am580 also induced PKCα expression but had no effect on PKCζ expression until well after the cells had stopped growing. Based on these results we conclude that retinoids arrest hormone-dependent human breast cancer cells following RARα-dependent induction of PKCα expression. The lack of retinoid effect on the hormone-independent MDA-MB-231 cell line could reflect the inability of retinoids to induce PKCα expression in these cells. Whether this is the result of insufficient levels of RARα or an unrelated defect in the PKCα gene (it is noteworthy that PKCα mRNA was detected in MDA-MB-231 cells, but not mature protein, see Figure 2 and 3) will be resolved in future studies.

CONCLUSION

The data presented in this first annual report support our hypothesis for a mechanism of RA-induced growth inhibition of T-47D cells. Induction of PKCα expression and concomitant repression of PKCζ expression following RA treatment are consistent with RA inducing growth arrest of T-47D cells. In contrast, retinoic acid had no effect on growth, or PKC expression in hormone independent, MDA-MB-231 breast cancer cells. The RARα-selective synthetic retinoid, Am580, was equally effective as retinoic acid at growth arrest and the induction of PKCα, but not reduction in PKCζ in T-47D cells. Addition of Gö6976, a selective inhibitor of conventional PKC, prevented the Am580 induced reduction in proliferation. In total, our interpretation is that retinoic acid arrests proliferation of T-47D cells following RARα dependent induction, and activation of PKCα. By manipulating the expression of PKCα, we have shown that expression and activity of PKCα is sufficient to exert growth inhibitory effects on T-47D cells.

Future work: we have described an important role for PKCα in mediating the anti-proliferative action of retinoids in human breast carcinoma cells. Our goal is to identify in more detail the role of PKCα in retinoid induced growth arrest of human breast carcinoma cells in terms of proto-oncogene expression and cell cycle progression. Currently we are establishing T-47D and MDA-MB-231 cells stably expressing PKCα, PKCζ, antisense PKCα and antisense PKCζ (see page 6; method section in specific aim 2). After stable transfection is established, we plan to complete Task 1d and 1e, Task 2 and Task 3 in each cell line including control T-47D and MDA-MB-231 cells. To extend our observation on RA induced PKCα expression, we also are currently establishing Stratagene's LacSwitch™ II inducible mammalian expression system with sense/ anti-sense PKCα and PKCζ cDNA in T-47D and MDA-MB-231 cells.

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Appendices

Figure 1-6, Attached.
At the end of figures, publication and meeting abstract produced during Sep,1996-Sep, 1997 were attached.

Figure legends

Figure 1. Retinoid regulation of human breast carcinoma cell proliferation. Hormone-dependent T-47D cells were plated at 15 x 10^4 cells/dish. After 24 hr retinoids were added to a final concentration of 0, 10^{-8} or 10^{-7} M from 1000x stock solutions in ethanol. Cells were trypsinized, stained with trypan blue and counted after the indicated intervals. Both retinoids, all-trans retinoic acid (**A**) and the RARα selective Am580 (**B**), inhibited T-47D cell proliferation without effecting viability (>90% of cells excluded trypan blue at all times). The hormone-independent human breast carcinoma cell line MDA-MB-231 was plated and treated in the same manner except the all trans retinoic acid (RA) was added at 10^{-6} M (**C**). Data shown (+/- SE) are from two independent experiments in which treatments were done in either triplicate (A and B) or duplicate (C). Day 1 represents the time of retinoid or solvent addition.

Figure 2. Protein Kinase C isozyme expression in T-47D and MDA-MB-231 cells. (A and B) Cell extracts (30μg protein/lane) from control (lanes 1, 5), 1 day (lanes 2, 6), 2 day (lanes 3, 7) and 5 day (lanes 4, 8) retinoic acid (10^{-6}M) treated T-47D (A) or MDA-MB-231(B) cells were subjected to SDS-PAGE/western blotting with PKC isozyme specific antibodies (affinity purified polyclonal PKCα; protein G-purified polyclonal PKCγ,δ,ε and ζ antibodies)(Gibco BRL, Inc., Gaithersburg, MD). The region of each gel shown was between the 67kDa and 93 kDa prestained molecular weight markers (New England Biolabs, Beverly, MA) run in adjacent lanes. Samples in lanes 5-8 were probed with antibodies that had been pre-incubated with immunizing peptides (1:3, antibody:peptide w/w). Arrows indicate the bands that were specifically competed for by the immunizing peptide. (C) Cell extracts (20 μg protein/lane) from control (lanes 2, 7), 1 day (lanes 3, 8), 2 day (lanes 4, 9)and 5 day (lanes 5, 10) retinoic acid (10^{-6} M) treated T-47D and MDA-MB-231 cells were subjected to SDS-PAGE/western blotting with PKCβ and γ specific antibodies (affinity purified polyclonal PKCβ antibody;protein G-purified polyclonal PKCγ antibody)(Gibco BRL, Inc., Gaitherburg, MD). As a positive control of PKCβ expression, F9 embryonal carcinoma cell extract (30μg protein/lane) was loaded in lanes 1 and 6 (Khuri et al., 1996). As a positive control of PKCγ expression, mouse brain PKC eluate from a DEAE-sepahrose column (30μg protein/lane) was loaded in lanes 1 and 6. Lanes 6-10 were run in parallel and incubate with antibodies that had been preincubated with immunizing peptide (1:3, antibody: peptide w/w). The region of each gel shown was between the 67 kDa and 93 kDa prestained molecular weight markers run in adjacent lanes.

Figure 3. PKCα and PKCζ mRNA expression in T-47D and MDA-MB-231 cells.
Total RNA (20μg protein/lane) from control (lanes 1,5), 1-day (lanes 2,6), 2 day (lanes 3,7), and 5 day (lanes 4,8) retinoic acid (10^{-6} M) treated T-47D (lanes 1-4) and MDA-MB-231 (lanes 5-8) cells were separated on 1.2% agarose gels, transferred to nylon membranes and hybridized to ^{32}P-labeled cRNA probes for either PKCα or PKCζ. PKCα probe detected a single transcript of ~5kb, and PKCζ probe detected primarily an 5.5kb mRNA.

Figure 4. Time and concentration dependent effects of retinoic acid and Am580 on PKCα and PKCζ expression in T-47D cells.
(A) T-47D cells were treated with ethanol solvent as control (lane 1), 10^{-9} M (lane 2), 10^{-8} M (lane 3), 10^{-7} M (lane 4) and 10^{-6} M (lane 5) retinoic acid for 5 days. Cell extracts (30μg protein/lane) were analyzed for PKCα and PKCζ expression by immunoblotting. (B) Cell extracts (30μg protein/lane) from control (lane 1), 10^{-9} M (lane 2), 10^{-8} M (lane 3), 10^{-7} M (lane 4) and 10^{-6} M (lane 5) Am580 treated T-47D cells for 1 day were analyzed for PKCα and PKCζ expression by western blotting as described in methods. In lanes 6-13, T-47D extracts from cells treated with 10^{-9} -10^{-6} M Am580 for 2 days (lanes 6-9) and for 5 days (lanes 10-13) were loaded.

Figure 5. Conventional PKC activity is required for the anti-proliferative response to retinoids.
T-47D cells were plated at 5 x 10^4 cells/dish. Cells were counted 24 hr later and treatments begun. Treatments were replenished after 48 hr. Control cells received solvent (0.05% DMSO), treated cells received either 10^{-8} M Am580, 500 nM Gö6976 or both. After 72 hr cells were trypsinized, stained with trypan blue and counted. The horizontal line represents the cell number at the time treatments were begun. * - indicates that the Am580 value is different significantly ($p<0.05$, Student's t-test) from the other values.

Figure 6. Stable expression of PKCα inhibits the proliferation of T-47D cells.

A. PKC isozyme expression in T-47D and PKCα transfected T-47D cells. Cell extracts (30μg protein/lane) from T-47D and PKCα transfected T-47D (αT-47D) were subjected to SDS-PAGE/western blotting with PKC isozyme specific antibodies (affinity purified polyclonal PKCα and β antibodies; protein G-purified polyclonal PKCγ, δ, ε, and ζ antibodies)(Gibco BRL, Inc., Gaithersburg, MD). The region of each gel shown was between the 67kDa and 93 kDa prestained molecular weight markers run in adjacent lanes.
B. T-47D or PKCα transfected T-47D (αT-47D) cells were plated at 8 x 10^4 cells/dish. After 24hr, treatments were begun. Media and supplements were replenished after 48hr. Control T-47D or αT-47D cells received solvent (0.05% DMSO), treated cells

received either 10^{-8} M trans retinoic acid, 50ng/ml TPA, or 500 nM cells Gö6976. After 72hr cells were trypsinized, stained with trypan blue, and counted. The horizontal line represents the cell number at the time treatments were begun. The values that do not share the same letter are significantly different at $P < 0.05$ (using Turkey's multiple comparison test, Sokal and Rohif, 1981, SAS 6.03, SAS Institute, Cary, NC).

Figure 1

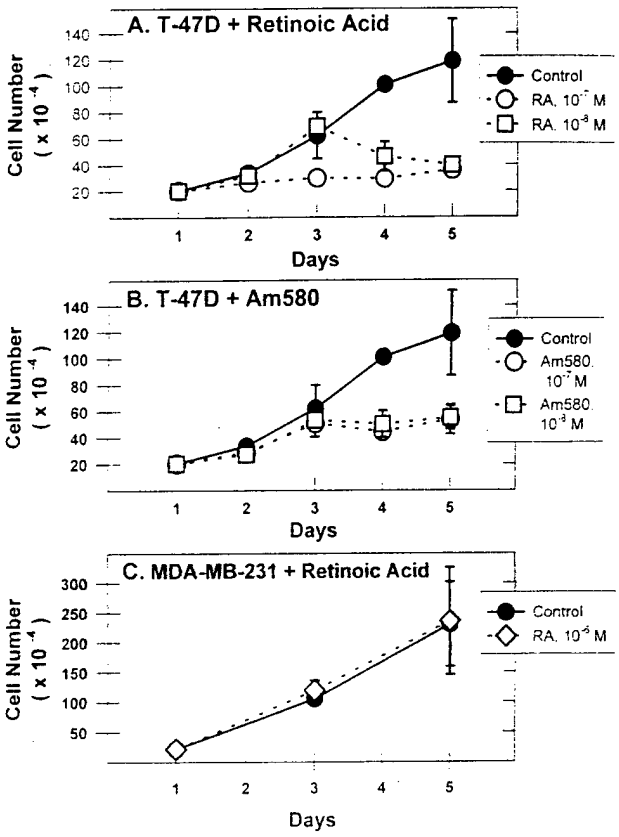

A. T-47D + Retinoic Acid
Cell Number (x 10 -4)
140
120
100
80
60
40
20
1
2
3
4
5
Days
Control
RA. 10-7 M
RA. 10-5 M
B. T-47D + Am580
Cell Number (x 10 -4)
Days
Control
Am580. 10-7 M
Am580. 10-5 M
C. MDA-MB-231 + Retinoic Acid
Cell Number (x 10 -4)
300
250
200
150
100
50
Days
Control
RA. 10-5 M

Figure 2

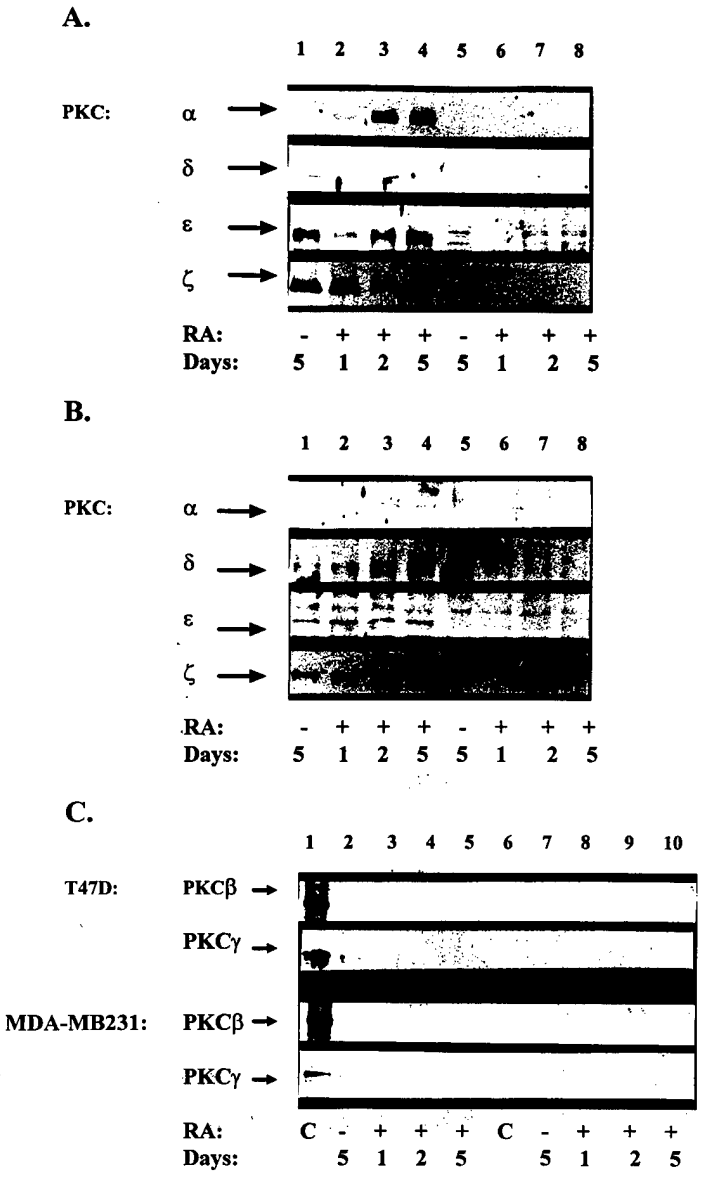

A.
1 2 3 4 5 6 7 8
PKC:
α
δ
ε
ζ
RA: - + + + - + + +
Days: 5 1 2 5 5 1 2 5
B.
1 2 3 4 5 6 7 8
PKC:
α
δ
ε
ζ
RA: - + + + - + + +
Days: 5 1 2 5 5 1 2 5
C.
1 2 3 4 5 6 7 8 9 10
T47D:
PKCβ
PKCγ
MDA-MB231:
PKCβ
PKCγ
RA: C - + + + C - + + +
Days: 5 1 2 5 5 1 2 5

Figure 3

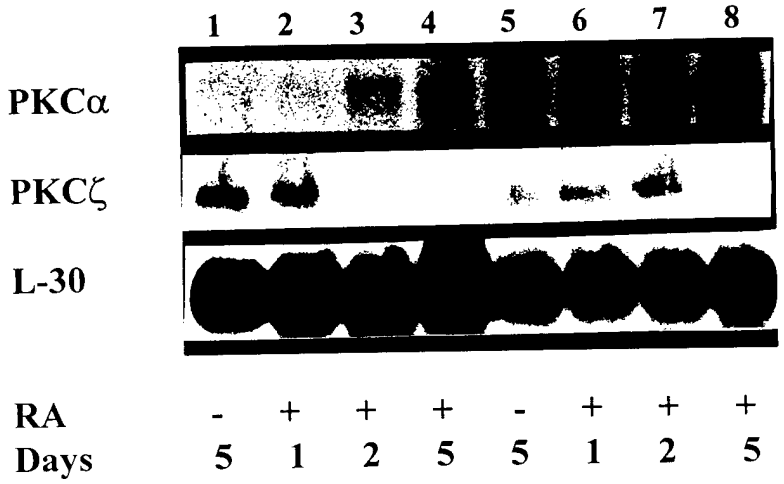
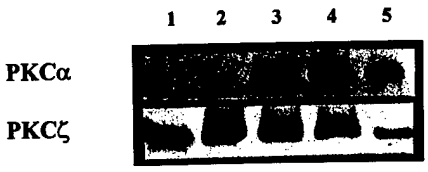


Figure 4

A.



RA	-	+	+	+	+
Con.(log M)	-	-9	-8	-7	-6
Days	5	5	5	5	5

B.

Am580	-	+	+	+	+	+	+	+	+	+	+	+	+
Con.(logM)	-	-9	-8	-7	-6	-9	-8	-7	-6	-9	-8	-7	-6
Days	2	1	1	1	1	2	2	2	2	5	5	5	5

Figure 5

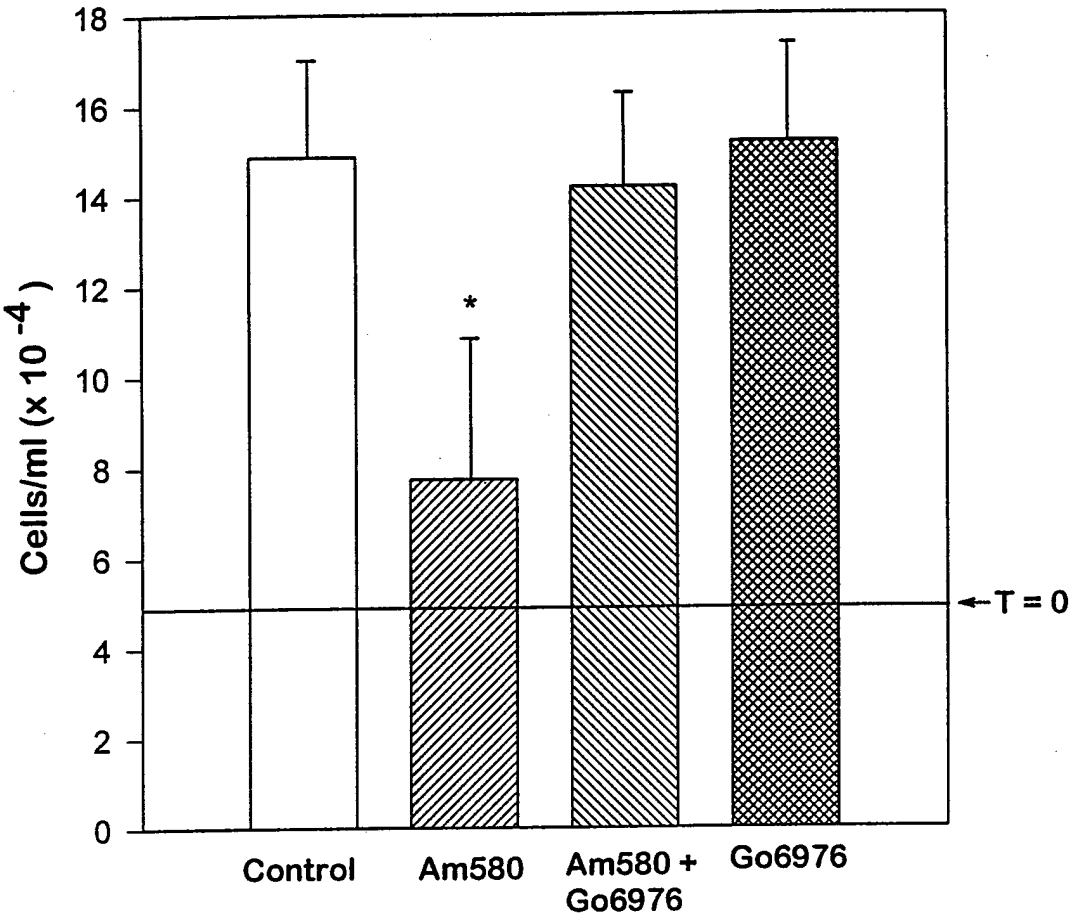
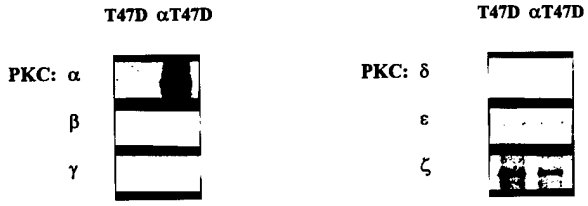
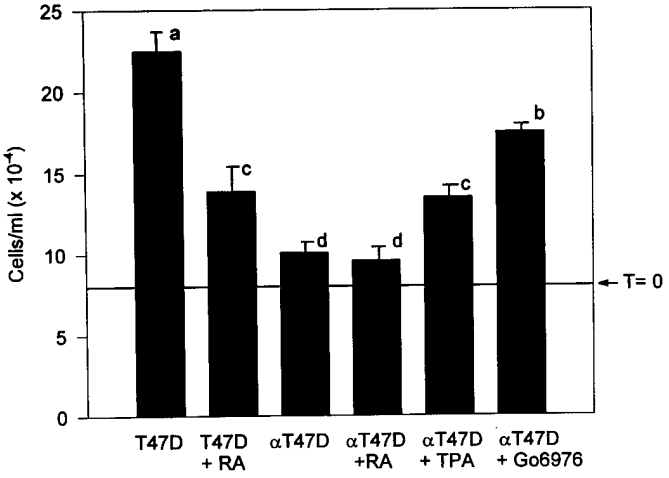

18
16
14
12
10
8
6
4
2
0
Cells/ml (x 10 -4)
*
←T = 0
Control
Am580
Am580 + Go6976
Go6976

Figure 6

A.


T47D αT47D
PKC: α
β
γ
T47D αT47D
PKC: δ
ε
ζ

B.


Cells/ml (x 10⁴)
25
20
15
10
5
0
a
b
c
c
d
d
← T= 0
T47D
T47D + RA
αT47D
αT47D +RA
αT47D + TPA
αT47D + Go6976

Bibliography

Meeting Abstract

88 th Annual Meeting; American Association For Cancer Research
(April 12-16, 1997; San Diego, CA)

Abstract # 451.
Distinct functions of protein kinase Cβ and Cα during retinoic acid induced differentiation of F9 cells.
Cho, Y., Klein, M., and Talmage, D.A. Institute of Human Nutrition, Columbia University, New York, NY 10032.

In a previous study, we demonstrated that retinoic acid (RA) and dibutyryl cyclic AMP induced differentiation into parietal endoderm was accompanied by a transition from protein kinase Cβ (PKCβ) to protein kinase Cα (PKCα) in F9 cells. The change in conventional PKC expression resulted in altered phorbol ester stimulation of genes. Specifically, we demonstrated that PKCβ activity was important for c-fos expression and PKCα activity led to expression of collagen IV, a marker of the parietal endoderm phenotype. To delineate further the roles of PKCα and β in F9 cell differentiation, we established cell lines stably expressing PKCα, PKCβ, anti-sense PKCα (As PKCα) or anti-sense PKCβ (As PKCβ) RNA. Increased PKCβ expression enhanced F9 proliferation, and both basal and phorbol ester induced c-fos expression. Inhibition of PKCβ expression (As-PKCβ) or constitutive expression of PKCα reduced F9 proliferation, and enhanced RA induced differentiation. The latter effect was apparent as both increased total expression and acceleration of RA-induced expression of laminins A, B1, B2, type IV collagen, and keratin 8 and 18. Re-expression of PKCβ in the parietal endoderm cell line, F9RA5, led to a gradual de-differentiation. Taken together, these studies demonstrate that RA regulation of PKC expression is a critical component of F9 embryonal carcinoma cell differentiation, and that PKCα and PKCβ play antagonistic roles in this process.

JOURNAL OF CELLULAR PHYSIOLOGY 172:306–313 (1997)

Retinoic Acid Induced Growth Arrest of Human Breast Carcinoma Cells Requires Protein Kinase Cα Expression and Activity

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Retinoic acid inhibits proliferation of hormone-dependent, but not hormone-independent breast cancer cells. Retinoic acid-induced changes in cellular proliferation and differentiation are associated with disturbances in growth factor signaling and frequently with changes in protein kinase C expression. PKCδ, ε, and ζ are expressed in both hormone-dependent (T-47D) and hormone-independent (MDA-MB-231) cell lines. Retinoic acid arrested T-47D proliferation, induced PKCα expression and concomitantly repressed PKCζ expression. The changes in PKCα and PKCζ reflect retinoic acid-induced changes in mRNA. In contrast, retinoic acid had no effect on growth, or PKC expression in MDA-MB-231 cells. Growth arrest and the induction of PKCα, but not the reduction in PKCζ, resulted from selective activation of RARα. In total, these results support an important role for PKCα in mediating the anti-proliferative action of retinoids on human breast carcinoma cells. **J. Cell. Physiol. 172:306–313, 1997.** © 1997 Wiley-Liss, Inc.

Retinoic acid (RA) inhibits the proliferation of many, but not all, human mammary carcinoma cell lines (Bollag et al., 1992). Several cytokines also inhibit the proliferation of human breast cancer cells (Bollag et al., 1992; Huslig et al., 1993), and there is growing evidence that cytokines, such as the interferons, potentiate the retinoic acid-induced inhibition of transformed epithelial cell proliferation (Bollag et al., 1992; Bollag, 1991; Hemmi et al., 1987). As a result, the combination of retinoids and interferons is a promising chemotherapeutic option in breast cancer (Bollag, 1991; Hemmi et al., 1987; Sparano and O'Boyle, 1992).

RA responsiveness appears to be limited to hormone-dependent cells (Butler and Fontana, 1992) and correlates with expression of the estrogen receptor (Fontana et al., 1990). The proliferative effect of estrogen on mammary cells has been linked to the production of mitogenic growth factors and increased expression of surface receptor tyrosine kinases (Daly et al., 1994; Halter et al., 1992). Growth factor binding to receptor tyrosine kinases activates multiple, interactive signaling pathways, most of which involve sequential activation of serine/threonine protein kinases. Two of the targets for receptor tyrosine kinases, phospholipase Cγ (PLCγ) and phosphatidylinositol 3-kinase (PtdIns 3-kinase), produce second messengers that activate multiple isozymes of protein kinase C (PKC). PLCγ, activated by tyrosine phosphorylation, hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol (DAG) and inositol trisphosphate (IP_3), which in turn, activate conventional and novel PKC isozymes. Phosphatidylinositol 3,4,5,-trisphosphate (PtdIns 3,4,5-P_3), produced after activation of PtdIns 3-kinase (Talmage et al., 1989; Stephens et al., 1993), can directly activate atypical PKCs (Nakanishi et al., 1993), and through poorly characterized means activate other serine/threonine protein kinases (e.g., $p70^{s6k}$, $p56^{Akt}$, and JNK/SAPK) (Chung et al., 1994; Franke et al., 1995).

Twelve distinct PKC isozymes are encoded by a family of at least 11 genes. These isozymes are divided into three groups based on sequence homology and cofactor requirement (Blobe et al., 1994): the conventional PKCs (α, βI, βII, and γ), the novel PKCs (δ, ε, μ, η, and θ), and the atypical PKCs (ι, λ, and ζ). Physiologically, conventional and novel PKCs are activated by the concerted action of phospholipid and the second messenger diacylglycerol (Blobe et al., 1994). Conventional PKCs also require calcium released from intracellular stores by IP_3 (Blobe et al., 1994). Atypical PKCs are calcium independent and are not activated by DAG but can be activated by the novel phospholipid, PtdIns 3,4,5-P_3, a product of PtdIns 3-kinase (Nakanishi et al., 1993). These observations support a model in which PKC isozymes are differentially activated in response to distinct growth factor or hormone activation of either PLC or PtdIns 3-kinase.

RA inhibits signaling between receptor tyrosine kinases and the nucleus (Leid et al., 1993; Talmage and

In this study we measured the level of seven of the PKC isozymes (α, β1/β2 together, γ, δ, ε, and ζ). The other isozymes have not been studied in these cells.

Contract grant sponsor: American Cancer Society; Contract grant number: CN134; Contract grant sponsor: U.S. Department of Army; Contract grant number: DAMD17-94-J-4100.

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Received 2 October 1996; Accepted 26 March 1997

Listerud, 1994). The components of these signaling pathways that serve as targets for retinoic acid have yet to be identified. At least two signaling pathways stimulated by receptor tyrosine kinases contain PKC family members. RA-induced differentiation of embryonal carcinoma and melanoma cells results in growth arrest, and is associated with changes in PKC expression (Rosenbaum and Niles, 1992; Khuri et al., 1996). Because of the relationship between the growth regulatory effect of RA on tumor cells and regulation of PKC expression, we compared the effect of retinoids on the pattern of PKC isozymes expressed in two breast cancer cell lines that are differentially responsive to RA. We demonstrated that all-trans retinoic acid, and a retinoic acid receptor-α (RARα) selective retinoid (Am580) induced PKCα expression in a time and concentration dependent manner that paralleled the effect of these retinoids on proliferation.

MATERIALS AND METHODS

Cell culture

T-47D human breast cancer cells (ATCC HTB133) were grown on plastic culture dishes in Roswell Park Memorial Institute (RPMI) medium 1640 supplemented with 10% fetal bovine serum (UBI, Saranac Lake, NY) and insulin (7.4 μg/ml). MDA-MB-231 human breast cancer cells (ATCC HTB26) were grown on culture dishes in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum. Cells were treated with 10^{-9} M–10^{-6} M all trans retinoic acid or Am580 dissolved in ethanol (final ethanol concentration was 0.1%), phorbol 12-myristate 13-acetate (50 ng/ml) (TPA), or 500 nM Gö6976 dissolved in DMSO (final concentration of DMSO was 0.05%) for up to 5 days.

To stably express PKCα in T-47D cells, pSVXα, encoding bovine PKCα (Borner et al., 1991) was transfected into the NIH3T3 GP + *env*Am12 packaging cell line (Markowitz et al., 1988) and colonies stably producing recombinant virus were isolated following selection for G418 resistance (250 μg/ml). T-47D cells were infected with freshly conditioned medium derived from subconfluent NIH3T3 GP + *env*Am12 cultures. Following infections, cultures were subjected to G418 (250 μg/ml) selection. Resistant cells were maintained in RPMI, 10% fetal bovine serum and 100 $\mu g \cdot ml^{-1}$ G418.

Measurement of relative proliferation

T-47D and MDA-MB-231 cells were plated at 15×10^4 cells/60 mm culture dish. Media was changed 24 hr later, at which time cells were treated with indicated concentrations of all-trans retinoic acid, Am580, TPA, or Gö6976. Media and experimental treatment were renewed every 72 hr. On the days indicated, cells were harvested and counted with a hemocytometer. Each sample was counted in duplicate, and each condition was done in duplicate or triplicate.

Western blot analysis for PKC isozyme expression

Cells were washed twice with cold phosphate buffered saline (PBS) and harvested in 500 μl of lysis buffer [20 mM Tris, pH 8, 150 mM NaCl, 10 mM sodium phosphate, 100 μM sodium vanadate, 100 μM ammonium molybdate, 10% glycerol, 1% nonidet P-40, and 0.1% sodium dodecyl sulfate (SDS)]. Lysates were cleared by centrifugation (10,000g for 5 min). Positive control extracts were prepared from F9 embryonal carcinoma cells for PKCβ, as previously described (Khuri et al., 1996) or mouse brain for PKCγ. Brain was homogenized in 20 mM Tris-HCl (pH 7.5), 10 mM EDTA, 1 mM EGTA, 0.5% NP40, and 0.4 mM phenylmethylsulfonyl fluoride for 10 min on ice. After removing the cell debris by centrifugation (12,000g, 15 min), cleared supernatants were applied to DEAE-sepharose columns (1 ml bed volume), washed with 15 column volumes of 20 mM Tris-HCl, 1 mM EDTA, and 0.1 mM EGTA and then PKC was eluted in 3 column volumes of the same buffer containing 90 mM NaCl. Protein concentrations were determined using Bradford's reagent (Bio-Rad, Hercules, CA). Samples were adjusted to equal protein concentration in SDS sample buffer and boiled for 5 min.

The protein extracts (30 μg/lane) were electrophoretically separated on 10% SDS-polyacrylamide gels and then transferred to Hybond-C extra nitrocellulose membranes (Amersham Corp., Chicago, IL) (Khuri et al., 1996). Membranes were blocked with 5% non-fat, dry milk in PBS at room temperature for 1 hr to reduce nonspecific antibody binding. Membranes were washed with PBS three times and incubated with PKC isozyme specific antibodies (0.5 μg/ml) (affinity purified polyclonal PKCα and β antibodies; protein G-purified polyclonal PKCγ, δ, ε, and ζ antibodies)(Gibco BRL, Inc., Gaithersburg, MD).[1] After incubation, membranes were washed in PBS containing 0.05% Tween 20 (PBST) three times and then incubated with the horseradish peroxidase coupled anti-rabbit IgG (1:7,500 dilution)(Amersham Corp., Chicago, IL) at room temperature for 2 hr. Immunoreactive proteins were visualized by enhanced chemiluminescence.

RNA isolation and northern blotting

Total RNA was isolated from treated cells as described by Dorsett et al. (1989). Cells were washed with PBS and then lysed with 7 M urea, 2% SDS, 350 mM NaCl, 1 mM EDTA, and 10 mM Tris-HCl, pH 8. Lysates were extracted once with phenol/chloroform (1:1) (TE equilibrated phenol, pH 8.0), once with chloroform, and nucleic acids were precipitated with 2–3 volumes 100% ethanol for 3 hr at −20°C. The precipitates were digested with DNase I (20 U/ml; Boehringer Mannheim, Indianapolis, IN) in 40 mM Tris-HCl pH 7.5, 10 mM NaCl, 6 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 20 U/ml placental RNase inhibitor (Boehringer Mannheim, Indianapolis, IN) at 37°C for 30 min, extracted with phenol/chloroform, and reprecipitated with ethanol. The final RNA was dissolved in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl, and 1% SDS, and yields were quantified as described by Sambrook et al. (1989). Typical yields were about 80–100 μg of total RNA from a 10 cm plate.

For northern blots, total RNA (20 μg/lane) was denatured by heating to 65°C for 5 min in 50% formamide and 1.1 M formaldehyde and resolved electrophoretically on 1.2% agarose gels containing 1.1 M formaldehyde and 40 mM MOPS (100 V for 3 hr). After electrophoresis, the gels were washed (1 × 20 min in 50 mM NaOH, 1 × 30 min in 10× SSC and 0.1 M Tris, pH 7.5, and 1 × 30 min in 10× SSC). The RNA samples were transferred to nylon membranes (Stratagene, La Jolla, CA) by diffusion in 10× SSC (overnight at room temper-

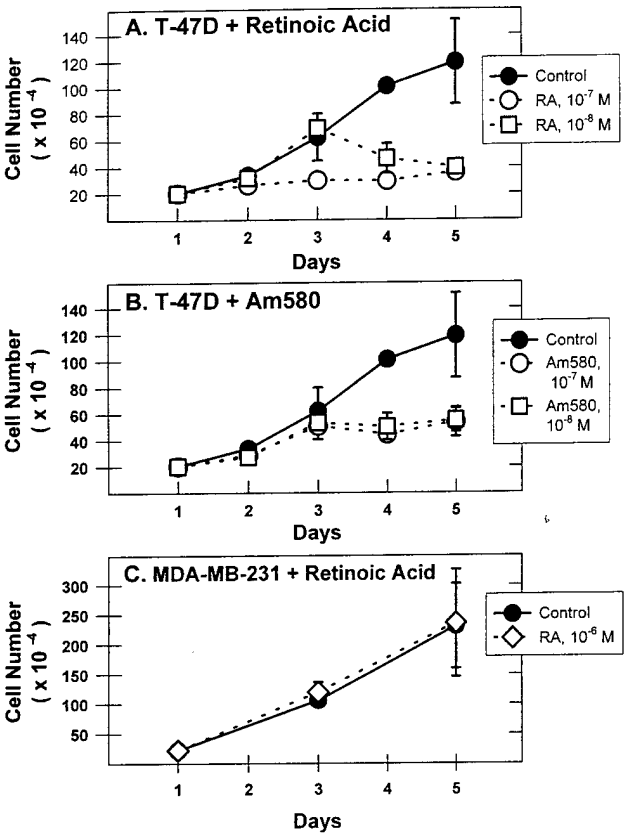

Fig. 1. Retinoid regulation of human breast carcinoma cell proliferation. Hormone-dependent T-47D cells were plated at 15×10^4 cells/dish. After 24 hr retinoids were added to a final concentration of 0, 10^{-8} or 10^{-7} M from 1,000× stock solutions in ethanol. Cells were trypsinized, stained with trypan blue, and counted after the indicated intervals. Both retinoids, all-trans retinoic acid (**A**) and the RARα selective Am580 (**B**), inhibited T-47D cell proliferation without affecting viability (>90% of cells excluded trypan blue at all times). The hormone-independent human breast carcinoma cell line MDA-MB-231 was plated and treated in the same manner except the all trans retinoic acid was added at 10^{-6} M (**C**). Data shown (±SE) are from two independent experiments in which treatments were done in either triplicate (A and B) or duplicate (C). Day 1 represents the time of retinoid or solvent addition.

ature), covalently cross-linked to the membranes with a Stratagene UV cross-linker, and hybridized overnight at 65°C with ^{32}P-cRNA probes, which were synthesized from either PKCα or PKCζ cDNA cloned into the pSP72 vector (Melton et al., 1984). Blots were washed twice at 65°C in 2× SSC/1% SDS and twice in 0.2× SSC/1% SDS. The blots were exposed to X-ray film at −80°C with intensifying screens.

RESULTS

Retinoids inhibit proliferation of breast carcinoma cells

Proliferation of the hormone dependent, T-47D human breast cancer line was inhibited by retinoids (Fig. 1A,B). All-trans retinoic acid (RA), 9-cis retinoic acid (not shown), and the RARα-selective synthetic retinoid, Am580 were equally effective at arresting growth of these cells. The similar dose response for growth arrest seen between RA and Am580, particularly at concentrations of 10^{-8} M where selectivity is greatest, argue for a primary role for RARα in regulating mammary cell proliferation. In contrast, the hormone-independent cell line, MDA-MB-231, was insensitive to RA, even at micromolar concentrations (Fig. 1C). Consistent with the ability of Am580 to inhibit T-47D proliferation, the lack of RA responsiveness of MDA-MB-231 cells has been explained partly by the low level of RARα expressed (Sheikh et al., 1994).

Effect of retinoic acid on PKC isozyme expression in breast carcinoma cells

In a recent study, we found that RA induced differentiation and growth arrest of F9 teratocarcinoma cells was accompanied by complex changes in the expression of PKC isozymes (Khuri et al., 1996). PKCα and PKCδ expression were clearly associated with changes in expression of proliferation and differentiation related genes (Khuri et al., 1996). Because of these findings, we examined the effect of RA on PKC isozyme expression in the RA sensitive T-47D cell line compared to the RA-insensitive MDA-MB-231 cell line. Untreated T-47D cells expressed the novel PKC isozymes, PKCδ and PKCε, and the atypical PKCζ (Fig. 2A), but not conventional PKCs (PKCα, β, or γ; Fig. 2A,C). When T-47D cells were treated with 10^{-6} M RA, PKCα expression was induced and concomitantly PKCζ expression decreased (PKCδ and PKCε expression were marginally decreased) (Fig. 2A). Increased PKCα expression was apparent within 1–2 days of retinoid addition (see also Fig. 4). By 5 days, PKCα was the predominant subtype expressed and PKCζ expression had dramatically decreased. Untreated MDA-MB-231 cells also expressed PKCδ, PKCε, and PKCζ, but not PKCα, PKCβ, and PKCγ (Fig. 2B,C). In contrast to T-47D cells, RA treatment of MDA-MB-231 cells had no effect on PKCα or PKCζ expression (PKCδ expression was marginally increased after 5 days)(Fig. 2B).

RA induced changes in PKCα and PKCζ expression in T-47D cells resulted from changes in mRNA levels (Fig. 3). Again, the induction of PKCα and the reduction in PKCζ expression was observed within 1–2 days of treatment. No change in either PKCα or PKCζ mRNA was seen in MDA-MB-231 cells. Curiously, PKCα mRNA was readily detected in these cells, despite the absence of measurable immunoreactive PKCα. Whether this indicates a defect in translation of this mRNA or mutations that lead to failure of this protein to be stably expressed is under further investigation.

Concentration dependence of retinoid regulation

The changes in PKCα and PKCζ expression following RA treatment coincided with the cessation of proliferation. This, along with the observation that neither changes in PKC expression nor growth arrest occurred in MDA-MB-231 cells, raised the possibility that these changes in PKC expression were responsible for the anti-proliferative effects of retinoids in T-47D cells. At low retinoid concentrations (10^{-8} M) growth arrest of T-47D cells was delayed (Fig. 1A,B). If the changes in PKC expression observed were related to the cessation of cell division, we reasoned that the time course of these changes should also be sensitive to the dose of

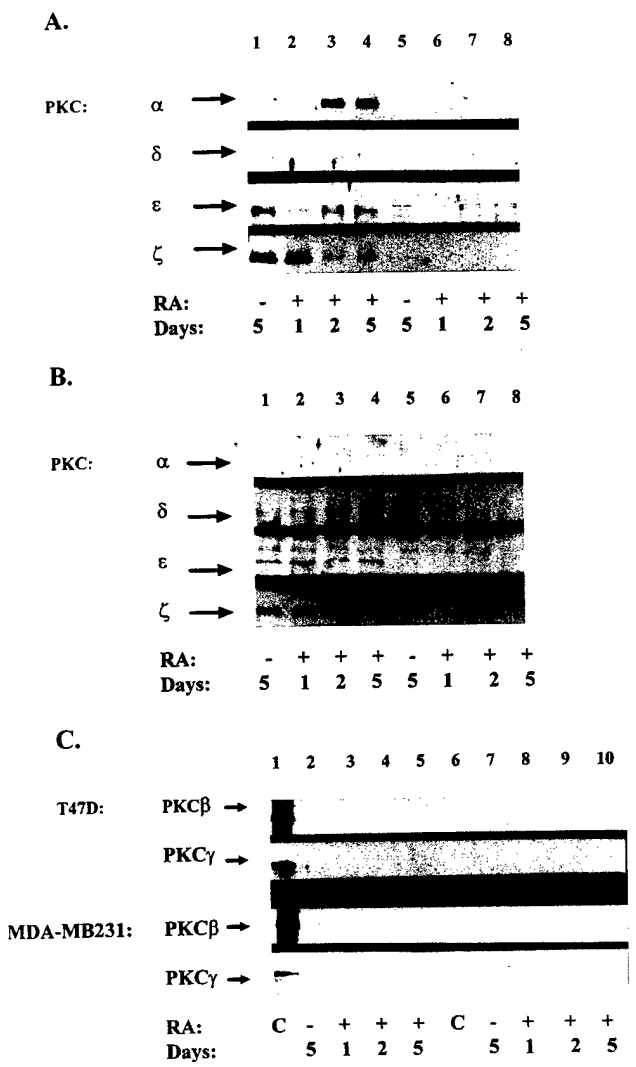

Fig. 2. Protein Kinase C isozyme expression in T-47D and MDA-MB-231 cells. **A,B:** Cell extracts (30 μg protein/lane) from control (lanes 1,5), 1 day (lanes 2,6), 2 day (lanes 3,7), and 5 day (lanes 4,8) retinoic acid (10^{-6}M) treated T-47D (A) or MDA-MB-231 (B) cells were subjected to SDS-PAGE/western blotting with PKC isozyme specific antibodies (affinity purified polyclonal PKCα antibody; protein G-purified polyclonal PKCγ, δ, ε, and ζ antibodies) (Gibco BRL, Inc., Gaithersburg, MD). The region of each gel shown was between the 67 kDa and 93 kDa prestained molecular weight markers (New England Biolabs, Beverly, MA) run in adjacent lanes. Samples in lanes 5–8 were probed with antibodies that had been pre-incubated with immunizing peptides (1:3, antibody:peptide w/w). Arrows indicate the bands that were specifically competed for by the immunizing peptide. **C:** Cell extracts (30 μg protein/lane) from control (lanes 2,7), 1 day (lanes 3,8), 2 day (lanes 4,9), and 5 day (lanes 5,10) retinoic acid (10^{-6}M) treated T-47D or MDA-MB-231 cells were subjected to SDS-PAGE/western blotting with PKCβ and γ specific antibodies (affinity purified polyclonal PKCβ antibody; protein G-purified polyclonal PKCγ antibody) (Gibco BRL, Inc., Gaithersburg, MD). As a positive control of PKCβ expression, F9 embryonal carcinoma cell extract (30 μg protein/lane) was loaded in lanes 1 and 6 (Khuri et al., 1996). As a positive control of PKCγ expression, mouse brain PKC eluate from a DEAE-sepharose column (30 μg protein/lane) was loaded in lanes 1 and 6. Lanes 6–10 were run in parallel and incubate with antibodies that had been preincubated with immunizing peptide (1:3, antibody: peptide w/w). The region of each gel shown was between the 67 kDa and 93 kDa prestained molecular weight markers run in adjacent lanes.

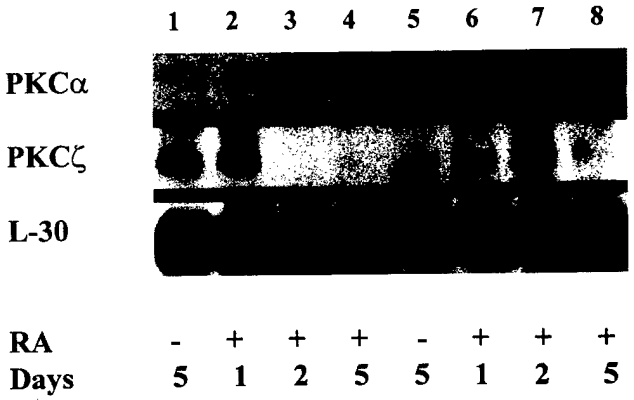

Fig. 3. PKCα and PKCζ mRNA expression in T-47D and MDA-MB-231 cells. Total RNA (20 μg/lane) from control (lanes 1,5), 1 day (lanes 2,6), 2 day (lanes 3,7), and 5 day (lanes 4,8) retinoic acid (10^{-6} M) treated T-47D (lanes 1–4) and MDA-MB-231 (lanes 5–8) cells were separated on 1.2% agarose gels, transferred to nylon membranes and hybridized to ^{32}P-labeled cRNA probes for either PKCα or PKCζ . PKCα probe detected a single transcript of ~5 kb, and PKCζ probe detected primarily a 5.5 kb mRNA.

RA. After 5 days, maximal expression of PKCα was seen with RA at concentrations of 10^{-8}–10^{-6} M (Fig. 4A). Slight elevations were typically seen with 10^{-9} M RA only after 5 days. In contrast, PKCζ expression remained unaffected at RA concentrations less than 10^{-6} M. These results indicate that RA is regulating expression of these two PKC isotypes via fundamentally different mechanisms and that decreased PKCζ is unlikely to contribute to growth arrest.

The data presented in Figure 1B, and by others (Sheikh et al., 1994; Talmage and Lackey, 1992) indicate that RARα is partially responsible for the antiproliferative effects of RA on human breast cancer cell lines. To extend the association between expression of PKCα and RARα-induced growth arrest, we examined the concentration and time dependent effect of the RARα selective ligand Am580, on PKC expression (Fig. 4B). PKCα was detected after 1 day of treatment with 10^{-8} M Am580. At 2 days, PKCα was seen in cells treated with 10^{-9} M Am580, and maximal expression was seen between 10^{-7} and 10^{-6} M Am580. High levels of PKCα expression were maintained after 5 days at 10^{-9} and 10^{-8} M. The higher concentrations of Am580 resulted in decreased PKCα expression after 5 days, indicating that PKCα expression was down-regulated by these higher concentrations of Am580. Compared to the rapid induction of PKCα by 10^{-9} – 10^{-8} M Am580, no decrease in PKCζ levels were seen after 2 days of treatment with 10^{-8} M Am580. Levels were marginally reduced at 2 days by the higher concentrations (10^{-7} or 10^{-6} M) of Am580. Even after 5 day, PKCζ was still expressed at low concentrations (10^{-9} or 10^{-8} M) of Am580 and intermediate (10^{-7} M) concentrations of RA. At low concentrations, Am580 is a selective agonist of RARα (kDa = 8×10^{-9} M for RARα, 1.1×10^{-6} M for RARβ and γ) (Delescluse et al., 1991). The sensitivity of T-47D proliferation and PKCα expression to low concentrations of Am580 indicate that both of these effects can be induced by RARα. In contrast, the decreased expression of PKCζ after only extended periods of treat-

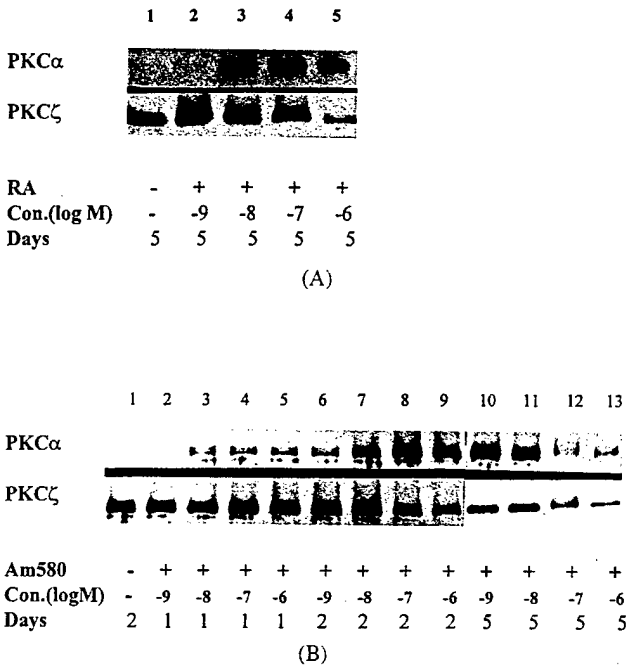

Fig. 4. Time and concentration dependent effects of retinoic acid and Am580 on PKCα and PKCζ expression in T-47D cells. **A:** T-47D cells were treated with ethanol solvent as control (lane 1), 10^{-9} M (lane 2), 10^{-8} M (lane 3), 10^{-7} M (lane 4), and 10^{-6} M (lane 5) retinoic acid for 5 days. Cell extracts (30 μg protein/lane) were analyzed for PKCα and PKCζ expression by immunoblotting. **B:** Cell extracts (30 μg protein/lane) from control (lane 1), 10^{-9} M (lane 2), 10^{-8} M (lane 3), 10^{-7} M (lane 4), and 10^{-6} M (lane 5) Am580 treated T-47D cells for 1 day were analyzed for PKCα and PKCζ expression by western blotting as described in Methods. In lanes 6–13, T-47D extracts from cells treated with 10^{-9}–10^{-6} M Am580 for 2 days (lanes 6–9) or for 5 days (lanes 10–13) were loaded.

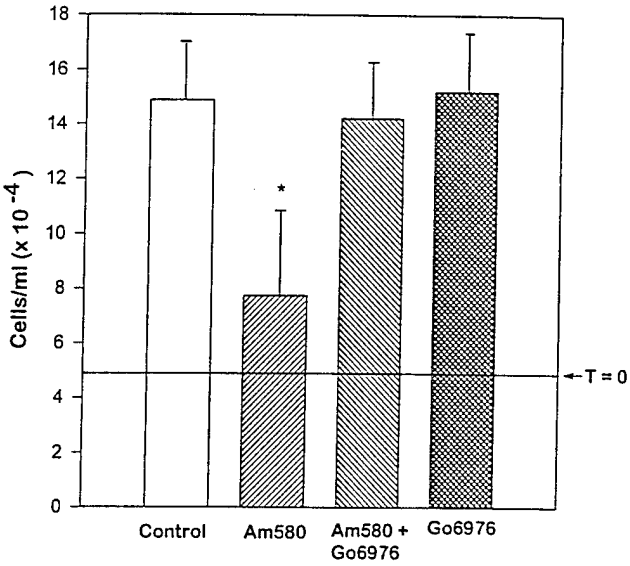

Fig. 5. Conventional PKC activity is required for the anti-proliferative response to retinoids. T-47D cells were plated at 5×10^4 cells/dish. Cells were counted 24 hr later and treatments begun. Treatments were replenished after 48 hr. Control cells received solvent (0.05% DMSO), treated cells received either 10^{-8} M Am580, 500 nM Gö6976, or both. After 72 hr cells were trypsinized, stained with trypan blue, and counted. The horizontal line represents the cell number at the time treatments were begun. * indicates that the Am580 value is different significantly ($P < 0.05$, Student's t-test) from the other values.

ment with high concentrations of RA or Am580 is not consistent with RARα-mediated regulation of this gene, or with a causal association between the loss of PKCζ expression and growth arrest.

PKCα activity is required for the anti-proliferative response to retinoids

Untreated T-47D cells did not express detectable levels of the conventional PKCs, PKCα, PKCβ, or PKCγ (Fig. 2A.C). Retinoids rapidly induced expression of PKCα but not PKCβ, or PKCγ and arrested proliferation in T-47D but not MDA-MB-231 cells. In order to determine if retinoic acid-induced PKCα plays an active role in mediating the anti-proliferative effect of retinoic acid, we assayed proliferation of T-47D cells in the presence of the synthetic indolocarbazole, Gö6976 which is a selective inhibitor of conventional PKCs (Martiny-Baron et al., 1993; Qatsha et al., 1993). Martiny-Baron et al. (1993) demonstrated that Gö6976 inhibits conventional PKCs (IC50 of PKCα = 2.3 nM, IC50 of PKCβ1 = 6.2 nM) but not other PKCs (no inhibition for PKCδ, ε, and ζ). T-47D cells were treated with 10^{-8} M Am580, 500 nM Gö6976, or both for 72 hr. Untreated cells underwent 1.5–2 population doublings during this period compared to less than 1 doubling for Am580 treated cells (Fig. 5). Addition of Gö6976 prevented the Am580 induced reduction in proliferation. Cells treated with retinoid and Gö6976 alone or in combination were fully viable; greater than 95% of cells excluded trypan blue. Therefore retinoid arrested proliferation of T-47D cells required the activity of conventional PKCs, presumably the retinoid-induced PKCα.

Stable expression of PKCα inhibits the proliferation of T-47D cells. In order to demonstrate further the functional role of PKCα in mediating the proliferative response of T-47D cells to retinoids, we isolated T-47D cells constitutively expressing PKCα (αT-47D) following infection with a recombinant retrovirus (pSVXα; Borner et al., 1991). SVXα infected T-47D cells (αT-47D) expressed PKCα in the absence of retinoic acid (Fig 6A). No additional alterations in PKCβ, γ, δ, ε, and ζ were seen in αT-47D cells (Fig. 6A), demonstrating that constitutive PKCα expression did not alter basal expression of other PKC isozymes.

T-47D and αT-47D cells were treated with 10^{-8} M trans retinoic acid, 50 ng/ml TPA or 500 nM Gö6976 for 72 hr. Untreated T-47D cells underwent 1.5–2 population doublings compared to less than 1 doubling for αT-47D cells. Constitutive expression of PKCα slowed T-47D proliferation more effectively than 10^{-8} RA. During the first 3 days of treatment addition of retinoic acid to αT-47D cells did not reduce proliferation further. Treatment of αT-47D cells with the selective inhibitor of the cPKCs, Gö6976 increased proliferation to near control levels (~75% of T-47D). Although less pronounced, chronic phorbol ester treatment, which downregulates multiple PKCs, also increased αT-47D proliferation (Fig. 6B).

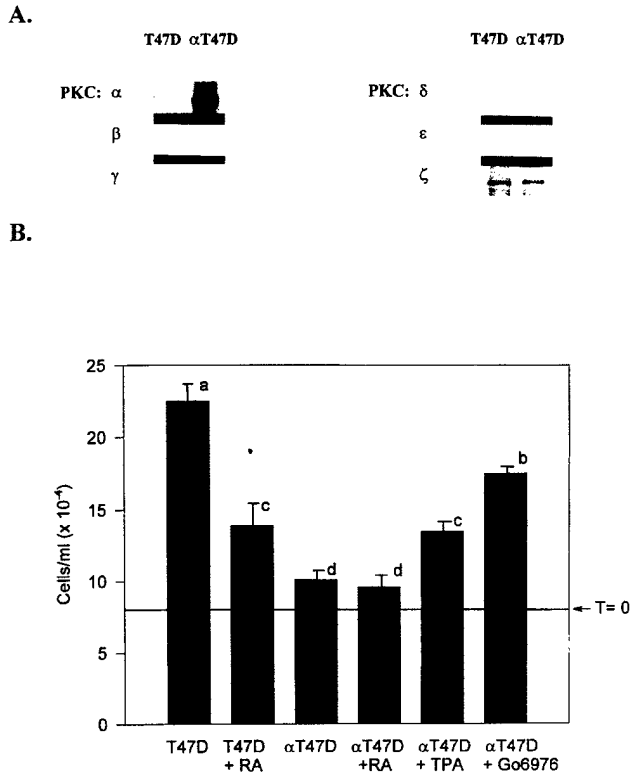

Fig. 6. Stable expression of PKCα inhibits the proliferation of T-47D cells. **A:** PKC isozyme expression in T-47D and PKCα transfected T-47D cells. Cell extracts (30 μg protein/lane) from T-47D and PKCα transfected T-47D (αT-47D) were subjected to SDS-PAGE/western blotting with PKC isozyme specific antibodies (affinity purified polyclonal PKCα and β antibodies; protein G-purified polyclonal PKCγ, δ, ε, and ζ antibodies) (Gibco BRL, Inc., Gaithersburg, MD). The region of each gel shown was between the 67 kDa and 93 kDa prestained molecular weight markers run in adjacent lanes. **B:** T-47D or PKCα transfected T-47D (αT-47D) cells were plated at 8×10^4 cells/dish. After 24 hr, treatments were begun. Media and supplements were replenished after 48 hr. Control T-47D or αT-47D cells received solvent (0.05% DMSO), treated cells received either 10^{-8} M trans retinoic acid, 50 ng/ml TPA, or 500 nM Gö6976. After 72 hr cells were trypsinized, stained with trypan blue, and counted. The horizontal line represents the cell number at the time treatments were begun. The values that do not share the same letter are significantly different at $P < 0.05$ (using Turkey's multiple comparison test, Sokal and Rohif, 1981, SAS 6.03, SAS Institute, Cary, NC).

DISCUSSION

The second messenger regulated PKCs are emerging as important components of retinoid responsiveness. On one level, Maciaszek et al (1994), and Tahayato et al. (1993) demonstrated synergistic activation of viral (SIV/HIV-1 LTRs) and cellular (the RARβ2 promoter) promoters, respectively, by retinoic acid and phorbol esters. Synergistic transactivation represents convergence of diverse signaling pathways on a common target. In the former case, retinoic acid, acting via RARs, was proposed to induce expression of a transcription factor that became functionally active following PKC dependent phosphorylation (Maciaszek et al., 1994). In the latter example, the common molecular target was proposed to be RAR itself, following retinoic acid binding and PKC-dependent phosphorylation (Tahayato et al., 1993).

A second level at which PKC and retinoid action intersect involves changes in PKC isozyme expression, frequently increased PKCα expression, that accompany retinoid-induced differentiation and growth arrest (Gruber et al., 1992; Khuri et al., 1996; Rosenbaum and Niles, 1992). In most cases the significance of altered PKC isozyme expression in achieving or maintaining the altered differentiated or proliferative state is unresolved. Here we have demonstrated that retinoids both induce PKCα expression and arrest the proliferation of a hormone-dependent human breast cancer cell line. Neither of these responses to RA were seen in a second, hormone-independent cell line. The synthetic nonglycosidic indolocarbazole, Gö6976, is a specific inhibitor of conventional PKCs with no significant inhibitory activity against novel and atypical PKCs (Martiny-Baron et al., 1993). Gö6976 abrogated the anti-proliferative effect of retinoids on T-47D cells. Since PKCα was the only conventional PKC detected in retinoid treated T-47D cells, we conclude that PKCα expression and activity is required for retinoid action. This conclusion was supporter by demonstrating that PKCα transfected T-47D cells grew at rates comparable to retinoid treated parental cells; a response partially reverted by the PKC inhibitor Gö6976, or by chronic phorbol ester treatment.

Other studies on human breast carcinoma cell lines revealed that the level of PKC expression is altered depending on estrogen receptor status (Borner et al., 1987) and that chronic exposure to phorbol ester results in growth arrest in G_0/G_1 and acquisition of a more differentiated phenotype (Darbon et al., 1990; Valette et al., 1987; Kennedy et al., 1992). As is frequently the case, it is difficult to determine if these effects of phorbol ester result from activation of conventional and/or novel PKCs, or result from PKC down-regulation following chronic activation, nor is it clear to what extent these responses are mediated by PKCα or other PKC isozymes. Recently Ways et al. (1995) reported a more "aggressive" phenotype resulting from PKCα expression in MCF-7 cells. Whether the difference in the effect of PKCα expression in our study compared to theirs results from the use of different parental cell lines is secondary to alterations in other PKCs or is because of a non-kinase dependent effect in MCF7 cells as suggested by Ways et al. (1995) is not clear.

In F9 embryonal carcinoma cells, retinoic acid-induced changes in conventional PKC expression, including PKCα induction, are related directly to changes in the regulation of cell cycle associated genes and markers of terminal differentiation (Khuri et al., 1996). Expression of PKCα in the absence of retinoic acid resulted in phorbol ester induced expression of the differentiation marker, type IV collagen. This report and the current study clearly establish PKCα as a key component of retinoid induced biological responses in multiple cell types.

The mechanism(s) by which PKCα inhibits T-47D cell proliferation is not known. Retinoid-induced growth arrest of T-47D cells also involves disrupted mitogenic signaling from receptor tyrosine kinases (Tighe and Talmage, in preparation). It is conceivable that PKCα-dependent phosphorylation of the EGF receptor (and/or similar receptors) desensitizes growth factor signaling (Cochet et al., 1984; Friedman et al.,

1984; Saloman, 1981; Lee and Weinstein 1978). Alternatively PKCα might indirectly regulate the expression of a gene whose product is actively involved in removing T-47D cells from the cell cycle.

In addition to inducing PKCα expression, retinoic acid also repressed PKCζ expression in T-47D cells. The functional relevance of this is not clear. Retinoid-dependent inhibition of PKCζ expression followed a time course distinct from PKCα induction and growth arrest, and was significantly reduced only in cells treated with high retinoic acid concentrations. Neither the kinetics of PKCζ repression nor the retinoid concentration dependence paralleled the effect of retinoids on proliferation of these cells. This, coupled with the ability of Gö6976 to block retinoid action, argues strongly against the functional importance in decreased PKCζ expression in mediating the retinoid anti-proliferative effects.

The extent to which different RARs regulate distinct versus redundant or overlapping biological responses is unclear. T-47D cells express both RARα and RARγ, and RARβ expression increases following prolonged RA treatment (Liu et al., 1996). Several observations strongly favor RARα as the mediator of RA growth regulation, and PKCα expression in these human mammary carcinoma cells (Sheikh et al., 1994; Valette et al., 1987; Kennedy et al., 1992). RARα is the major RAR subtype expressed in T-47D cells but is present at low levels in MDA-MD-231 cells (Sheikh et al., 1994). Expression of RARα cDNAs in MDA-MB-231 cells results in increased sensitivity to the growth inhibiting effects of RA (although they remain considerably less responsive than T-47D cells) (Sheikh et al., 1994). The synthetic retinoid Am580 arrested T-47D proliferation at concentrations ($<10^{-8}$ M), at which it demonstrates greatest selectivity for RARα (Delescluse et al., 1991). At these concentrations Am580 also induced PKCα expression but had no effect on PKCζ expression until well after the cells had stopped growing. Based on these results we conclude that retinoids arrest hormone-dependent human breast cancer cells following RARα-dependent induction of PKCα expression. The lack of retinoid effect on the hormone-independent MDA-MB-231 cell line could reflect the inability of retinoids to induce PKCα expression in these cells. Whether this is the result of insufficient levels of RARα or an unrelated defect in the PKCα gene (it is noteworthy that PKCα mRNA was detected in MDA-MB-231 cells, but not mature protein; see Figs. 2 and 3) will be resolved in future studies.

ACKNOWLEDGMENTS

This research was supported by grants from the American Cancer Society (CN134), the American Institute for Cancer Research, the U.S. Department of Army (DAMD17-94-J-4100), the Lucille P. Markey Charitable Trust, and the Columbia Presbyterian Cancer Center.

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