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Regulation of Retinoid X Receptor Responsive Element-Dependent Transcription in T Lymphocytes by Ser/Thr Phosphatases: Functional Divergence of Protein Kinase C (PKC)θ and PKCα in Mediating Calcineurin-Induced Transactivation[1]

Mohammad Ishaq,[2] Ming Fan,[3] Kip Wigmore, Arunasri Gaddam, and Ven Natarajan

T lymphocyte activation signals regulate the expression and transactivation function of retinoid X receptor (RXR) α through an interplay of complex signaling cascades that are not yet fully understood. We show that cellular Ser/Thr protein phosphatases (PPs) play an important role in mediating these processes. Inhibitors specific for PP1 and PP2A decreased basal expression of RXRα RNA and protein in T lymphocyte leukemia Jurkat cells and prevented activation-induced RXRα accumulation in these cells. In addition, these inhibitors attenuated the RXR responsive element (RXRE)-dependent transcriptional activation in transient transfection assays. Inhibitors of calcineurin (CN), by contrast, did not have any effect on the basal RXRα expression and even augmented activation-induced RXRα expression. Expression of a dominant-active (DA) mutant of CN together with a DA mutant of protein kinase C (PKC)θ, a novel PKC isoform, significantly increased RXRE-dependent transcription. Expression of catalytically inactive PKCθ or a dominant-negative mutant of PKCθ failed to synergize with CN and did not increase RXRE-dependent transcription. Expression of a DA mutant of PKCα or treatment with PMA was found to attenuate PKCθ and CN synergism. We conclude that PP1, PP2A, and CN regulate levels and transcriptional activation function of RXRα in T cells. In addition, CN synergizes with PKCθ to induce RXRE-dependent activation, a cooperative function that is antagonized by the activation of the conventional PKCα isoform. Thus, PKCθ and PKCα may function as positive and negative modulators, respectively, of CN-regulated RXRE-dependent transcription during T cell activation. ***The Journal of Immunology*****, 2002, 169: 732–738.**

Activation of T cells through interaction of cognate Ag with the TCR/CD3 complex, cross-linking with anti-CD3 Abs, or treatment with phorbol esters results in a cascade of signaling events. These events lead to IL-2 production and either T cell proliferation or inhibition of proliferation and activation-induced apoptosis (AICD),[3] depending on the requirements of the specific signaling outcome (1–6). Among the major biochemical pathways that are activated during T cell activation are protein kinase C (PKC), a family of Ser/Thr protein kinases that play a crucial role in IL-2 production and control of cellular growth (7, 8). Recent studies have identified a Ca^{2+} independent PKCθ isoenzyme as a novel PKC isoform that selectively associates with T cell synapse, a supramolecular activation complex that consists of TCR, CD28, LFA-1, other signaling molecules and associates with specialized domains called membrane rafts (9–11). PKCθ has been shown to synergize with calcineurin (CN) in activating a number of regulatory elements in the IL-2 promoter and induce IL-2 production (12–15). In addition, the two enzymes have been shown to cooperate and induce Fas ligand expression during AICD (16).

Retinoid X receptors (RXRs) play a central role as nuclear transcription factors by homodimerization or through heterodimerization with many other members of the retinoid and steroid receptor family of transcription factors (17–23). The importance of RXRs in T lymphocyte signal transduction is beginning to be understood. We have recently shown that RXRα levels are differentially regulated during activation signals that lead to T cell proliferation and signals that result in the inhibition of cellular proliferation and AICD (24, 25). These studies also uncovered the role of mitogen-activated protein kinase (MAPK) pathways in the regulation of RXRα-dependent transcriptional activation of the RXR responsive element (RXRE)-containing promoters and identified extracellular signal-regulated kinase and c-Jun N-terminal kinase (JNK) pathways as positive and negative transcriptional regulators, respectively (24). From these studies, it became apparent that T lymphocyte activation modulates the expression and transactivation

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[4] Abbreviations used in this paper: AICD, activation-induced cell death; PKC, protein kinase C; CN, calcineurin; RXR, retinoid X receptor; MAPK, mitogen-activated protein kinase; RXRE, RXR responsive element; JNK, c-Jun N-terminal kinase; PP, protein phosphatase; OA, okadaic acid; 9-CRA, 9-*cis* retinoic acid; CsA, cyclosporin A; CA, cantharidin; Tau, tautomycin; ET, endothal thioanhydride; DA, dominant active; DN, dominant negative; NA, norkadone.

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function of RXRα through an interplay of complex kinase cascades that are not yet fully understood.

Activation of MAPKs is a transient process. Inactivation of these enzymes by protein phosphatases is highly regulated and has been shown to be conducted by phosphatases like protein phosphatase (PP) 1 and PP2A (26–29). CN, also known as PP2B, is a Ca^{2+}-calmodulin dependent PP which dephosphorylates NFAT for translocation to the nucleus and IL-2 transcription (30, 31). In the present study, we show that cellular Ser/Thr PPs play an important role in the regulation of RXRα expression and RXRα-dependent transcriptional activation in T lymphocytes. We also found that CN synergizes with PKCθ in regulating RXRE-dependent transcriptional activation, a cooperative function that is antagonized by activated PKCα.

Materials and Methods

Cells and treatments

The T lymphocyte leukemia Jurkat cell line (clone E6-1) was obtained from American Type Culture Collection (Manassas, VA). Jurkat cells were maintained in RPMI 1640 medium (BioWhittaker, Frederick, MD) supplemented with 10 mM HEPES buffer, 2 mM L-glutamine, 60 μg/ml gentamicin, and 10% FBS (HyClone Laboratories, Logan, UT). PHA, PMA, okadaic acid (OA), norkadone (NA), and 9-*cis* retinoic acid (9-CRA) were obtained from Sigma-Aldrich (St. Louis, MO), and were used at 2.5 μg/ml, 50 ng/ml, 100 nM, 100 nM, and 1 μM, respectively. Cyclosporin A (CsA) (Biomol, Plymouth Meeting, PA), rottlerin, cantharidin (CA) (Calbiochem, San Diego, CA), tautomycin (Tau), and endothal thioanhydride (ET) (Alexis Biochemicals, San Diego, CA) were used at 2.5 μg/ml, 25 μM, 50 μM, 500 nM, and 5 μM, respectively.

RNase protection assay and semiquantitative RT-PCR

RNase protection assay and semiquantitative RT-PCR were performed as described previously (25).

Western blot

Protein extracts were electrophoresed in a 10% NuPAGE Bis Tris gel using NuPAGE MOPS-SDS running buffer (NOVEX, San Diego, CA), and transferred to a polyvinylidene difluoride membrane using an XCell Blot module (NOVEX). The membrane was blocked with Blocker Blotto (Pierce, Rockford, IL) and incubated overnight at 4°C with appropriate Ab. The protein was detected using the ECL Western blotting detection system from Amersham Pharmacia Biotech (Piscataway, NJ).

Nuclear run-on transcription assay

Nuclear run-on was performed with Jurkat cells by the procedure described previously (24).

Transfections

Transcriptional activity of RXRα was studied by transfection using RXRE-containing luciferase reporter plasmid, TKCRBPII-Luc as described earlier (24). TKCRBPII-M-Luc was generated by cloning a DNA fragment containing mutations in the RXR binding site, in the TK-Luc vector. The plasmid pCMX-hRXRα has been described earlier (24). Dominant-active (DA) PKCθ (A148E), dominant-negative (DN) PKCθ (K409R), DA PKCα (A25E)-expressing plasmids, and the pEF4 HisA empty vector were provided by Dr. A. Altman (La Jolla Institute of Allergy and Immunology, San Diego, CA). The plasmid encoding the hemagglutinin (HA)-tagged DA mutant of calcineurin (HA−CnΔCaM−AI) was obtained from Dr. A. Altman with permission from Dr. M. Karin (University of California, San Diego, CA). Jurkat cells (10^7) were transfected by electroporation using a Gene Pulser II (Bio-Rad, Hercules, CA) at 0.250 kV and 975 μF as described (24). After transfection, the cells were incubated in the medium for 24 h. Cells were then incubated with the indicated reagents and time periods before harvest and determination of luciferase activity using the luciferase assay system (Promega, Madison, WI). Transfection efficiency was normalized to protein concentrations in the extracts as described (24).

Results

Regulation of RXRα expression in T cells by Ser/Thr PPs

In the present study, we have dissected the role of Ser/Thr phosphatases PP1, PP2A, and CN in the expression of RXRα in T cells.

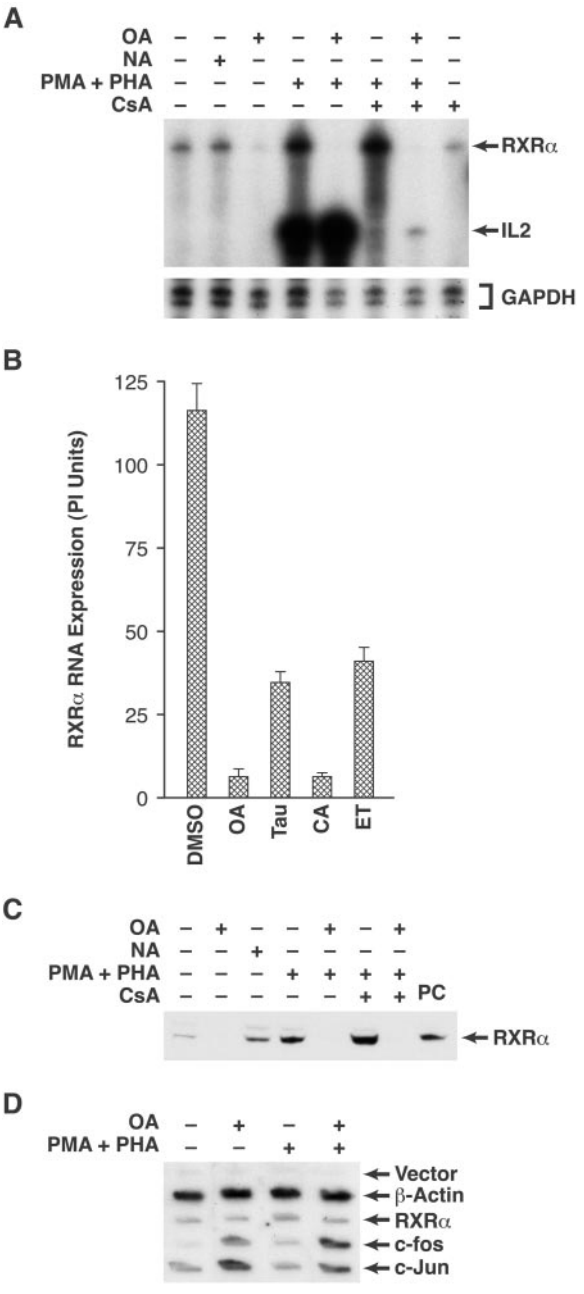

FIGURE 1. OA induces loss of basal and activation-induced RXRα expression in Jurkat cells. *A,* Jurkat cells were treated as indicated for 8 h. Total RNA was isolated and subjected to RNase protection analysis using RXRα and IL-2-specific probes as described (24). GAPDH was used as internal control for normalizing the RNA concentrations. For clarity, a shorter x-ray exposure time is shown for GAPDH at the bottom of the figure. RNA concentration was also normalized to the ribosomal RNA content in the samples (data not shown). *B,* Inhibition of RXRα expression with PP2A-specific inhibitors, CA and ET, and PP1-specific inhibitor, Tau. Total RNA was extracted from Jurkat cells treated as indicated for 8 h and RXRα RNA was quantitated using semiquantitative RT-PCR. PI, phosphoimager units. *C,* Nuclear extracts were prepared and 25 μg of protein were subjected to SDS-PAGE. After transfer to a membrane, RXRα protein was detected by RXRα (D-20) Ab using an ECL Western blotting detection system as described. PC, in vitro translated human RXRα protein used as positive control. This experiment is a representative of three independent experiments. *D,* Nuclear run-on was performed with Jurkat cells as described (24). Equivalent amounts of radioactive RNA were hybridized to nylon membranes on which 10 μg of linearized and denatured indicated plasmids were slot-blotted. The membranes were washed and the transcriptional activity was quantitated using a bio-imaging analyzer (Bas 1000; Fuji, Stamford, CT).

When Jurkat cells were treated with 100 nM OA, a PP1 and PP2A specific inhibitor, there was a marked loss of the basal RXRα mRNA levels within 8 h of treatment (Fig. 1*A*). Treatment with NA, an inactive analog of OA, did not have any effect on the RXRα expression. Inhibition of RXRα expression was also observed with PP2A-specific inhibitors CA and ET, as well as PP1-specific inhibitor Tau (Fig. 1*B*). These results indicate that active PP1 and PP2A are necessary to maintain the basal RXRα levels in T cells. We next studied the effect of OA on the activation-induced up-regulation of RXRα in these cells. Consistent with our previous findings (24), activation of Jurkat cells with PMA + PHA resulted in induction of RXRα expression. However, when the treatment was conducted in the presence of OA, there was nearly complete inhibition of RXRα mRNA (Fig. 1*A*) up-regulation. OA did not show any inhibitory effect on the activation-induced expression of IL-2 mRNA, but instead enhanced its expression (Fig. 1*A*). We have shown previously that treatment of Jurkat cells with CsA, a specific inhibitor of CN, does not change the basal levels of RXRα expression, but enhances activation-induced RXRα expression (24). We next studied the effect of CsA on the OA-induced loss of RXRα expression during T cell activation by treating the cells with PMA + PHA + CsA for 8 h in the presence or absence of OA. Fig. 1*A* shows that addition of CsA did not prevent the OA-induced inhibition of RXRα up-regulation by PMA + PHA.

Western blot analysis (Fig. 1*C*) with nuclear extracts, made from Jurkat cells after various treatments, showed that changes in the RXRα mRNA levels reflected in the corresponding changes in the levels of RXRα protein.

To define the transcriptional or posttranscriptional mechanisms involved in the loss of RXRα mRNA with OA, we measured the transcription of RXRα mRNA 8 h after treatment with OA, using the nuclear run-on transcription assay (Fig. 1*D*). The levels of RXRα mRNA synthesis did not show any significant change after the treatment with OA. However, under similar conditions, the mRNAs of two AP-1 binding proteins, c-*jun* and c-*fos*, and RXRα mRNA from PMA + PHA-treated cells showed significant increase in transcription. These results indicate that the loss of RXRα expression during OA treatment may involve posttranscriptional mechanisms.

Together, these data indicate that PP1 and PP2A inhibitors not only decrease basal RXRα expression, but also inhibit RXRα levels that are induced during activation of T lymphocytes. Unlike PP1 and PP2A inhibitors, CN inhibitors do not influence basal RXRα levels and show an additive effect on the levels of RXRα induced during activation. When used together, OA not only inhibits activation-induced RXRα induction but also neutralizes CsA action.

Inhibition of PP1 and PP2A attenuates RXRE-dependent transcription independent of RXRα levels

To investigate whether PP1 and PP2A inhibition-induced loss of RXRα expression reflected in the corresponding loss of RXRE-dependent transcription, transcriptional activity was studied in Jurkat cells by transient transfection assay using the TKCRBP-II-Luc reporter (24). Fig. 2*A* shows the dose-dependent loss of endogenous RXRE mediated transcription by OA. At 10 nM OA, the concentration which inhibits only PP2A, the inhibition of RXRE-dependent transcription was significant (33%). At 100 nM, the concentration at which both PP2A and PP1 are inhibited, transcription was inhibited by 90%. PP1-specific inhibitor Tau and PP2A specific inhibitors CA and ET also inhibited RXRE-dependent

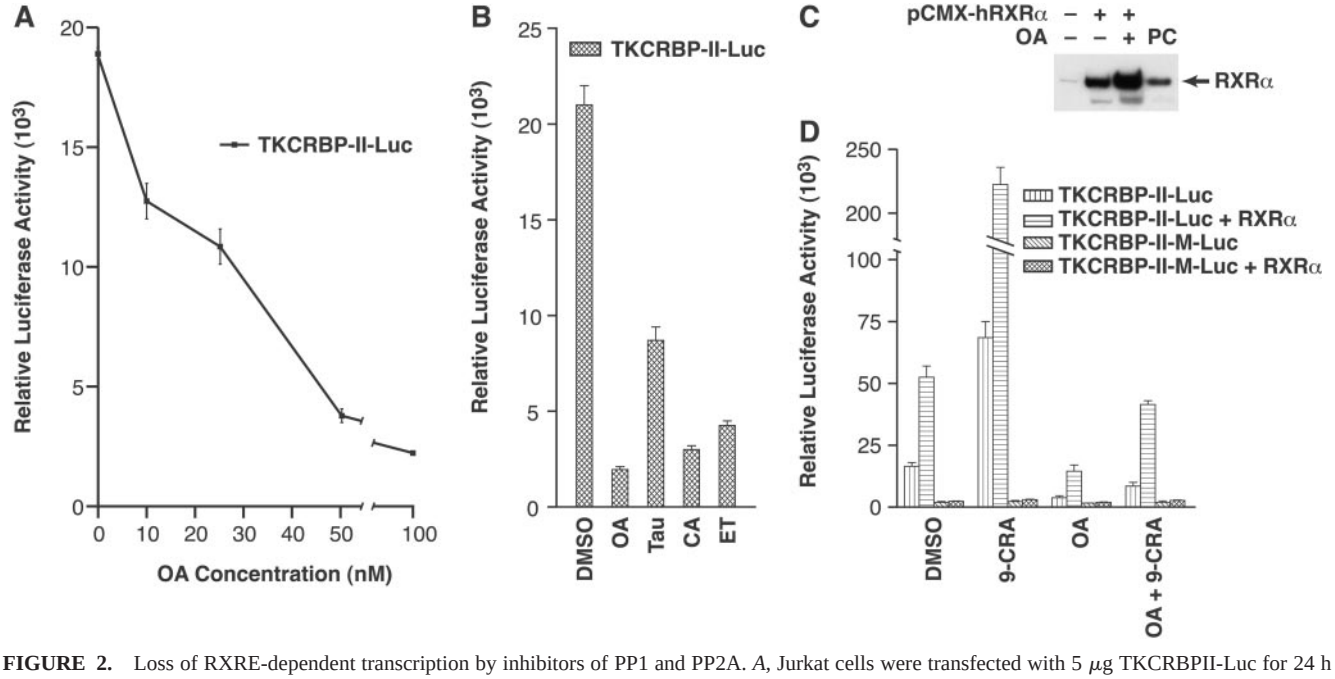

FIGURE 2. Loss of RXRE-dependent transcription by inhibitors of PP1 and PP2A. *A*, Jurkat cells were transfected with 5 μg TKCRBPII-Luc for 24 h and then treated for 8 h with indicated concentrations of OA. *B*, Jurkat cells were transfected with 5 μg TKCRBPII-Luc for 24 h and then treated for 8 h with PP2A-specific inhibitors, CA and ET, and PP1 specific inhibitor, Tau. Cells were harvested and luciferase activity was measured as described in *Materials and Methods*. The values represent the mean of three independent experiments with SE calculated for each value. *C*, Twenty-five micrograms of nuclear extract, prepared from TKCRBPII-Luc and TKCRBPII-Luc + pCMX-hRXRα-cotransfected cells after 8 h of OA treatment was subject to Western blot analysis using RXRα-specific Abs as described in *Materials and Methods*. PC, in vitro-translated human RXRα protein used as positive control. *D*, Jurkat cells were transfected with 5 μg TKCRBP-II-Luc or TKCRBPII-M-Luc either in the presence or absence of 2.5 μg of pCMX-hRXRα plasmid. After 24 h, cells were treated for 8 h with OA either in the presence or absence of 9-CRA. Cells were harvested and luciferase activity was measured as described in *Materials and Methods*. The values represent the mean of three independent experiments with SE calculated for each value.

transcription (Fig. 2*B*). These data are consistent with the dependence of RXRE-mediated transcription on active cellular PP1 and PP2A in Jurkat cells.

We next investigated the effect of OA on the transcription driven by exogenously transfected RXRα and also studied its effect on ligand-independent and -dependent RXRE-mediated transcription. Jurkat cells were transfected with TKCRBP-II-Luc reporter and pCMX-hRXRα (a CMV promoter-driven human RXRα-expressing plasmid) constructs for 24 h and then treated with 100 nM OA for 8 h in the presence or absence of 9-CRA. Western blot analysis (Fig. 2*C*) confirmed the expression of RXRα protein in the transfected cells. OA treatment was found to enhance CMV-driven RXRα expression in the transfected cells. Luciferase reporter assay (Fig. 2*D*) demonstrated a considerable increase in the RXRE-dependent transcription in pCMX-hRXRα-transfected cells. Remarkably, OA treatment of pCMX-hRXRα-transfected cells was found to markedly decrease this transcription. The loss of RXRE-dependent transcription was independent of the presence of 9-CRA, indicating that both ligand-independent and -dependent transcription were affected by treatment with OA. These results show that inhibition of PP1 and PP2A led to the modification of RXRα function and inhibition of transcription even in the presence of high levels of RXRα.

PKCθ synergizes with CN to induce RXRE-dependent transcription

We have previously shown that CN may have a role in the regulation of RXRα levels during T cell activation (24). However, the role of this phosphatase in RXRE-mediated transcription is unknown. Recent studies have shown that activated CN synergizes with a novel member of PKC isoenzyme PKCθ and induces IL-2 gene transcription (10). To investigate the contribution of CN in the regulation of RXRE-dependent transcription, Jurkat cells were cotransfected with TKCRBP-II-Luc and DA-CN, either in the presence of wild-type PKCθ, DA-PKCθ, DN-PKCθ, or the empty vector. Fig. 3 shows that expression of CN alone did not influence the RXRE-mediated transcriptional activity. However, if the transfection was performed in the presence of DA-PKCθ and DA-CN, there was a significant up-regulation of transcriptional activity. This synergistic cooperation was observed with both ligand-dependent and -independent transactivation of both endogenous and exogenously expressed RXRα (Fig. 3). Transfection with DA-PKCθ alone, in the absence of DA-CN, only marginally increased the RXRE-dependent transcription. Wild-type PKCθ, DN-PKCθ, or empty vector failed to synergize with DA-CN and did not induce transcription. In subsequent experiments, expression of DN-PKCθ was found to interfere with the DA-CN and DA-PKCθ cooperation and resulted in the inhibition of RXRE-dependent transcription (Fig. 4).

CsA is a known inhibitor of CN (31). Rottlerin, a Ca^{2+}-independent PKC isoform inhibitor, which was earlier known to inhibit PKCδ has recently been used to inhibit PKCθ in T cells (16, 32–34). We used these inhibitors to confirm the specificity of CN and PKCθ synergism-induced RXRE-dependent transcription by treating Jurkat cells with CsA or rottlerin, 24 h after transfection with TKCRBPII-Luc, DA-CN, and DA-PKCθ. The results (Fig. 4*A*) show that both these compounds inhibited the induction of RXRE-dependent transcription indicating that activated CN and PKCθ indeed participate and mediate RXRE-dependent transcription. CsA and rottlerin did not have any significant effect on the expression levels of DA-PKCθ and DA-CN proteins as shown by Western blot analysis of the transfected cell extracts (Fig. 4*B*).

Taken together, these data indicate that CN plays a pivotal role in modulating RXRE-dependent transcription in T cells by cooperating with PKCθ and this synergism leads to positive regulation of RXRE-dependent transcription. Our data, however, do not rule out the role of other PKC isoforms in CN-induced RXRE-dependent transcription.

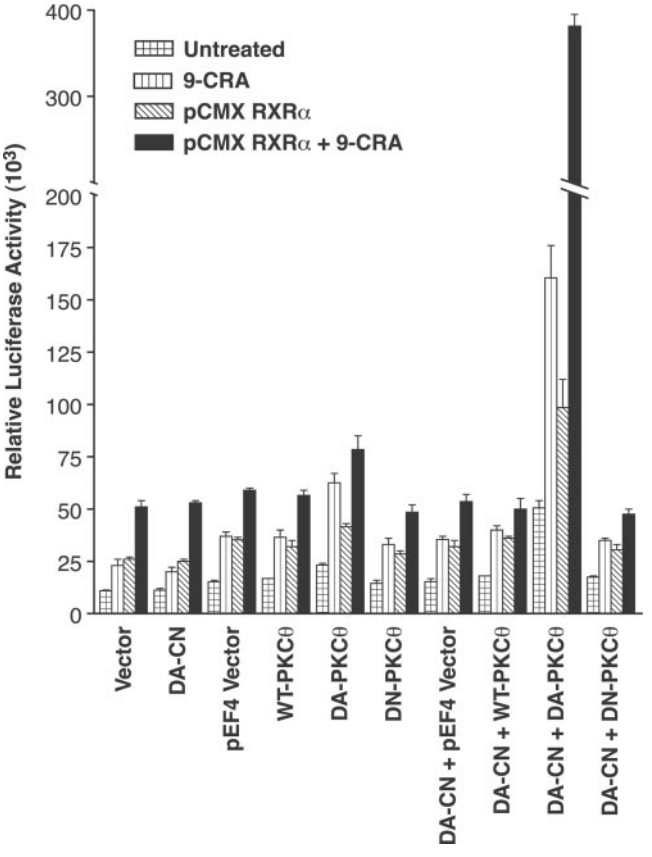

FIGURE 3. Induction of RXRE-dependent transcription by CN and PKCθ synergism. Jurkat cells were treated for 12 h with 9-CRA, 24 h after transfection with 5 μg TKCRBP-II-Luc or TKCRBP-II-Luc + 2.5 μg pCMX-RXRα plasmids in the presence of 5 μg of indicated plasmid constructs. Cells were harvested and luciferase activity was measured. The values represent the mean of three independent experiments with SE calculated for each value.

Inhibition of CN and PKCθ synergism by PKCα

PMA is known to synergize with ionomycin or DA-CN to induce IL-2 promoter and is also known to increase CN and PKCθ cooperation-induced NF-κB and IL-2 promoter activities (35). To study whether PMA had a similar effect on CN- and PKCθ-induced RXRE-dependent transcription, Jurkat cells were treated with PMA 24 h after transfection with TKCRBP-II-Luc and DA-CN plasmids, either in the presence of DA-PKCθ or the empty vector. Fig. 5*A* shows that PMA, even at a concentration as low as 1 ng/ml, not only inhibited basal levels of RXRE-dependent transcription, but also inhibited transcription induced by CN-PKCθ cooperation. Treatment with PMA did not have any significant effect on the expression levels of DA-PKCθ and DA-CN proteins as shown by Western blot analysis of the transfected cell extracts (Fig. 5*B*). To understand the mechanism of transcriptional inhibition, it was reasoned that PMA might antagonize RXRE-dependent transcription through activation of a conventional PKCα isoform, which may have an inhibitory effect on CN-PKCθ-induced RXRE-dependent transcription. To test this hypothesis, a DA-PKCα expressing plasmid was transfected in Jurkat cells together with TKCRBPII-Luc and DA-CN plasmids either in the presence of

DA-PKCθ or empty vector. Results show that expression of DA-PKCα significantly inhibited the CN- and PKCθ-induced RXRE-dependent transcription in a concentration-dependent manner, in addition to its inhibitory effect on basal RXRE-dependent transcription (Fig. 6*A*). This inhibition was also observed with both ligand-dependent and exogenously expressed RXRα (data not shown). Cotransfection with DA-PKCα had no significant effect on the expression levels of DA-PKCθ or DA-CN (Fig. 6*B*). These data demonstrate that activated PKCα has a negative regulatory role in modulating RXRE-dependent transcription induced by PKCθ and CN synergism. Inhibitory effect of PMA on this synergy may be a result of PKCα activation induced by this compound.

Discussion

Coordinated control of protein kinase and phosphatase cascades is an important feature of T cell response to antigenic stimulation. Recent studies have revealed that multiple complexes, which contain both kinase and phosphatase activities, are essential for the regulation and specificity of the T cell signaling pathways (27). Although the role of phosphorylation, in modulating the activities of many transcription factors during T cell stimulation, has been well recognized, little is known about the role of phosphorylation and dephosphorylation in the regulation of expression and transcriptional function of RXRα in these cells. Our earlier studies (24) have emphasized the importance of MAPK pathways in the maintenance of normal functioning of RXRE-dependent transcription during T cell activation. In the present study, we have explored the role of cellular Ser/Thr PPs PP1, PP2A, and CN in the expression and transactivation function of RXRα. We have shown that active PP1 and PP2A are essential not only in maintaining the basal levels of RXRα, but also in maintaining RXRα expression induced during T cell activation. Although the mechanism of this regulation remains unknown, transcriptional run-on studies have pointed out that posttranscriptional mechanisms may contribute to the loss of RXRα expression when Jurkat cells are treated with PP1 and PP2A inhibitor OA. The 3′-untranslated region of RXRα mRNA contains a number of AU-rich sequences (25). Such sequences are known to have a role in mRNA decay (36). Whereas the role of these sequences in the stability of RXRα mRNA remains to be studied, other factors may also contribute to the instability of RXRα mRNA during OA treatment.

PP1 and PP2A are known to be essential for cell cycle regulation, and a tight balance between kinase and phosphatase activities controls the decision between cell survival and apoptosis (26–28). We have previously shown that the levels of RXRα are regulated in T cells during cell cycle transitions, proliferation, and also under conditions that lead to the inhibition of proliferation or AICD (24, 25). Because PP1 and PP2A inhibitors attenuate both the basal as

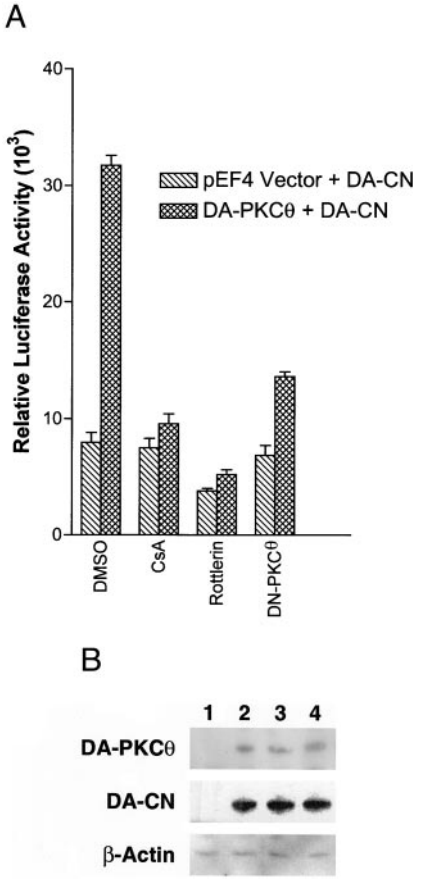

FIGURE 4. Specificity of CN and PKCθ cooperation in inducing RXRE-dependent transcription. *A,* Jurkat cells were treated for 12 h with CsA or rottlerin, 24 h after transfection with 5 μg TKCRBP-II-Luc plasmid in the presence of 5 μg of indicated plasmid constructs. Cells were harvested and luciferase activity was measured. The values represent the mean of three independent experiments with SE calculated for each value. *B,* Cell extracts from empty vector (*lane 1*), and DA-PKCθ + DA-CN (*lanes 2–4*)-transfected cells were subject to Western blot analysis as indicated using target-specific Abs. *Lanes 3* and *4* contained extracts from transfected cells treated with CsA and rottlerin, respectively.

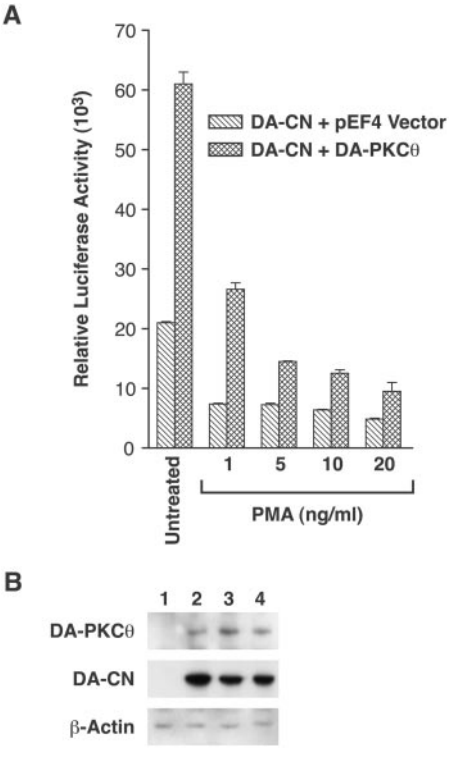

FIGURE 5. Inhibition of CN and PKCθ synergism-induced RXRE-dependent transcription by PMA. *A,* Jurkat cells were treated for 12 h with indicated concentrations of PMA, 24 h after transfection with 5 μg TKCRBP-II-Luc plasmid in the presence of 5 μg indicated plasmid constructs. Cells were harvested and luciferase activity was measured. The values represent the mean of three independent experiments with SE calculated for each value. *B,* Cell extracts from empty vector (*lane 1*) and DA-PKCθ + DA-CN (*lanes 2–4*)-transfected cells were subject to Western blot analysis as indicated using target-specific Abs. *Lanes 3* and *4* contained extracts from transfected cells treated with 1 and 20 ng/ml PMA, respectively.

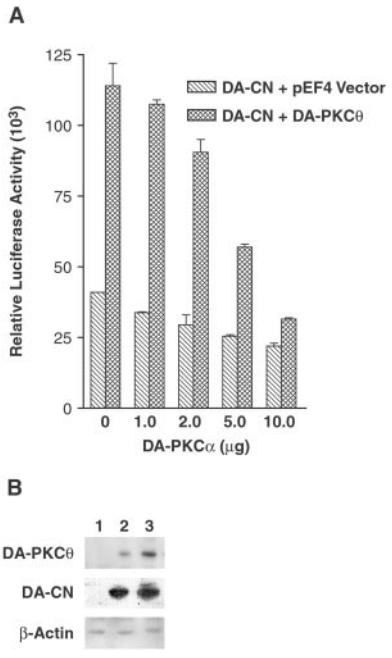

FIGURE 6. CN and PKCθ synergism-induced RXRE-dependent transcription is inhibited by activated PKCα. *A*, Jurkat cells were transfected with 5 μg TKCRBP-II-Luc plasmid in the presence of various concentrations of indicated plasmid constructs. Cells were harvested after 36 h and luciferase activity was measured. The values represent the mean of three independent experiments with SE calculated for each value. *B*, Cell extracts from empty vector (*lane 1*), DA-PKCθ + DA-CN (*lane 2*), and DA-PKCθ + DA-CN + DA-PKCα-transfected cells were subject to Western blot analysis as indicated using target-specific Abs.

well as activation-induced expression of RXRα, it is evident that PP1 and PP2A are important physiological regulators of RXRα expression during T cell signaling.

We have demonstrated that inhibition of PP1 and PP2A interfered with both ligand-independent and -dependent RXRE-mediated transactivation. This inhibition was observed with both endogenous RXRE-dependent transcription and exogenously expressed RXRα-dependent transcription. Remarkably, the decrease in RXRE-mediated transactivation by OA correlated with the loss of RXRα levels only in cells, which expressed endogenous RXRα. OA did not inhibit CMV-driven RXRα expression but was instead found to induce RXRα levels in pCMX-RXRα-transfected Jurkat cells, perhaps, through its direct induction of CMV promoter. These results suggest that loss of endogenous RXRE-dependent transcription by OA treatment may in part be a result of its inhibitory effect on the accumulation of endogenous RXRα protein, and also through its direct or indirect effect on transcriptional activity of RXRα protein. OA-induced inhibition of RXRE-dependent transactivation may involve posttranslational modification of RXRα and/or a cofactor(s), which are essential in mediating the transactivation.

PPI and PP2A inhibition by OA is known to induce the JNK pathway in Jurkat cells (37). Activation of the JNK pathway was recently shown to inhibit RXRE-dependent transcription in Jurkat (24) and COS-7 cells (38). OA-induced activation of the JNK pathway might contribute to the loss of RXRE-dependent transcription in these cells. PP1 and PP2A have been shown to inhibit PKCα activity by inhibiting autophosphorylation (39), suggesting that OA-induced inhibition of PP1 and PP2A may interfere with RXRE-dependent transcription through an increase of PKCα activation. This was supported by our data showing that activated PKCα inhibited RXRE-dependent transcription (Fig. 6, see below).

CN and PKCθ have recently been shown to act as essential players in the activation of the *IL-2* gene. A synergistic cooperation between the two has been shown to activate a number of IL-2 promoter-associated transcription factors such as NFAT, NF-κB, and CD28RE (9–15). In addition, CN and PKCθ synergy has also been reported to induce Fas ligand expression in T cells during AICD (16). In this study, we have reported that this novel signaling cross-talk between CN and PKCθ is also operating to modulate RXRα-mediated transcription in Jurkat cells. The activated CN plays an essential role as a positive transcriptional regulator of RXRE-dependent transcription in T cells through its synergy with PKCθ. This cross-talk between seemingly unrelated signaling cascades, involving CN, PKCθ, and RXRα, represents a novel mechanism T lymphocytes use to modulate RXRE-dependent transcription. Our data, however, do not rule out the role of other PKC isoforms in CN-induced RXRE-dependent transcription.

PMA is known to synergize with DA-CN and induce NFAT and IL-2 promoters in transient transfection assays, and this effect has been attributed to the induction of PKCθ by PMA treatment (35). In this study, we have shown that PMA does not synergize with DA-CN to induce RXRE-dependent transcription but instead down-regulates basal and CN-PKCθ synergy-induced transactivation. To explore the molecular mechanism that might explain this inhibitory response to PMA, we hypothesized that PKCα, a conventional PKC isoform activated by PMA in addition to the novel PKCθ isoenzyme, may act as a negative regulator of RXRE-dependent transcription. Our results have revealed that expression of a DA mutant of PKCα not only inhibited the basal levels of RXRE-dependent transcription, but also attenuated CN-PKCθ synergy-induced RXRE-mediated transcription. Together, these data are consistent with the existence, in T cells, of a regulatory mechanism in which PKCθ and CN cooperate to activate RXRE-mediated transcription, a phenomenon that is negatively regulated by PKCα. Whether PMA-induced inhibition of RXRE-dependent transcription is solely due to PKCα activation remains to be studied. This study does not rule out the possibility that other PMA-induced PKC isoforms also contribute to this inhibition.

It is apparent that CN and PKCθ signaling cross-talk, which was initially discovered to be a hallmark of IL-2-promoter-dependent transcriptional activation, may also be involved in many other signaling pathways in T cells. We have provided direct evidence supporting the involvement of CN and PKCθ cooperation in RXRE-mediated signaling. The functional divergence of PKCα and PKCθ, in regulating RXRE-mediated transcription, emphasizes the importance of multiple PKC isoforms in modulating RXRα-dependent signaling in T lymphocytes.

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