Activation of Protein Kinase C by All-trans-retinoic Acid*

Received for publication, February 12, 2003, and in revised form, May 12, 2003
Published, JBC Papers in Press, June 12, 2003, DOI 10.1074/jbc.M301523200

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All-trans-retinoic acid (RA) is a potent inducer of leukemia cell proliferation and induces differentiation of acute promyelocytic leukemia cells in vitro and in vivo. For RA to induce its biological effects in target cells, binding to specific retinoic acid nuclear receptors is required. The resulting complexes bind to RA-responsive elements (RAREs) in the promoters of RA-inducible genes to initiate gene transcription and to generate protein products that mediate the biological effects of RA. In this report, we provide evidence that a member of the protein kinase C (PKC) family of proteins, PKCβ, is activated during RA treatment of the NB-4 and HL-60 acute myeloid leukemia cell lines as well as the MCF-7 breast cancer cell line. Such RA-dependent phosphorylation was also observed in primary acute promyelocytic leukemia cells and resulted in activation of the kinase domain of PKCβ. In studies aimed at understanding the functional relevance of PKCβ in the induction of RA responses, we found that pharmacological inhibition of PKCβ (but not of other PKC isoforms) diminished RA-dependent gene transcription via RAREs. On the other hand, overexpression of a constitutively active form of the kinase strongly enhanced RA-dependent gene transcription via RAREs. Gel shift assays and chromatin immunoprecipitation studies demonstrated that PKCβ associated with retinoic acid receptor-α and was present in an RA-inducible protein complex that bound to RAREs. Pharmacological inhibition of PKCβ activity abrogated the induction of cell differentiation and growth inhibition of NB-4 blast cells, demonstrating that its function is required for such effects. Altogether, our data provide strong evidence that PKCβ is activated in an RA-dependent manner and plays a critical role in the generation of the biological effects of RA in malignant cells.

All-trans-retinoic acid (RA) is a potent inducer of cell differentiation and growth arrest of malignant cells in vitro and in vivo (1–6). This agent has potent effects against acute promyelocytic leukemia blast cells, and its introduction in the clinical management of the disease has dramatically changed the outcome of this historically fatal subtype of acute leukemia (5). RA and other retinoids have been shown to inhibit cell growth or to promote programmed cell death of neoplastic cells of diverse origin (7–14). The molecular mechanisms that regulate the induction of the biological effects of retinoids include a series of signaling events that are initiated by the binding of retinoids to specific receptors in the nucleus of target cells. Two families of retinoid receptors have been identified so far: retinoic acid receptors (RARs) (types α, β, and γ), which are activated by both RA and 9-cis-retinoic acid, and retinoid X receptors (RXRs) (types α, β, and γ), which are activated only by 9-cis-retinoic acid (15–17). RA binds to the nuclear RARs and induces the formation of RAR-RXR heterodimers, which associate with specific DNA-binding sequences present in the promoters of RA-responsive genes called retinoic acid-responsive elements (RAREs). Such binding of RA nuclear complexes to promoter RAREs results in initiation of transcription of genes whose protein products mediate RA biological responses (15–17).

In addition to the induction of formation of RAR-RXR complexes, RA induces a variety of other cellular effects that appear to play a role in the generation of its effects on target cells. Such mechanisms via which retinoids induce their biological effects on malignant cells include inhibition of activation of the AP-1 protein via a CBP (cAMP-responsive element-binding protein)-regulated mechanism (18, 19), modulation of histone acetylation (20), and up-regulation of transforming growth factor-β2 and insulin-like growth factor-binding protein-3 expression (21).

In conclusion, the RA-induced activation of the AP-1 complex appears to play a role in the generation of its effects on target cells. Such mechanisms via which retinoids induce their biological effects on malignant cells include inhibition of activation of the AP-1 protein via a CBP (cAMP-responsive element-binding protein)-regulated mechanism (18, 19), modulation of histone acetylation (20), and up-regulation of transforming growth factor-β2 and insulin-like growth factor-binding protein-3 expression (21).

Retinoids also regulate the activation of members of different groups of MAPKs. It has been previously shown that they inhibit activation of the c-Jun N-terminal kinase (22), and such inhibition appears to be required for the induction of retinoid
responses (22). On the other hand, RA induces activation of the MAPK ERK2 (23) as well as activation of the p38 MAPK (24). The activation of ERK2 mediates positive regulatory effects in the induction of retinoid responses, and its function appears to be essential for RA-dependent differentiation of HL-60 cells (23). On the other hand, activation of the p38 MAPK exhibits negative regulatory effects on the induction of differentiation of NB-4 cells by RA (24), and pharmacological inhibitors of this kinase promote the anti-leukemic effects of RA in vitro (24).

The protein kinase C (PKC) family of proteins is a multigene family of at least 12 serine/threonine kinases that participate in signal transduction events and are classified into three groups based on the differences in their structure and regulatory domains as well as differences in their activation requirements (25). The protein members of this family of kinases exhibit serine kinase activities and, upon their activation, regulate phosphorylation/activation of other serine kinases, resulting in signals that ultimately mediate multiple biological responses. The tissue distribution of PKC isoforms varies considerably, with PKCα, PKCδ, and PKCζ being widely expressed, whereas most of the other isoforms are selectively expressed in different types of cells and tissues (25). PKCδ belongs to the group of novel PKC isoforms, which are Ca2+-independent and are activated by phorbol esters, diacylglycerol, and phosphatidylserine (26). Previous studies have shown that this kinase plays important roles in the induction of anti-proliferative and pro-apoptotic responses in response to DNA-damaging agents and ionizing radiation (27, 28). Consistent with this, it has been demonstrated that overexpression of its catalytically active fragment is capable of inducing apoptosis of target cells (29).

In this study, we provide evidence that PKCδ is activated during treatment of acute promyelocytic leukemia and breast cancer cell lines with RA. Our data demonstrate that this PKC isoform forms complexes with RARα and binds to RAREs. Such a function of PKCδ plays a critical role in RA-dependent transcriptional regulation, as evidenced by the fact that inhibition of PKCδ kinase activity blocks RA-dependent gene transcription via RAREs. Consistent with this, pharmacological inhibition of PKCδ diminishes induction of cell differentiation of acute promyelocytic leukemia blast cells by RA and blocks RA-dependent suppression of cell growth, underscoring the critical role that this PKC isoform plays in the induction of RA responses.

EXPERIMENTAL PROCEDURES

Cells and Reagents—The RA-sensitive human acute promyelocytic leukemia cell line NB-4 and the acute myeloid leukemia cell line HL-60 were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics. MCF-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics. Polyclonal antibodies against PKCδ and Stat1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody against PKCδ phosphorylated/activated at Thr505 was obtained from New England Biolabs Inc. (Beverly, MA). An antibody against Stat1 phosphorylated at Ser727 was obtained from Upstate Biotechnology, Inc. The PKCδ inhibitor rottlerin and the PKCδ inhibitor Go 6976 were obtained from Calbiochem. Peripheral blood mononuclear cells were isolated from the peripheral blood of a patient with acute promyelocytic leukemia, after obtaining informed consent approved by the Institutional Review Board of Northwestern University.

Cell Lysis, Immunoprecipitations, and Immunoblotting—Cells were treated with RA (final concentration of 1 μM) for the indicated times and lysed in phosphorylation lysis buffer as described previously (30–32). Immunoprecipitations and immunoblotting using an ECL method were performed as described previously (30–32). Briefly, cells were treated for the indicated times with retinoic acid and were then lysed in phosphorylation lysis buffer. Cell lysates were immunoprecipitated with anti-PKCδ antibody, and immunoprecipitates were then incubated three times with phosphorylation lysis buffer and two times with kinase buffer (25 mM Tris–HCl (pH 7.4), 5 mM MgCl2, 0.5 mM EDTA, 1 mM dithiothreitol, 20 μM of phosphatidylserine, and 20 μM ATP) and resuspended in 30 μl of kinase buffer containing 5 μg of histone H1 as an exogenous substrate, to which 20–30 μCi of [γ-32P]ATP was added. The reaction was incubated for 15–30 min at room temperature and terminated by the addition of SDS sample buffer. Proteins were analyzed by SDS-PAGE, and phosphorylated histone H1 was detected by autoradiography.

Mobility Shift Assays—Gel shift and supershift assays were performed as described previously (35–37). Briefly, nuclear extracts from untreated or RA-treated cells were incubated with or without double-stranded oligodeoxynucleotides corresponding to a DBS RARE sequence (AGGTAGGGTTTCAAGAAAGTTCACCTC) in the presence or absence of unlabeled oligonucleotide. Supershift assays using antibodies against PKCδ or RARα were performed as described previously (35–37).

Luciferase Reporter Assays—MCF-7 cells were transfected with a β-galactosidase expression vector and a RARE-luciferase plasmid (38) using the Superfect transfection reagent (Qiagen Inc.) following the manufacturer’s recommended procedure. Forty-eight hours after transfection, triplicate cultures were either left untreated or treated with RA for 16 h in the presence or absence of pharmacological inhibitors of the different PKC isoforms. The cells were preincubated with Go 6976 (2.5 nM), LY 379196 (50 nM), rottlerin (5 μM), and PKCδ pseudosubstrate (50 μM), which are specific inhibitors for PKCα (29), PKCη/θ (40), PKCδ (33, 34), and PKCζ (41), respectively, prior to the addition of RA to the cultures. The cells were then washed twice with cold phosphate-buffered saline; and after cell lysis, luciferase activities were measured following the protocol of Promega. The measured luciferase activities were normalized for β-galactosidase activity for each sample. In other experiments, cells were transfected with a SV40 luciferase construct; and 48 h after transfection, triplicate cultures were either left untreated or treated with interferon (IFN)-α (5000 units/ml), RA, rottlerin, or combinations of the these agents. In the experiments in which the effects of overexpression of wild-type or constitutively active PKCδ on RARE-dependent gene transcription were evaluated, the cells were transfected with the pcDNA3-PKC-WT or the pcDNA3-PKC-CAT construct, which encodes a truncated protein in which the catalytic domain (CAT) of PKC is preserved and the regulatory N-terminal domain is deleted, thereby generating a constitutively active catalytic domain (provided by Dr. J.-W. Soh, Columbia University College of Physicians and Surgeons, New York, NY) (42).

Chromatin Immunoprecipitation (ChIP) Assays—ChIP assays were performed essentially as described previously (44, 45). ChIP DNA was input as a control and the template for PCR using RB DNA RARE forward (5′-CAG AGA AAC AGC CAG-3′) and reverse (5′-CAT GGG CAG CTC CAG AAG-3′) primers.

Flow Cytometric Analysis—Flow cytometric studies were performed using a FACScan (Becton Dickinson, San Jose, CA) as in our previous study (24). Briefly, NB-4 cells were treated with Me2SO or RA in the presence or absence of 1 μM rottlerin for 5 days, and cell differentiation was determined by staining with anti-CD11b monoclonal antibody. The anti-CD11b monoclonal antibody and a matched isotype control were purchased from Coulter Immunotech.

RESULTS

We first determined whether treatment of cells with RA induces activation of PKCδ in the NB-4 acute promyelocytic leukemia cell line, which expresses the t(15;17) translocation. NB-4 cells were incubated in the presence or absence of RA for different times and subsequently lysed in phosphorylation lysis buffer. After cell lysis, total lysates were resolved by SDS-PAGE and immunoblotted with an antibody against PKCδ phosphorylated at Thr505. As shown in Fig. 1, RA treatment of NB-4 cells induced strong phosphorylation of PKCδ, which was time-dependent, with the intensity of the signal being strong at 12 h of RA treatment and gradually declining to base-line levels at 48–72 h (Fig. 1A). Stripping and reprobing the same blot demonstrated that equal amounts of PKCδ protein were detectable prior to and after RA treatment, indicating that RA treatment does not affect the levels of PKCδ protein expression (Fig. 1B). Similarly, phosphorylation of the PKCδ protein was induc-
RA induces phosphorylation and activation of PKCδ in acute promyelocytic leukemia cells. A, NB-4 cells were treated with RA (ATRA) for the indicated times. Equal amounts of total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against PKCδ phosphorylated at Thr505. B, the blot shown in A was stripped and reprobed with an antibody against PKCδ. C, isolated peripheral blood mononuclear cells, from a patient with acute promyelocytic leukemia, were incubated in the presence or absence of RA for the indicated times. The cells were lysed and equal amounts of total cell lysates (100 μg/ml) were analyzed by SDS-PAGE and immunoblotted with an antibody against PKCδ phosphorylated at Thr505. D, NB-4 cells were incubated for 24 h in the presence or absence of 1 μM RA as indicated. Cell lysates were immunoprecipitated (IP) with anti-PKCδ antibody, and immunoprecipitates were subjected to an in vitro kinase assay using histone H1 as an exogenous substrate. Phosphorylated proteins were detected by autoradiography.

RA Activates Protein Kinase Cδ

It is well established that retinoids induce their biological effects by regulating gene transcription for proteins that mediate cell differentiation, cell cycle arrest, and/or apoptosis of target neoplastic cells (15–17). Such RA-dependent gene transcription is regulated by binding of retinoid-receptor complexes to RAREs present in the promoters of sensitive genes (15–17). As our data demonstrated that PKCδ was activated during treatment of cells with RA, we sought to determine whether it plays a role in RA-dependent transcriptional regulation. We first examined whether H-7, a nonspecific pan-PKC inhibitor, inhibits RA-dependent gene transcription. We performed experiments in which MCF-7 cells were transfected with a plasmid containing an RARE-luciferase construct and treated with RA in the presence or absence of H-7. As shown in Fig. 3A, H-7 significantly abrogated RA-dependent RARE-mediated luciferase activity, suggesting that PKC activity is required for RA-dependent gene transcription. We subsequently determined whether rottlerin, a specific inhibitor of PKCδ (25, 33, 34, 49–51), exhibits negative regulatory effects on RA-inducible transcriptional activation. MCF-7 cells were transfected with the RARE-luciferase construct and treated with RA in the absence or presence of rottlerin to inhibit PKCδ. The RA-dependent increase in RARE-dependent gene transcription was blocked when cells were pretreated with rottlerin (Fig. 3B). On the other hand, the Go 6976 inhibitor, which selectively inhibits PKCα (39), and the LY 379196 inhibitor, which selectively inhibits PKCδ (40), had no effects on RA-dependent luciferase activity (Fig. 3B). Similarly, a PKCδ pseudosubstrate (41) had no significant effects on transcriptional regulation via RAREs (Fig. 3B), further establishing the specificity of the process.

Previous studies have established that Stat1 is up-regulated in an RA-dependent manner and that such up-regulation of Stat1 appears to be responsible for the induction of the synergistic effects that RA and interferons exhibit in malignant cells (52–55). As pharmacological inhibition of PKCδ blocked RARE-
independent experiments for each panel are shown. B. MCF-7 cells were transfected with an RARE-luciferase construct. Forty-eight hours after transfection, the cells were preincubated for 60 min in the presence or absence of RA (1 μM), and luciferase activity was measured. Data are expressed as fold increase in response to RA treatment over control untreated samples for each condition. The means ± S.E. of three independent experiments for each panel are shown.

As our data indicated a role for PKCδ in RA-mediated gene transcription and modulation of Stat1 protein expression, we sought to determine whether such inhibition also blocks up-regulation of Stat1 protein expression by RA. NB-4 cells were incubated with RA for 24 or 48 h; the cells were lysed; and total cell lysates were analyzed by SDS-PAGE and immunoblotted with an antibody against Stat1 phosphorylated at Ser727 (Fig. 4A) or against Stat1 (Fig. 4B). Consistent with previous reports (50–53), significantly higher levels of Stat1 were detectable in RA-treated samples (Fig. 4B). Also, there was an increase in the level of Stat1 phosphorylated at Ser727 (Fig. 4A) (24), likely reflecting the increase in the levels of Stat1 protein induced by RA. Treatment of cells with rottlerin decreased the levels of RA-dependent, serine-phosphorylated Stat1 (Fig. 4A) as well as total Stat1 protein (Fig. 4B). Thus, based on these findings, it is likely that PKCδ is required for the induction of RA-dependent expression of Stat1, suggesting that it plays a role in the induction of the synergistic effects of RA and interferons.

To definitively establish the role of PKCδ in RARE-dependent gene transcription, we determined whether overexpression of wild-type or constitutively active PKCδ enhances RA-dependent transcriptional regulation. MCF-7 cells were transfected with constructs for either wild-type PKCδ (pcDNA3-PKCδ-WT) (Fig. 5A) or constitutively active PKCδ (pcDNA3-PKCδ-CAT) (Fig. 5B) and the DR5 RARE-luciferase plasmid. The cells were subsequently incubated in the presence or absence of RA, and luciferase assays were performed. Overexpression of wild-type PKCδ resulted in substantial enhancement of RA-dependent gene transcription (Fig. 5A). Such an enhancement was abrogated when cells were treated with rottlerin, demonstrating the specificity of the process (Fig. 5A). On the other hand, overexpression of constitutively active PKCδ increased luciferase activity at the base line (prior to RA treatment) (Fig. 5B) and resulted in further enhancement of RA-dependent RARE-mediated gene transcription (Fig. 5B).

As our data indicated a role for PKCδ in RA-mediated gene transcription and modulation of Stat1 protein expression, we sought to determine whether its function is essential for the induction of the synergistic effects of RA and IFN-α. We have previously shown that PKCδ is activated by the type I IFN receptor and that such activation is required for type I IFN-dependent gene transcription via INF-stimulated response or GAS elements (33). As RA up-regulates Stat1 expression in a PKCδ-dependent manner, we examined whether pretreatment of cells with RA enhances IFN-α-inducible gene transcription via GAS elements and, if so, whether PKCδ activity is required for such effects. MCF-7 cells were transiently transfected with the 8XGAS-luciferase construct and subsequently treated with IFN-α or a combination of IFN-α and RA. As expected, treatment of cells with IFN-α resulted in induction of GAS-driven
luciferase activity (Fig. 6). Combined treatment of the cells with RA and IFN-α resulted in substantially higher levels of luciferase activity, whereas concomitant treatment of cells with rottlerin abrogated the IFN-α and RA synergistic effects (Fig. 6), strongly suggesting that PKC activity is required for the generation of such responses.

To further understand the mechanisms by which PKC regulates RA-dependent gene transcription, we examined whether, during RA stimulation, PKC associates with and forms complexes with other proteins that bind to RAREs. We performed gel shift assays using a double-stranded DR5 RARE oligonucleotide. As expected, treatment of NB-4 cells with RA resulted in the induction of several complexes that bound RAREs (Fig. 7).
oligonucleotide (Fig. 7 A), demonstrating the specificity of the binding. Some of the bands detected in the gel shift assay were supershifted by anti-PKCα/H9254 antibody, but not by control nonimmune rabbit IgG, indicating that the PKCα/H9254 protein participates in the formation of RARE-binding regulatory complexes (Fig. 7 A). As expected, the RA-dependent DNA-binding complexes were also supershifted by anti-RARα antibody (Fig. 7 B). Consistent with these findings, in studies using nuclear extracts from RA-treated NB-4 cells, we found that the PML-RARα fusion protein was co-immunoprecipitated by anti-PKCα antibody in an RA-dependent manner (Fig. 8, A and B). Most importantly, when ChIP assays were performed, we found that PKCα was present in a complex that bound to RAREs in an RA-dependent manner in NB-4 cells (Fig. 9). These findings provide very strong evidence that PKCα associates with RARs and likely modulates RA-dependent gene transcription via direct interaction with the RAR-RARα complex.

In further studies, we sought to examine the biological relevance of RA-dependent activation of PKCα in cells of acute promyelocytic leukemia origin. We determined the effects of inhibition of the PKCα pathway on the induction of RA-dependent cell differentiation of NB-4 cells using an approach that we employed in previous studies (24). Cells were treated with RA in the presence or absence of rottlerin, and the induction of differentiation was determined by staining the cells with anti-CD11b antibody. Concomitant treatment with rottlerin partially reversed the RA-dependent CD11b expression (Fig. 10), indicating that PKCα activity is essential for the induction of differentiation of NB-4 blast cells to granulocytes.

In parallel studies, we examined whether pharmacological inhibition of PKCα reverses the induction of the suppressive effects of RA on cell proliferation. NB-4 cells were incubated with RA in the presence or absence of rottlerin or pharmacologically inhibited PKCα with rottlerin or other PKC isoforms (Fig. 11). The results showed that PKCα inhibition reverses the growth inhibitory effects of RA on NB-4 cells, while other PKC isoforms were not inhibited. Cell proliferation was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.

In summary, the results presented here provide strong evidence that PKCα associates with RARs and likely modulates RA-dependent gene transcription via direct interaction with the RAR-RARα complex. The inhibition of PKCα activity reverses the growth inhibitory effects of RA on NB-4 cells, while other PKC isoforms are not inhibited. These findings have important implications for the development of new therapeutic strategies for treating acute promyelocytic leukemia.
logical PKC inhibitors that selectively block activation of other isoforms. Consistent with previous reports (24), RA inhibited the growth of NB-4 cells in a dose-dependent manner. Such an inhibition was reversed by concomitant treatment of cells with rottlerin (Fig. 11). On the other hand, Go 6976 and LY 37916 had no significant effects, indicating that PKCa and PKCδ do not play a role in the generation of the growth inhibitory effects of RA in NB-4 cells (Fig. 11).

DISCUSSION

The PKC family of serine/threonine kinases includes several members that play important roles in signaling for various cytokine receptors in mammalian cells (25, 51, 55–58). The classification of distinct members of the PKC family in different isoform groups relies on the requirements that the different isoforms exhibit for activation of their kinase domains. One group includes PKC isoforms that require increases in intracellular calcium for their activation. The members of this group, which are also activated by the traditional PKC activators, the phorbol esters, are defined as the conventional PKC isoforms. The three known conventional PKC isoforms are PKCa, PKCb, and PKCd (25, 51, 55–58). The second group of PKC isoymes is the group of novel PKC isoforms, which do not require Ca2+ for their activation, but are activated by phorbol esters (25, 51, 55–58). PKCδ, PKCe, PKCh, PKCd, and PKCy are included in this group. Finally, a third group of atypical PKC isoforms exists, whose members are Ca2+-independent and are insensitive to phorbol esters. PKCγ and PKCd are the two known atypical PKC isoforms (25, 51, 55–58).

The different isoforms of the PKC family participate in signaling cascades for various cytokine and growth factor receptors. Extensive studies have shown that these kinases play critical roles in the regulation of several important cellular responses such as differentiation, cell growth, and apoptosis (25, 51, 55–58). It is of interest that different PKC isoforms mediate different responses and, in some cases, appear to exhibit opposing effects on cell proliferation and apoptosis. For instance, PKCe exhibits oncogenic properties and promotes cell proliferation (59), whereas PKCδ mediates antiproliferative and pro-apoptotic signals (28, 59–65). Similarly, PKCd and PKCδ exhibit antagonistic effects on the transformation of cells by the epidermal growth factor receptor, with PKCd promoting epidermal growth factor-transforming activity and PKCδ inhibiting such a transformation and functioning as a tumor suppressor gene (64).

Our finding that PKCδ participates in the generation of RA responses and regulates induction of cell differentiation and antiproliferative effects is consistent with the previously described capacity of this PKC isoform to mediate growth-suppressive signals. It is of particular interest that this kinase is also activated by interferons and regulates IFN-dependent gene transcription via modulation of serine phosphorylation of Stat1 (33). Interferons are growth inhibitory cytokines that exhibit synergistic effects with retinoids in the generation of cell differentiation and growth suppression (66–70). It is noteworthy that RA not only augments the transcription of interferon-responsive genes, but also causes increased synthesis and secretion of IFN-α itself (54), raising the possibility of an autocrine loop mediating Stat1 activation. Our data indicate that, in addition to its involvement in the induction of RA-dependent responses, PKCδ is required for the generation of the synergistic effects of IFN-α and RA on gene transcription. Such regulatory effects on transcription via GAS elements are likely mediated by the RA-inducible, PKCδ-dependent up-regulation of Stat1 protein expression. Such effects, beyond mediating IFN-α and RA synergy, may be important for retinoic acid sensitivity, as a recent study demonstrated that, in certain cases, retinoic acid resistance is associated with lack of IFN-α synthesis and Stat1 induction (69).

Our data also establish that PKCδ is present in RA-RAR nuclear complexes that bind to RAREs. This is demonstrated by gel shift and supershift assays, co-immunoprecipitation experiments, and ChIP assays. Previous studies had implicated a PKC isoform in retinoic acid-dependent gene transcription, as evidenced by the fact that depletion of cellular PKC by prolonged treatment with 12-O-tetradecanoylphorbol-13-acetate leads to loss of ligand-dependent transcription (72). Such an effect could be directly linked to loss of DNA-binding activity of complexes containing RARα, but the identity of the PKC isoform involved was unknown at the time (72). Other studies have demonstrated that PKCa- or PKCy-dependent phosphorylation of RARα at Ser157 correlates with decreased ability of human RARα to heterodimerize with human RXRα, resulting in decreased transcriptional activity (73). As other studies have established that different PKC isoforms have opposing effects in the induction of certain responses, it is possible that PKCδ acts as a positive modulator of RARE-dependent gene transcription and opposes the effects of PKCa and/or PKCy. A similar phenomenon appears to occur in the regulation of the RXRs in T-lymphocytes, in which case PKCe synergizes with calcineurin to induce RXR-dependent activation, whereas such activation is antagonized by the PKCδ isoform (74). Independent of the precise mechanisms involved, our findings provide strong evidence for a novel function of PKCδ in the induction of RA responses. Future studies should examine whether induction of PKCδ activity also occurs in response to other retinoids and whether other PKC isoforms antagonize the effects of PKCδ on RA-dependent transcriptional regulation.

At this time, the precise upstream regulatory events that ultimately result in PKCδ activation are not known. The phosphorylation/activation of PKCδ by RA may reflect engagement of an inside-out signaling loop following the formation of RA-RAR complexes or could be regulated by other early biochemical cellular events induced by RA. There is accumulating evidence that serine/threonine kinases regulate activation of RARs via modulation of their phosphorylation status and, recently, the phosphatidylinositol 3′-kinase pathway was shown to exhibit effects on the phosphorylation, degradation, and transcriptional activity of RARα (75). Interestingly, retinoic acid-dependent neuronal tissue differentiation (76), as well as induction of expression and activation of tissue transglutaminase, is phosphatidylinositol 3′-kinase-dependent (77). Studies in other systems have also shown that PKCδ is activated downstream of phosphatidylinositol 3′-kinase via the kinase PDK1 (71, 78). It is therefore possible that the RA-dependent pathway, which ultimately facilitates RARE-dependent transcription, involves sequential activation of a phosphatidylinositol 3′-kinase/PDK1/PKCδ cascade, but this hypothesis remains to be determined in future studies.

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RA Activates Protein Kinase Cα