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Protein Kinase C Modulates Regulation of the *CYP1A1* Gene by the Aryl Hydrocarbon Receptor*

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Transcriptional activation of the human *CYP1A1* gene by halogenated and polycyclic aromatic hydrocarbons is mediated by the aryl hydrocarbon receptor (AhR) complex, a ligand-dependent transcription factor. A competent AhR comprises at least two components following nuclear translocation and DNA binding, the AhR and the AhR nuclear translocator (Arnt) protein, whose combined action on human *CYP1A1* gene transcription is shown to be dependent upon functional protein kinase C (PKC). In the present study, we examined the effects of phorbol 12-myristate 13-acetate, a potent PKC activator, on the ligand-induced transcriptional activation of the *CYP1A1* gene and cellular function of the AhR in human HepG2 101L cells. The 101L cells carry a stable transgene consisting of 1800 bases of 5′-flanking DNA and the promoter of the human *CYP1A1* gene linked to the firefly luciferase structural gene (Postlind, H., Vu, T. P., Tukey, R. H. & Quattrochi, L. C. (1993) *Toxicol. Appl. Pharmacol.* 118, 255–262). Pretreatment of cells with 12-myristate 13-acetate enhanced ligand-induced *CYP1A1* gene expression 2–3-fold. Inhibition of PKC activity blocked directly the transcriptional activation and the transactivation of the *CYP1A1* gene, indicating a role for PKC in the AhR-mediated transcriptional activation process. However, the DNA binding activities of the *in vitro* activated and the induced nuclear AhR as measured by electrophoretic mobility shift analysis were not affected when *CYP1A1* transcription was inhibited, indicating the actions of PKC to be a nuclear event that works in concert with or precedes AhR binding to the gene. These results illustrate that PKC is absolutely essential for the cellular and molecular events that control induction of *CYP1A1* gene transcription.

Exposure to environmental contaminants such as polycyclic aromatic hydrocarbons, which are found in places such as cigarette smoke and smog, as well as halogenated derivatives like 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)[1] and some polychlorinated biphenyls (PCBs), leads to the induction in animals of cytochromes P450 1A1 and 1A2 (1, 2). The process of transcriptional activation of these cytochromes is felt to proceed in large part through a signaling process involving ligand-dependent activation of the aryl hydrocarbon receptor (AhR). The current thinking regarding properties of the ligand and AhR-directed gene transcription is based upon both cellular and molecular studies that have focused primarily on the inducible expression of the *CYP1A1* gene. The cytosolic AhR belongs to the family of basic helix-loop-helix (bHLH) proteins and is occupied in the cytoplasm of the cell with hsp90, which is uncoupled from the receptor in the presence of ligand (3–5). The ligand-activated AhR migrates rapidly to the nucleus, where it associates with the bHLH AhR nuclear translocator (Arnt) protein and then binds to specific enhancer sequences flanking the *CYP1A1* gene, dioxin responsive elements (DREs). DNA binding of the dimeric AhR·Arnt to the DREs initiates the recruitment of transcriptional factors followed by induction of the *CYP1A1* gene.

It was originally believed that the movement of the ligand-activated AhR to the nucleus was a process dependent upon the Arnt protein (6). However, recent observations demonstrate that Arnt is a nuclear protein and most likely participates only in the nucleus to facilitate ligand-dependent AhR binding to DNA (7). The release of hsp90 from the cytosolic AhR is initiated by ligand binding (8), a process that is then followed by rapid transport of the AhR to the nucleus. Yet little is known regarding the cellular characteristics of the AhR that initiate transcriptional activation of target genes. It is known that the AhR is a phosphoprotein, and the *in vitro* treatment of cytosol or of induced nuclear extract with either acid or alkaline phosphatase abolishes the specific binding of the AhR to its responsive DNA element (9–12). Our laboratory has recently demonstrated that the acute treatment of mice with phorbol esters dramatically reduces the ligand-induced nuclear accumulation of the AhR, an event that was concordant with lowered *Cyp1a-1* and *Cyp1a-2* transcription rates (13). In addition, prolonged treatment of tissue culture cells with phorbol esters and the down-regulation of PKC activities inhibits ligand induced accumulation of *CYP1A1* mRNA (9). While it has been suggested that ligand binding does not directly influence the phosphorylation state of the AhR (12, 15), PKC activity may play a central role in additional cellular processes that coordinate the AhR·Arnt complex in facilitating gene regulation. Since the AhR is a heterodimer complex that is composed of both cytosolic and nuclear proteins, the actions of PKC could be targeting cellular events that facilitate its activity both in the cytosol as well as in the nucleus.

We have recently developed a sensitive tissue culture cell line that can be used to study the intracellular events in the AhR-mediated expression of the human *CYP1A1* gene (16). The 101L cells are derived from the human hepatoma cell line, HepG2, and carry a stably integrated human *CYP1A1* pro-

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[1] The abbreviations used are: TCDD, tetrachlorodibenzo-*p*-dioxin; 3MC, 3-methylcholanthrene; PCB, polychlorinated biphenyls; PMA, phorbol 12-myristate 13-acetate; bHLH, basic helix-loop-helix; AhR, aryl hydrocarbon receptor; Arnt, AhR nuclear translocator; hsp90, 90-kDa heat shock protein; DRE, dioxin-responsive element; PKC, protein kinase C; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; RLU, relative light units; GRE, glucocorticoid responsive element.

moter and the 5′-flanking DNA driving the firefly luciferase structural gene. In the present study, we have utilized 101L cells as a tool to investigate the effects of phorbol 12-myristate 13-acetate (PMA), a model phorbol ester and PKC activator, on the biological function of the cytosolic and nuclear AhR complex and its contribution to the induction of the *CYP1A1* gene.

MATERIALS AND METHODS

Chemicals and Biochemicals—TCDD was obtained from Chemsyn Science Laboratories (Lenexa, KS). PCBs were kindly supplied by Dr. Steven Safe (Texas A & M University). 3-Methylcholanthrene (3MC), PMA, and staurosporine were purchased from Sigma. Chelerythrine chloride, H89, and 4α-phorbol 12,13-didecanoate were from LC Laboratories (Woburn, MA). Luciferin was obtained from Analytical Luminescence Laboratory (Ann Arbor, MI). [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham Life Science. All tissue culture media supplies and Geneticin (G-418) were purchased from Life Technologies, Inc. The remaining laboratory chemicals were of the highest quality available and were purchased from Fisher Scientific and Sigma.

Cell Culture and Transient Transfections—The human 101L cells are stable cells derived from the human hepatoma cell line HepG2, into which the human *CYP1A1* promoter and 5′-flanking sequences, fused to the firefly luciferase gene, were stably integrated (16). The 101L cells were grown as monolayers at 37 °C in 95% air and 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 0.4 mg/ml G-418. HepG2 cells were grown under the same condition with no G-418 supplemented. Solutions of the chemicals that were added to the media were first dissolved in Me_2SO. After the addition to the media, the Me_2SO concentration never exceeded 0.3% (v/v). The concentrations of chelerythrine chloride (10 μM) and staurosporine (200 nM) used were derived from dose-response experiments evaluating the ability of these agents to inhibit *CYP1A1* gene transcription. Cell viability was determined by a colorimetric assay with a tetrazolium salt that measures living cells and can be performed using a scanning multiwell spectrophotometer (17). The concentrations of staurosporine and chelerythrine chloride are in agreement with previous experiments using these agents (10, 18, 19).

The pCMV/GRDBD/AhR, pCMV/GRDBD/Arnt, and p(GRE)$_2$T105Luc plasmids were kindly provided by Dr. Lorenz Poellinger (Huddinge University Hospital, Sweden). The plasmids pCMV/GRDBD/AhR and pCMV/GRDBD/Arnt consist of N-terminal zinc finger DNA-binding domains of the glucocorticoid receptor linked to C-terminal amino acids 83–805 of the AhR and C-terminal amino acids 128–774 of the Arnt, respectively (20). With a deleted bHLH domain, each construct is able to express and function independently, and the ligand-driven responses can be monitored by the GRE-driven luciferase activity from cotransfection with the p(GRE)$_2$T105Luc reporter plasmid. HepG2 cells were seeded at 1.8×10^{-6} cells/six-well plate and were transfected with 6.5 μg/well total plasmid DNA by the calcium phosphate precipatation method (21). Twenty-four hours after transfection, cells were treated with the inducer for 16 h followed by analysis of luciferase activity.

Luciferase Assay—Luciferase activity was determined by the methods described previously with minor modifications (22, 23). After washing the cells with phosphate-buffered saline, 101L cells were harvested in a lysis buffer containing 1% Triton, 25 mM Tricine, pH 7.8, 15 mM $MgSO_4$, 4 mM EDTA, and 1 mM dithiothreitol (DTT). Cell lysates were centrifuged at 14,000 rpm in a microcentrifuge for 10 min at 4 °C, and the supernatants were used for luciferase and protein assays. Luciferase assays were carried out by mixing 10 μl of the cell extracts with 300 μl of reaction mixture, which contained 15 mM potassium phosphate buffer, pH 7.8, 15 mM $MgSO_4$, 2 mM ATP, 4 mM EDTA, 25 mM Tricine, and 1 mM DTT. Reactions were started by adding 100 μl of luciferin (0.3 mg/ml) dissolved in 0.1 M potassium phosphate buffer, pH 7.8. Light output was measured for 10 s at 25 °C using a Monolight 2001 luminometer (Analytical Luminescence Laboratory), and the luciferase activity is expressed as relative light units (RLU)/μg of protein. The protein content was determined according to Bradford (24) using the Bio-Rad protein assay method (Emeryville, CA).

Preparation of Nuclear and Cytosolic Proteins—Nuclear extracts from 101L cells were isolated as described previously (25), and all the procedures were performed at 4 °C. After washing the tissue culture plates twice with HEPES buffer (10 mM, pH 7.5), the treated 101L cells were collected by scraping into MDH buffer (3 mM $MgCl_2$, 1 mM DTT, 25 mM HEPES, pH 7.5) and homogenized with a Dounce homogenizer. The homogenate was centrifuged at 1000 × *g* for 3 min, and the pellet was washed with MDHK buffer (3 mM $MgCl_2$, 1 mM DTT, 25 mM HEPES, pH 7.5, 0.1 mM KCl) three times. The pellet was then lysed in HDK buffer (25 mM HEPES, pH 7.5, 1 mM DTT, 0.4 M KCl) and centrifuged at 105,000 × *g* for 60 min, and the supernatant was designated as nuclear extract.

For *in vitro* AhR activation experiments, cytosols from PMA and staurosporine-treated 101L cells were prepared as described previously (26). Briefly, the washed cells were collected by scraping and incubated for 15 min in HED buffer (25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT). The cells were homogenized with a Dounce homogenizer, and the homogenate was then immediately diluted 1:1 with HED2G buffer (25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT, 20% glycerol). After centrifugation, the 105,000 × *g* supernatant was designated as cytosolic extract. The cytosolic receptor was activated by incubating with 20 nM TCDD for 2 h at 22 °C.

Electrophoretic Mobility Shift Assay (EMSA)—A complementary pair of synthetic DNA oligonucleotides containing the sequence 5′-GATCCGGCTCTTGTCACGCAACTCCGAGCTCA-3′ and 5′-GATCTGAGCTCGGAGTTGCGTGAGAAGAGCCG-3′ (the 27-base pair AhR binding site of DRE3, designated here as "DRE oligonucleotide" (27)) were synthesized, annealed, and labeled at their 5′ ends by using T4 polynucleotide kinase and [γ-^{32}P]ATP. DNA binding was measured using an EMSA, which was performed as described by Denison *et al*. (28). The binding reactions contained nuclear protein (10 μg) or activated cytosolic protein (40 μg), 2.4 μg of poly(dI-dC), 1 μg of salmon sperm DNA, and 1×10^6 cpm of ^{32}P-labeled double-stranded DRE in a final volume of 30 μl of binding buffer (25 mM HEPES, pH 7.5, 1.5 mM EDTA, 1 mM DTT, and 10% glycerol (v/v)). To determine the specificity of binding to DRE, a 200-fold molar excess of unlabeled DRE oligonucleotide was used. DNA-protein complexes were separated under nondenaturing conditions on a 6% polyacrylamide gel using 1 × TBE (89 mM Tris borate, 89 mM boric acid, 2 mM EDTA) as a running buffer. The gels were then dried, and protein-DNA complexes were visualized by autoradiography.

RESULTS

Effects of Various AhR Agonists on CYP1A1 Gene Induction in 101L Cells—It has previously been demonstrated that the treatment of 101L cells with AhR ligands such as TCDD, 3MC, and omeprazole leads to a rapid and dose-dependent induction of the *CYP1A1*-luciferase activity (16, 23). To examine the sensitivity of these cells to well characterized AhR ligands such as other halogenated aromatic hydrocarbons (29), a series of PCBs were examined for their ability to induce *CYP1A1* gene transcription. The levels of induction were compared with those of TCDD and 3MC. Consistent with previous studies, TCDD is the most potent inducer of the *CYP1A1* gene transcription as shown in Fig. 1. The PCBs induce transcription in a dose-dependent fashion, and the order of potency from most active to weakest was 3,3′,4,4′,5-pentachlorobiphenyl > 3,3′,4,4′,5,5′-hexachlorobiphenyl > 2,3,4,4′,5-pentachlorobiphenyl > 3,3′,4,4′-tetrachlorobiphenyl > 2,3,3′,4,4′,5-hexachlorobiphenyl. At a concentration of 10 μM, 2,3,3′,4,4′,5-hexachlorobiphenyl was the weakest inducer, displaying approximately a 40-fold induction of luciferase activity, while 3,3′4,4′,5-pentachlorobiphenyl generated a 130-fold increase over Me_2SO-treated cells. These results indicate that 101L cells are a sensitive biological tool to examine the actions of AhR ligands on the stimulation of gene transcription. In turn, events that might impact on AhR function can now be accurately correlated with the biological role of the AhR.

Effects of Phorbol Esters and PKC on CYP1A1 Transcriptional Activity—Using a number of the different AhR ligands, experiments were developed to examine the actions of phorbol esters on ligand-dependent gene transcription. The first series of experiments were designed to examine the effect of phorbol esters on the halogenated aromatic hydrocarbon induction of *CYP1A1* gene transcription. The treatment of 101L cells for 18 h with TCDD and the other PCBs generated differing degrees of *CYP1A1* gene activation, as measured by reporter gene activity (Fig. 2). However, when the cells were pretreated with PMA for 3 h followed by an 18-h exposure to the TCDD or various PCBs, PMA caused a synergistic increase in *CYP1A1* gene transcription in all of the structurally related AhR ligands

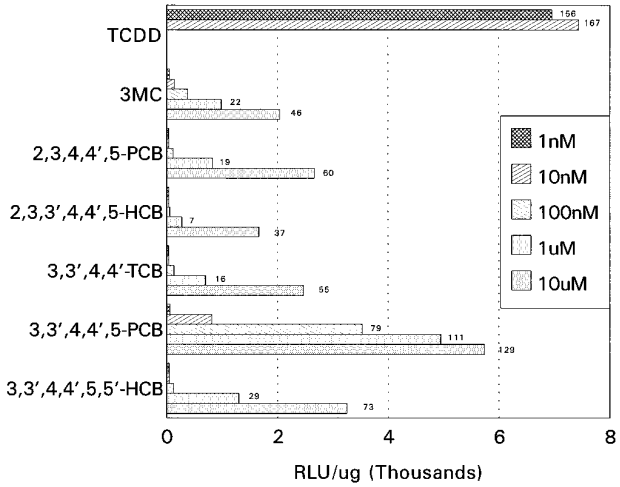

FIG. 1. ***CYP1A1* gene transcription induced by AhR ligands.** Several structurally related PCBs were evaluated for their ability to induce *CYP1A1* gene transcription in 101L cells, and the levels were compared with the induction by 3MC and TCDD. Each reagent was added to a growing monolayer of 101L cells in Me_2SO, and the cells were harvested for luciferase activity 18 h later. Luciferase activity is expressed as RLU/μg of protein. The results reported at each dose represent the average of three separate determinations, with each enzyme assay conducted in duplicate. The -fold induction is indicated *above* each *bar graph*. *PCB*, polychlorinated biphenyls; *HCB*, hexachlorobiphenyl; *TCB*, tetrachlorobiphenyl. The final concentration of each compound is shown in the *key* to the *right*.

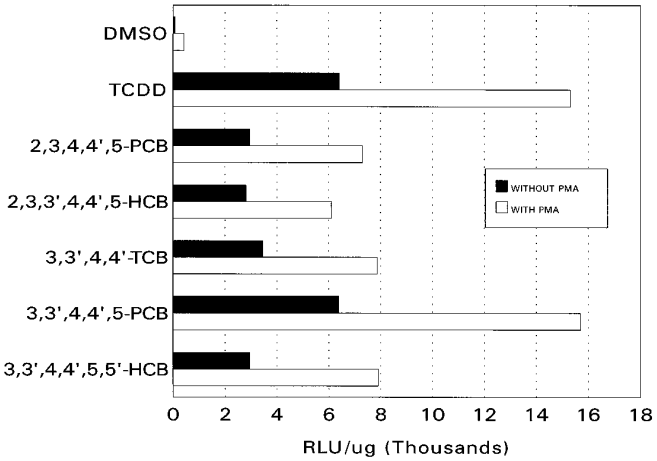

FIG. 2. **Effects of PMA on *CYP1A1* gene transcription in 101L cells.** For these experiments, 101L cells were pretreated with 125 nM PMA for 3 h followed by an 18-h treatment of TCDD (10 nM) and several structurally related PCBs (10 μM). Luciferase activity was measured, and the activity was expressed as RLU/μg of protein. The values represent the average of three individual determinations. *DMSO*, Me_2SO.

we tested. Depending on the ligand tested, PMA facilitated up to a 2–2.5-fold increase in transcriptional activity. Therefore, the actions of PMA appear to affect the functional properties of the AhR. In addition, 4α-phorbol 12,13-didecanoate, an inactive phorbol ester derivative, at concentrations between 50 nM and 1 μM showed no effect on the TCDD-induced transcriptional activation of the *CYP1A1* gene (data not shown). Since one of the principal actions of active phorbol esters leads to modulation of PKC activity, this result suggests that the actions of PMA on AhR-mediated gene transcription in tissue culture may be modulated through a PKC-directed mechanism.

To examine the direct actions of PKC activity on *CYP1A1* gene transcription, inhibitions of cellular protein kinase activities were examined. Protein kinase inhibitors were added to 101L cells for 1 h followed by TCDD treatment for 3 h. As in other experiments, a 3-h exposure to TCDD induced gene transcription 50-fold. The specific PKA inhibitor H89 (30), showed no effect on the TCDD-induced or PMA-enhanced *CYP1A1* gene transcription at the concentration up to 1 μM (Fig. 3). However, the PKC inhibitors, staurosporine and chelerythrine chloride, at concentrations that have been reported to inhibit general PKC activity (18, 19, 31), completely blocked AhR ligand-induced *CYP1A1* gene transcription (Fig. 4, *A* and *B*). Similar results were also observed with the PKC inhibitor calphostin C (data not shown).

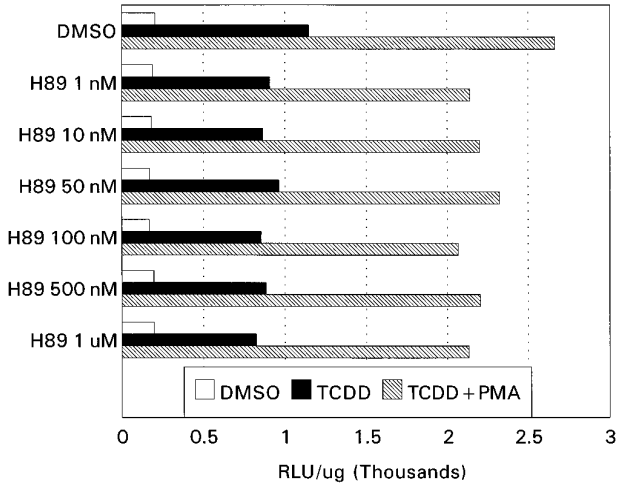

FIG. 3. **Effects of PKA inhibitor H89 on *CYP1A1* gene transcription.** The 101L cells were pretreated with various concentrations of H89 as indicated, followed by a 3-h treatment of 1 nM TCDD and/or 125 nM PMA. The cells were then harvested, and luciferase activity was determined. Luciferase activity is expressed as RLU/μg of protein. The values represent the average of two individual determinations. At these concentrations, there was no cell toxicity associated with the PKA or H89 treatment. *DMSO*, Me_2SO.

Effects of PMA and PKC Activity on Cytosolic AhR Function—CYP1A1-induced gene transcription by AhR ligands is entirely dependent upon binding of the ligand-activated AhR complex to enhancer sequences flanking the promoter. To determine if the changes in DNA binding of the AhR complex paralleled the changes we have observed in transcriptional activity, EMSAs were performed to measure the direct binding of the activated AhR to DRE. The cytosolic AhR, which is coupled with hsp90, can be activated *in vitro* to a DNA binding species by incubating cytosol with ligand. To determine whether the actions of PMA and PKC on 101L cells influence the ability of the cytosolic AhR to bind ligand and then to associate with DNA, 101L cells were treated with PMA for 3 h or staurosporine for 4 h followed by the preparation of cytosolic extracts. Cytosolic preparations were then incubated with TCDD, and the ability of the ligand-activated cytosolic AhR to bind to DNA was analyzed by EMSA. As shown in Fig. 5, PMA treatment of 101L cells did not significantly increase the activated DNA binding of the cytosolic AhR (*lane 7 versus lane 3*). Similar results were obtained with staurosporine treatment. At concentrations of staurosporine that completely blocked TCDD-directed *CYP1A1* gene transcription, there was no effect on the ability of the activated cytosolic AhR to associate with DNA (*lane 5 versus lane 3*). The treatment of cells with both staurosporine and PMA did not impact on the ability of the cytosolic AhR to associate with DNA. These results indicate that the actions of PKC has a limited impact on the ability of ligand to activate the cytosolic AhR to a functional DNA binding species. Similar conclusions have been made in experiments designed to inhibit PKC activity *in vitro*, followed by analysis of AhR activation and DNA binding to enhancer sequence (15).

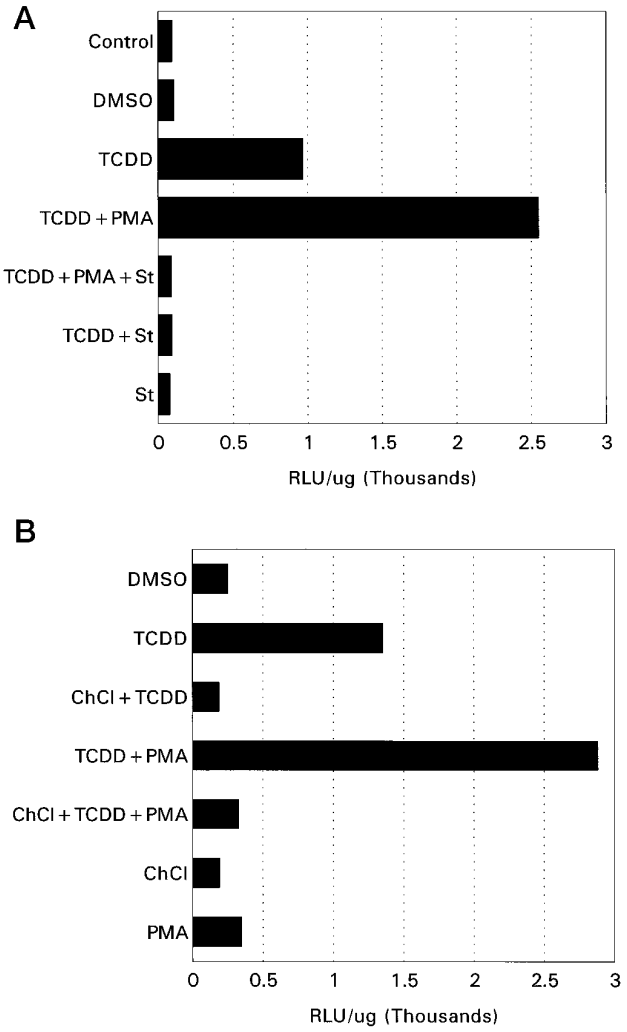

FIG. 4. **Effects of PKC inhibitors staurosporine and chelerythrine chloride on *CYP1A1* gene transcription.** The 101L cells were pretreated with 200 nM of staurosporine (*St*) for 1 h, as shown in *A*, and with 10 μM chelerythrine chloride (*ChCl*) for 1 h, as shown in *B*, followed by the addition of 1 nM TCDD and/or 125 nM PMA for 3 h. After incubation, the cells were harvested, and luciferase activity was determined. Luciferase activity is expressed as RLU/μg of protein. The values represent the average of two individual determinations. In all experiments, there was no detectable cell toxicity. *DMSO*, Me_2SO.

The Actions of PMA and PKC on Nuclear DNA Binding to Enhancer Sequences—EMSAs were also used to measure the accumulation of TCDD-induced nuclear AhR complex as measured by direct binding to DRE. In this experiment, 101L cells were pretreated with staurosporine for 1 h followed by a 3-h treatment with TCDD and/or PMA. The nuclear extracts were then prepared, and EMSAs were conducted. As shown in Fig. 6, TCDD induced accumulation of the nuclear AhR levels (*lane 3*), as determined by DNA binding activity. When 101L cells were treated with staurosporine for 1 h, transcriptional activation by TCDD was blocked. However, staurosporine (*lane 6*) did not alter the TCDD-induced nuclear AhR levels at the concentrations that blocked transcriptional activity. In addition, the actions of PMA on TCDD-directed nuclear DNA binding were not altered. Therefore, the nuclear DNA binding profiles are not concordant with those of *CYP1A1* gene transcription (Fig. 4). These results suggest that the nuclear uptake of the AhR is independent of PKC and that the actions of PKC on the induction of *CYP1A1* gene transcription are a nuclear event.

Effects of Staurosporine on the Transactivation of AhR and Arnt—The AhR and Arnt proteins have been shown independently to transactivate reporter gene constructs (20, 32). To examine the actions of staurosporine on the components of the AhR complex, chimeric constructs were used for the transactivation experiments. Both the AhR and Arnt cDNAs lacking the coding region of the N-terminal bHLH domains were fused to a functional human glucocorticoid receptor (GRDBD) containing the DNA-binding domains. These chimerics upon transfection have been shown to regulate transcription of glucocorticoid responsive elements independently of their bHLH partner protein (20, 32). In these experiments, the DBD fusion proteins bind to the GRE element flanking a GRE-driven luciferase reporter plasmid. Similar to previous studies (20, 32), the pGRDBD·Arnt is constitutively expressed at high levels and is not responsive to TCDD treatment, whereas the expression of pGRDBD·AhR is dependent upon ligand treatment (Fig. 7). Both TCDD-induced transactivation by the pGRDBD·AhR and the constitutive transactivation by the pGRDBD·Arnt chimeric proteins were blocked by staurosporine treatment, indicating that the nuclear transactivation event is dependent upon PKC activity. This result supports previous experiments that indicate a role for PKC in the transcriptional activation of the *CYP1A1* gene. In addition, these results also suggest that both components of the AhR complex are dependent upon PKC, which supports previous studies indicating that phosphorylation of both AhR and Arnt is critical for DNA binding activity (10).

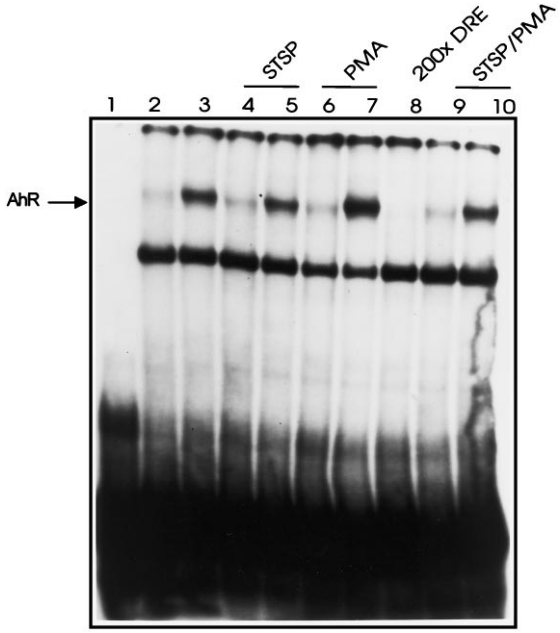

FIG. 5. **The effects of staurosporine on DNA binding activity of the AhR as determined following *in vitro* activation.** The 101L cells were pretreated with either 200 nM staurosporine (*STSP*) for 1 h, 125 nM PMA for 3 h, or staurosporine for 1 h followed by PMA for 3 h. At the end of the treatments, cytosol extracts were prepared and incubated with ^{32}P-labeled DRE and 20 nM TCDD (*lanes 3*, *5*, *7*, and *10*) or Me_2SO (*lanes 2*, *4*, *6*, and *8*) at room temperature for 3 h. The activated AhR and the binding profiles were then analyzed by EMSAs. *Lane 1* is probe alone, and the position of the activated AhR is indicated on the *left*.

DISCUSSION

AhR mediates the biological actions of ligands such as polychlorinated dibenzo-*p*-dioxins, polycyclic aromatic hydrocarbons, benzimidazoles, and bioflavonoids. The cellular events that underlie AhR-mediated gene transcription involve a series of dynamic steps that bring together several cellular and nuclear proteins, one of which appears to be PKC. In the cytosol of the cell, the AhR is coupled with the 90-kDa heat shock protein

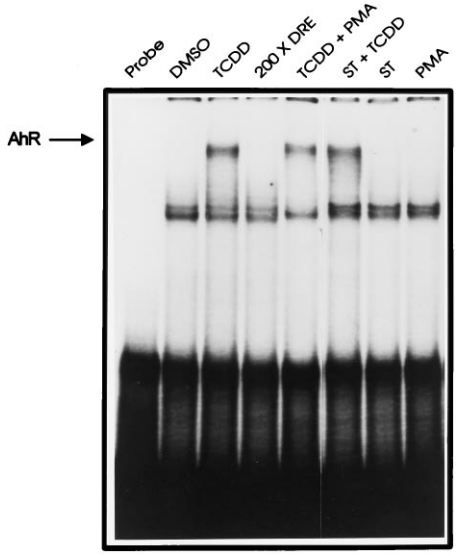

FIG. 6. **DNA binding activity of the nuclear AhR following treatment with TCDD, PMA, and staurosporine.** The 101L cells were pretreated with 200 nM staurosporine for 1 h followed by a 3-h treatment of 1 nM TCDD and/or 125 nM PMA. As outlined under "Materials and Methods," nuclear extracts were prepared, 10 μg of protein was incubated with labeled DRE sequence, and the proteins that associate with this sequence were analyzed by EMSA. Oligonucleotide competition experiments were performed in the presence of a 200-fold excess of DRE sequence. The *arrow* indicates the TCDD-inducible protein-DNA complex. *DMSO*, Me_2SO.

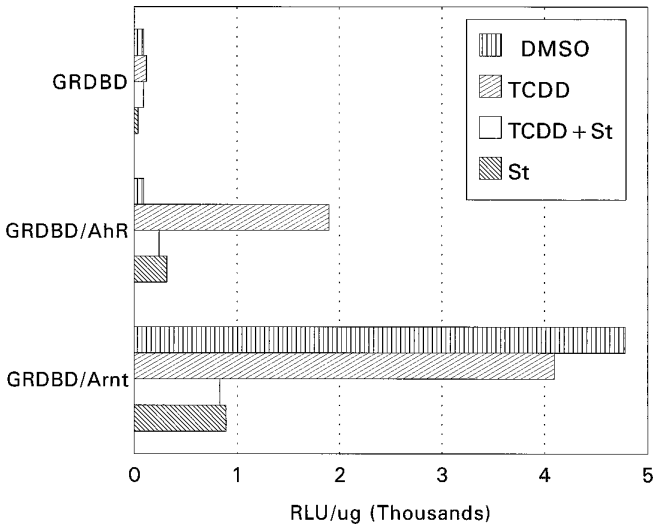

FIG. 7. **Effects of staurosporine on transactivation of AhR and Arnt.** The HepG2 cells were transiently transfected with either pGRDBD/AhR or pGRDBD/Arnt along with the GRE-driven reporter plasmids. Twenty-four hours after transfection, the 101L cells were pretreated with 200 nM staurosporine (*STSP*) for 1 h followed by a 16-h treatment with TCDD. The cell lysate was then isolated, and transactivation activity was analyzed by luciferase activity. Luciferase activity is expressed as RLU/μg of protein. The values represent the average of two individual determinations. With the above experiments, there was no detectable cell toxicity. *DMSO*, Me_2SO.

(hsp90), which is released from the receptor following ligand binding (3, 5, 33), a process that is dependent upon PKA (32). The liganded AhR migrates to the nucleus, where it associates with its partner protein, Arnt. This dimeric protein complex recognizes DREs of target genes such as *CYP1A1* and *CYP1A2* and activates promoter-specific transcription. In 101L cells, we can directly monitor AhR function by its ability to stimulate *CYP1A1* gene transcription as measured by firefly luciferase activity (16), and we can also quantitate AhR concentration in the nucleus by its ability to bind to specific enhancer sequences. In addition, the activated form of the AhR can also be quantitated *in vitro* using cytosol, and it is possible to examine the cellular influences on the ability of ligand to stimulate hsp90 release and transform the AhR·Arnt complex into a species that binds DNA. By studying a series of AhR-mediated events, such as gene transcription, nuclear DNA binding, and ligand activation of the receptor, it has been possible to conclude that ligand-induced gene transcription, and not cytosolic AhR activation, is dependent upon PKC activity.

Exposure of 101L cells to PMA, a potent PKC activator, dramatically enhances transcriptional activation of the *CYP1A1* gene induced by various AhR ligands (Figs. 1 and 2). This experiment indicates that the actions of PMA affect either the functional properties of the AhR or participate in modulating the actions of the AhR. When 101L cells were pretreated with PKC inhibitors for 1 h, transcriptional activation of TCDD-inducible as well as the PMA-enhanced *CYP1A1* gene transcription was completely blocked (Fig. 4). Since PKA inhibition did not affect transcriptional activation (Fig. 3), phosphorylation events carried out through PKC are assumed to underlie the induction process. Transcriptional inhibition was not reflective of AhR binding to enhancer sequences, either with activated cytosol or with DNA binding of ligand-stimulated nuclear receptor. It has been proposed that actions of PKC may participate in the events that lead to nuclear uptake of the AhR (34). This theory is based upon observations that the treatment of mouse hepatoma Hepa-1 cells with staurosporine blocks the appearance of TCDD-stimulated induction of the AhR to the nucleus, as measured by EMSA. Other studies have shown that staurosporine treatment of Hepa-1 cells does lead to a decrease in ligand binding to cytosolic AhR as well as affect total cellular AhR levels (35), all of which may impact on nuclear receptor levels of the AhR complex. However, using human HepG2 cells, staurosporine has no effect on inhibiting the nuclear accumulation of the AhR within the very rapid time period that it blocks transcription (Fig. 6). These results indicate that the actions of PKC in modulating *CYP1A1* gene transcription are occurring independently from cellular and molecular events that modulate cellular activation of the AhR, nuclear transport and DNA binding to enhancer sequences.

The accelerated rate of *CYP1A1* transcription by phorbol esters and the linkage to PKC activity could involve signal transduction processes. Interestingly, AhR ligands are known to induce PKC activity (34, 36) as well as AP-1 activity (37), and the latter is a cellular event controlled by PKC signaling mechanisms in the cell. The activation of PKC leads to the recruitment the Jun/Fos, the AP-1 family of transcription factors (38). The AP-1 family of proteins belong to the class of basic leucine zipper proteins that bind DNA as dimers. These dimers may be homodimers, but they can also be heterodimers formed between two members of the Jun family or between Jun and Fos. Formation of AP-1 complexes, in response to the activation of PKC, could act to bind to specific DNA sequences and, in conjunction with the AhR, promote transcriptional activation. In addition, these proteins can participate in cross-talk between different regulatory pathways and can act independently via mechanisms that do not require binding to DNA consensus sequences (39, 40). In light of observations that PKC has little immediate impact on the biochemical properties of the AhR, it could be imagined that the activation of AP-1 activity by AhR ligands serves a central role in facilitating the inducible expression of the *CYP1A1* gene by the AhR.

An alternative explanation for the actions of PKC could be that it modulates the phosphorylation patterns of complexes that make up the transcriptional initiation complex. A common

response to extracellular signals is the rapid programmed changes in the rates of gene expression, a process that is brought about by the activation of transcription factors through changes in phosphorylation states. Interestingly, it has recently been demonstrated that the interactions of AhR·Arnt with enhancer sequences are associated with binding of other constitutively expressed transcription factors to the enhancer as well as the promoter (41, 42). These changes lead to alterations in chromatin structure, an event that precedes transcription. Since phosphorylation is believed to modulate the activity of many transcriptional factors (43), cellular signaling events carried out following activation of PKC may be essential in promoting gene responses initiated by the actions of the AhR. It is possible that phosphorylation may facilitate protein-protein interactions between the nuclear AhR and other transcription factors to promote transcriptional initiation. Since there are several AhR enhancer sequences that exist upstream of the *CYP1A1* gene, the AhR may work cooperatively with other activated transcriptional factors to produce the synergistic effect on gene expression (41, 44, 45).

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Protein Kinase C Modulates Regulation of the *CYP1A1* Gene by the Aryl Hydrocarbon Receptor

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