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Protein Kinase C Activity Is Required for Aryl Hydrocarbon Receptor Pathway-Mediated Signal Transduction

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ABSTRACT

The role of protein kinase C (PKC) in the human aryl hydrocarbon receptor (hAhR) signal transduction pathway was examined in cell lines stably transfected with pGUDLUC6.1, in which luc$^+$ is solely controlled by four dioxin-responsive elements (DREs). These cell lines, P5A11 and HG40/6, were derived from HeLa and HepG2 cells respectively. Simultaneous treatment of these cells with 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin (TCDD) and phorbol-12-myristate-13-acetate (PMA) enhanced *trans*-activation of the reporter construct several-fold relative to cells treated with TCDD alone. PKC inhibitors block the PMA effect and hAhR-mediated signal transduction, demonstrating these processes require PKC activity. Examination of other independently generated, HeLa-derived cell lines stably transfected with pGUDLUC6.1 demonstrates the PMA effect in P5A11 cells is not a clonal artifact. Transient transfections indicate the PMA effect is not due to a luciferase message/gene product stabilization mechanism or stimulation of the basal transcription machinery. Examination of cytosolic preparations demonstrates PKC stimulation or inhibition does not alter hAhR and hAhR nuclear translocator protein levels or TCDD-induced down-regulation of hAhR levels. Similarly, examination of nuclear extracts indicated PKC stimulation or inhibition does not alter nuclear AhR levels or hAhR/hAhR nuclear translocator protein heterodimer DRE-binding activity as assessed by electrophoretic mobility shift assay. These results demonstrate a PKC-mediated event is required for the hAhR to form a functional transcriptional complex that leads to *trans*-activation and that the DRE is the minimal DNA element required for PMA to enhance AhR-mediated *trans*-activation.

Halogenated polycyclic aromatic hydrocarbons are environmental contaminants of concern from a human health perspective due to their widespread distribution (Fernandez-Salguero *et al*., 1996). The prototypic compound used to study the toxic response to halogenated polycyclic aromatic hydrocarbons is TCDD. TCDD binds with high affinity to the AhR to generate the activated receptor; which is responsible for nearly all of the biological effects of dioxin (Fernandez-Salguero *et al*., 1996). The biological/toxic effects of TCDD on mammals include thymic atrophy, teratogenesis, a slow wasting syndrome, tumor promotion, and chloracne (Poland and Knutson, 1992). Null *ARNT* allele mice were not viable past embryonic day 10.5, were defective in angiogenesis, and had impaired development (Maltepe *et al*., 1997), whereas null *Ahr* allele mice were viable and fertile but had slowed early growth and hepatic defects (Fernandez-Salguero *et al*., 1995; (Schmidt *et al*., 1996). The phenotypes of these null allele mice demonstrate both the AhR and its heterodimerization partner, the ARNT, play essential roles in development and normal cellular metabolism.

Before TCDD exposure, the AhR is found predominantly in a cytoplasmically localized 9S complex containing the AhR, two HSP90 molecules, and a protein of currently unknown function, hepatitis B virus X-associated protein 2 (Chen and Perdew, 1996; Meyer *et al*., 1998). This 9S, ligand-activated, AhR complex then traverses the nuclear membrane, HSP90 dissociates, and the AhR forms a 6S heterodimer with the ARNT. The AhR/ARNT heterodimer is capable of binding to DREs and altering transcription of dioxin-responsive genes (Swanson *et al*., 1993). Genes that are regulated by the AhR

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ABBREVIATIONS: PKC, protein kinase C; hAhR, human aryl hydrocarbon receptor; DRE, dioxin-responsive element; TCDD, 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin; PMA, phorbol-12-myristate-13-acetate; hARNT, human aryl hydrocarbon receptor nuclear translocator protein; HSP90, 90-kDa heat shock protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EMSA, electrophoretic mobility shift assay; PAS, PER/ARNT/SIM (periodicity/aryl hydrocarbon receptor nuclear translocator protein/simple-minded); DAG, *sn*-1,2-diacylglycerol; βNF, β- naphthoflavone; 8-bromo-cAMP, 8-bromoadenosine-cAMP; Bis I, bisindolylmaleimide I·HCl; Chel, chelerythrine chloride; FBS, fetal bovine serum; PBS, phosphate-buffered saline; α-MEM, α-minimum essential media; mAb, monoclonal antibody; 4-O-methyl-PMA, 4-*O*-methyl-phorbol-12-myristate-13-acetate; PKA, protein kinase A; TRE, 12-*O*-tetradecanoylphorbol-13-acetate-responsive element; BSA, bovine serum albumin; DMSO, dimethylsulfoxide; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate.

include CYP4501A1, the glutathione-*S*-transferase Ya subunit, NAD(P)H:quinone reductase, plasminogen activator inhibitor 2, and interleukin 1 (Poland and Knutson, 1992).

The AhR and ARNT are members of the PAS family of transcription factors. DNA binding and heterodimerization of the AhR and ARNT involve the amino-terminal portions of these proteins, which contain basic and helix-loop-helix motifs and a PAS domain (Reisz-Porszasz *et al.*, 1994; Ma *et al.*, 1995). In addition, the AhR contains a HSP90 and ligand-binding domain within the PAS domain (Perdew and Bradfield, 1996). The carboxyl-terminal regions of the AhR and ARNT act as transcription activation domains; however, in the AhR/ARNT heterodimer, the AhR transcription activation domain is dominant (Ko *et al.*, 1996). The AhR and ARNT are both phosphoproteins with phosphorylation of the AhR seeming to occur predominately on its carboxyl-terminal half (Perdew GH, unpublished observations; Mahon and Gasiewicz, 1995). Furthermore, the AhR and ARNT proteins are both phosphorylated on threonine residues, suggesting a serine/threonine kinase may be involved directly or indirectly in the regulation of the functions of these proteins (Perdew GH, unpublished observations).

A variety of data obtained from *in vitro* experiments, whole-animal studies, and eukaryotic cell culture experiments suggest the serine/threonine kinase PKC, which is activated by the DAG analog PMA, plays an important role in the regulation of the AhR signal transduction pathway (Nishizuka, 1995). Treatment of mice topically with PMA or AhR ligands revealed CYP1A1 induction was down-regulated in PMA-treated mice (Reiners *et al.*, 1992). Similarly, treatment of mice subcutaneously with PMA or AhR ligands revealed CYP1A1 induction was down-regulated in PMA-treated mice (Okino *et al.*, 1992). Pretreatment of human keratinocytes and MCF-7 human breast cancer cells with PMA before TCDD exposure resulted in decreased P4501A1 expression relative to cells treated with TCDD alone (Moore *et al.*, 1993; Berghard *et al.*, 1993); interestingly, longer pretreatments of MCF-7 cells with PMA resulted in superinducibility of CYP1A1 by TCDD. Chen and Tukey (1996) examined the effects of PMA on the ligand-induced transcriptional activation of the AhR in human HepG2 101L cells, which are stably transfected with 1904 bases of 5′-flanking DNA from the promoter of the human CYP1A1 gene fused to the firefly luciferase structural gene. Pretreatment of 101L cells with PMA resulted in a 2–3-fold enhancement in the induction of the stably transfected reporter construct relative to cells treated with TCDD alone; in addition, PKC inhibitors blocked this PMA-induced enhancement of luciferase expression and the ability of TCDD to induce the luciferase reporter construct. These data suggest the PKC pathway impinges on the AhR regulatory circuit and that the effects of PMA vary depending on the tissue type, cell line, or reporter system examined.

Overall, little is known about the ability of protein kinases to regulate the activity of the AhR or ARNT. However, studies conducted by Chen and Tukey (1996) and by Carrier *et al.* (1992) have made significant contributions toward understanding the role of PKC in these processes by demonstrating PKC activity is required for AhR-mediated signal transduction. In addition, in the study of Chen and Tukey, they eliminated some candidate mechanisms by which PKC could be involved in AhR-mediated signal transduction. The data presented here demonstrate a PKC-mediated event is required for the hAhR to form a functional transcriptional complex that leads to *trans*-activation and that stimulation of PKC with PMA leads to an enhancement in TCDD-induced, AhR-mediated *trans*-activation relative to cells treated with TCDD alone. Importantly, the DRE is found to be the minimal DNA element required for PMA to enhance AhR-mediated *trans*-activation.

Materials and Methods

Reagents. Glycerol, acrylamide, and bisacrylamide were purchased from Research Organics (Cleveland, OH). Goat anti-mouse IgG (Fc specific) antibodies, biotin-SP-conjugated goat anti-mouse IgG (Fc specific), and streptavidin were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Na[^{125}I] was obtained from Amersham Life Science (Cleveland, OH). The use of Iodo-Beads (Pierce Chemical, Rockford, IL) in the iodination of either goat anti-mouse IgG (Fc specific) or streptavidin was as described by the manufacturer. PVDF membrane was obtained from Millipore (Bedford, MA). Restriction enzymes and T4 DNA ligase were from New England Biolabs (Beverly, MA). [γ-^{32}P]ATP was from Amersham Life Science. BSA, βNF, DMSO, phorbol, 4-O-methyl-PMA, PMA, 8-bromo-cAMP, Bis I, and Chel were from Sigma Chemical (St. Louis, MO). TCDD was obtained from Steven Safe, Texas A & M University (College Station, TX). All other reagents, unless otherwise specified, were obtained from Sigma Chemical.

Plasmids. The pCI-neo, pGL3-Control, and pGL3-Basic vectors were from Promega (Madison, WI). The pREP4ΔEBNA-1 vector (a gift from Dr. D. M. Wojchowski) is a derivative of pREP4 (InVitrogen, Carlsbad, CA) in which the *EBNA-1* and *OriP* loci have been deleted by *Avr*II and *Xba*I restriction and religation of these compatible ends. The pGUDLUC6.1 vector (a gift from Dr. M. S. Denison, University of California, Davis) was prepared by subcloning a 1810-bp *Hin*dIII fragment isolated from the plasmid pGUDLUC1.1 into the *Hin*dIII site located 5′ to *luc*$^+$ in the pGL3-Basic vector (Garrison *et al.*, 1996). This 1810-bp, *Hin*dIII fragment contains a portion of the mouse mammary tumor virus long terminal repeat, inclusive of the viral promoter but lacking functional glucocorticoid-responsive elements, as well as an 480-bp fragment isolated from the 5′-flanking region of the murine CYP1A1 gene that contains four DREs and no other known or identifiable response elements (Lee *et al.*, 1984). This 480-bp fragment confers TCDD responsiveness on the mouse mammary tumor virus viral promoter and a luciferase reporter gene (Garrison *et al.*, 1996). All plasmids were propagated in *Escherichia coli* strain DH5α (*supE*44 ΔlacU169 (ϕ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1).

Cell culture and transient transfections. FBS was obtained from Hyclone Laboratories (Logan, UT). OPTI-MEM was from Life Technologies (Gaithersburg, MD). Trypsin/EDTA, PBS, and α-MEM were from Sigma Chemical (St. Louis, MO). HeLa (a gift from Dr. Jeffery Ross, McArdle Laboratory for Cancer Research, Madison, WI) and HepG2 (American Type Culture Collection, Rockville, MD) cells were grown in α-MEM supplemented with 10% FBS, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin at 37° in 94% air/6% CO_2. Stably transfected cell lines were propagated in growth media containing 75 μg/ml hygromycin (Calbiochem-Novabiochem International, San Diego, CA) as the selective agent. HeLa cells to be transiently transfected were grown to 80% confluency in 60 × 15-mm tissue culture plates and transfected with 3 μg of DNA/dish using LipofectAMINE (Life Technologies) as described by the manufacturer.

Luciferase assays. Cells were grown as described to 80% confluency in 24-well tissue culture trays and washed twice with PBS before lysis. Cell lysates were prepared and assayed for luciferase activity using the Promega Luciferase Assay System as specified by the manufacturer. Light output was measured for 15 sec at 25° using

a TD-20*e* Luminometer (Turner Designs, Sunnyvale, CA) and luciferase activity was expressed as relative light units/μg of protein. Protein content was determined using the BCA protein assay (Pierce Chemical).

Generation of the P5A11, P2D12, and P1B12 cell lines. HeLa cells were grown as described previously, in 100 × 20-mm tissue culture dishes to 80% confluency. The ProFection Mammalian Transfection System (Promega) was used, as described by the manufacturer, to transfect cells by the calcium phosphate method with 20 and 6 μg of *Xmn*I-linearized pGUDLUC6.1 and pREP4ΔEBNA-1, respectively. At 24 hr after transfection, cells were split 1:8 into 100 × 20-mm tissue culture dishes and allowed to proliferate for 96 hr without selection. Stably transfected cells were selected with growth media containing 300 μg/ml hygromycin for 3 weeks; media was changed every 72 hr. Surviving after expansion were screened for stable integration of pGUDLUC6.1 by incubation with growth media containing 100 μM βNF for 24 hr. After βNF treatment, cells were lysed and assayed for luciferase activity as described earlier.

Generation of the HG40/6 cell line. HepG2 cells were grown as described in 100 × 20-mm tissue culture dishes to 80% confluency. The ProFection Mammalian Transfection System (Promega) was used, as described by the manufacturer, to transfect cells by the calcium phosphate method with 5 and 20 μg of pCI-neo and *Xmn*I-linearized pGUDLUC6.1, respectively. At 40 hr after transfection cells were split 1:40 into 100 × 20 mm tissue culture dishes and allowed to proliferate for 24 hr without selection. Stably transfected cells were selected with growth media containing 600 μg/ml G418 for 3 weeks; media was changed every 72 hr. Surviving colonies after expansion were screened for stable integration of pGUDLUC6.1 by incubation with growth media containing 10 nM TCDD for 24 hr. After TCDD treatment, cells were lysed and assayed for luciferase activity as described earlier.

Preparation of cellular extracts. P5A11 cells were grown as described to 80% confluency in 175-cm^2 tissue culture flasks, washed twice with PBS, and serum fasted for 14 hr before treatment. Immediately before the addition of compounds, BSA was added to a final concentration of 5 mg/ml. Cells treated with the PKC inhibitors Bis I or Chel were preincubated with these compounds for 15 min before exposure to DMSO, TCDD, or PMA. Cells were incubated with the compounds for 4 or 1.5 hr before the preparation of cytosolic or nuclear extracts, respectively. Cells were harvested using trypsin/EDTA, washed twice with PBS, and homogenized in MENG-Mo [25 mM MOPS, 2 mM EDTA, 0.02% NaN_3, 10% glycerol, and 20 mM $Na_2MoO_4{\cdot}2H_2O$, pH 7.4] with 40 strokes in a Dura-Grind Dounce tissue homogenizer (Wheaton Instruments, Millville, NJ) at 4°. Cell disruption was confirmed by microscopic examination of lysates. Nuclear pellets were isolated by centrifugation of the cell lysates at 1,000 × *g* for 15 min. The supernatant was centrifuged at 100,000 × *g* for 30 min to obtain the cytosolic fraction. The nuclear pellet was resuspended and washed three times with MENG-Mo and once with MENG-Mo containing 50 mM NaCl. High salt nuclear extracts were prepared by suspension of the nuclear pellet in an equal volume of MENG-Mo containing 1 M NaCl, incubation of this suspension at 4° for 1 hr, and then centrifugation at 100,000 × *g* for 1 hr. Total protein in cytosolic and nuclear fractions was determined with the BCA protein assay (Pierce Chemical).

Quantitative assay for the AhR and ARNT proteins. Protein samples were separated by tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis on an 8.0% polyacrylamide tricine gel, after which the proteins were electrophoretically transferred to polyvinylidene difluoride membrane (Millipore). Transfers were performed at 8 V for 6 hr in a Genie electroblot unit (Idea Scientific, Minneapolis, MN) in transfer buffer [20 mM Tris, 185 mM glycine, 20% (v/v) methanol]. After protein transfer, the membrane was blocked with 3% (w/v) BSA in PBS containing 10 mM Na_2HPO_4, 150 mM NaCl, and 0.5% (v/v) Tween 20 for 30 min at 25°. The blots were rinsed once in blot wash buffer consisting of 0.1% (w/v) BSA in PBS containing 0.5% (v/v) Tween 20. The AhR- (Rpt 1) and ARNT- (2B10) specific mAbs used in these experiments were generated as described previously (Hord and Perdew, 1994; Perdew *et al*., 1995). Blots were incubated with mAb Rpt 1 (0.5 μg/ml) or 2B10 for 1 hr at room temperature, followed by three 10-min rinses with blot wash buffer. mAb Rpt 1- or 2B10-probed blots then were incubated with ^{125}I-labeled goat anti-mouse IgG (0.5 μg/ml, Fc fragment specific) or biotinylated goat anti-mouse IgG (0.5 μg/ml, Fc fragment specific) for 1 hr at 25°, followed by three 10-min rinses with blot wash buffer. Blots probed with biotinylated goat anti-mouse IgG then were incubated with [^{125}I]streptavidin (1.0 μg/ml) for 1 hr at 25°, followed by three 10-min rinses with blot wash buffer. Probed blots were dried, and visualization was performed by autoradiography or with a Bio-Rad (Hercules, CA) GS-363 Molecular Imager System PhosphorImager system. Quantification of ^{125}I-labeled bands in phosphoimaged blots was performed using Molecular Analyst software (version 1.4; BioRad).

DRE-specific EMSA. DRE-specific EMSAs were performed with high salt nuclear extracts isolated from P5A11 cells treated with DMSO, TCDD, PMA, and Chel as described. The assay system used essentially was as described previously (Singh *et al*., 1996). Wild-type DRE oligonucleotides of sequences 5′-GATCTGGCTCTTCT-CACGCAACTCCG-3′ and 3′-ACCGAGAAGAGTGCGTTGAGGC-CTAG-5′ were gifts from Dr. M. S. Denison. The final composition of the assay mix was 25 mM HEPES, pH 7.5, 10% (v/v) glycerol, 100 mM NaCl, 21.6 ng/μl poly(dI/dC), 5 mM dithiothreitol, 4 mM $MgCl_2$, 4 mM spermidine, and 2.5% (w/v) CHAPS. Nuclear extracts (5.3 μg of protein) were incubated with the assay mix for 15 min at 25°, followed by the addition of 0.1875 ng of ^{32}P-end-labeled, wild-type DRE and incubation for an additional 15 min at 25°. The specificity of the binding was assessed by incorporating 100-fold molar excess of unlabeled, wild-type DRE into the assay system. The final assay volume was 25 μl for all samples. Then, 2.5 μl of 0.25% (w/v) xylene cyanol in 20% (w/v) Ficoll was added to each sample. Samples were loaded onto a nondenaturing 4% polyacrylamide TAE gel and separated by electrophoresis. Gels were dried and visualized with a BioRad GS-363 Molecular Imager System PhosphorImager and Molecular Analyst software. Quantification of shifted bands was performed using the Molecular Analyst software.

Results

Effects of TCDD, PMA, and PMA structural analogs on hAhR-mediated *trans*-activation in P5A11 cells. The P5A11 cell line was generated by stably transfecting HeLa cells with the pGUDLUC6.1 minimal DRE/luciferase reporter construct to examine rapidly the effects of various biologically active compounds on AhR-mediated signal transduction. To confirm the TCDD responsiveness of the stably transfected minimal DRE/luciferase reporter construct pGUDLUC6.1 and to begin to examine the role PKC plays in AhR-mediated signal transduction, P5A11 cells were treated with TCDD, PMA, and PMA structural analogs. In an attempt to examine the early effects of PKC stimulation on the AhR responsive luciferase reporter a short, 4-hr time course was selected. Dose-response experiments demonstrate that the stably transfected pGUDLUC6.1 reporter construct is *trans*-activated in a saturating, TCDD dose-dependent manner in the P5A11 cell line and that the saturated response begins at 1 nM TCDD (Long WP, unpublished observations). Dose-response experiments in which P5A11 cells were treated with increasing doses of PMA, a PKC activator, in the presence of 1 nM TCDD demonstrated that the stably transfected reporter is *trans*-activated in a saturating, PMA dose-dependent manner and that saturation is reached at 81 nM PMA (Long WP, unpublished observations). It is important to

note that this value is in the range in which PMA typically is found to stimulate PKC *in vivo* (Iizuka *et al.*, 1989; Chen and Tukey, 1996). To examine further the effects of PMA on *trans*-activation of the DRE/luciferase reporter construct, P5A11 cells were treated with DMSO, 1 nM TCDD, 81 nM PMA, 81 nM phorbol, or 81 nM 4-O-methyl-PMA as indicated (Fig. 1) and assayed for luciferase. Treatment of P5A11 cells with 1 nM TCDD results in a 3.8-fold increase in *trans*-activation of the reporter construct relative to cells treated with DMSO alone (Fig. 1). Simultaneous treatment of P5A11 cells with 1 nM TCDD and 81 nM PMA results in a 3.5- fold increase in *trans*-activation of the reporter construct relative to cells treated with 1 nM TCDD alone (Fig. 1) (i.e., PMA is enhancing AhR-mediated *trans*-activation of the reporter construct; also referred to as the "PMA effect"). Phorbol, the biologically inactive, parent compound of PMA, and 4-O-methyl-PMA, which weakly stimulates PKC (Iizuka *et al.*, 1989), were used at concentrations of 81 nM to determine whether the phorbol ring structure of PMA was responsible for the PMA effect in P5A11 cells. Fig. 1 demonstrates that P5A11s treated with 81 nM phorbol or 81 nM 4-O-methyl-PMA alone had only basal expression levels of reporter relative to DMSO-treated controls. As expected, substitution of 81 nM phorbol for 81 nM PMA and cotreatment of cells with 1 nM TCDD results in reporter levels identical to those in cells treated with 1 nM TCDD alone (Fig. 1). In addition, as expected, substitution of 81 nM 4-O-methyl-PMA for 81 nM PMA in cells treated with 1 nM TCDD resulted in an enhanced *trans*-activation effect intermediate to that seen in P5A11s treated with 1 nM TCDD and 81 nM PMA (Fig. 1). Furthermore, the PKA activator 8-bromo-cAMP had no effect on AhR-mediated *trans*-activation (Pray-Grant M, unpublished observations; Hei *et al.*, 1991). These results demonstrate that the P5A11 cell line is a sensitive, TCDD-responsive model system suitable for examination of the role of PKC in AhR-mediated signal transduction. These data also confirm that the phorbol ring structure of PMA is not responsible for the PMA effect but that instead the 12-myristate and 13-acetate moieties of PMA, which mimic the conformation of the acyl side chains in *sn*-1,2-diacylglycerol and thus stimulate PKC activity, seem to be responsible for the PMA effect. Last, these data suggest that PMA/DAG-binding PKC isoforms, but not PKA, impinge on the AhR pathway.

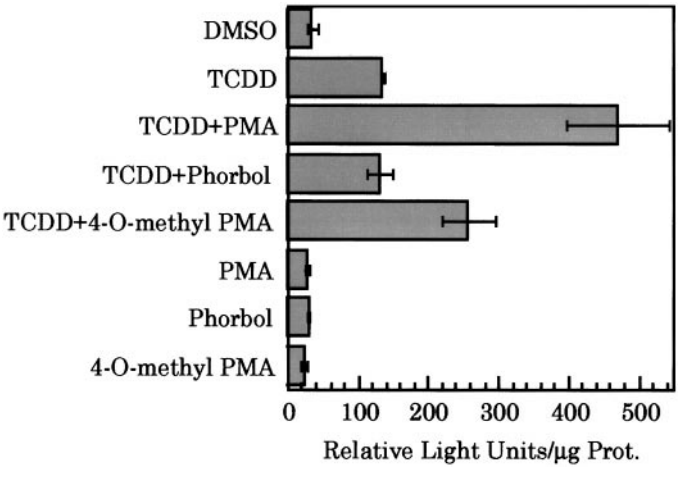

Fig. 1. Effects of TCDD, PMA, and structural analogs of PMA on DRE/luciferase reporter *trans*-activation in P5A11 cells. P5A11 cells were grown to 80% confluency and serum fasted in α-MEM for 14 hr before treatment, and BSA was added to a final concentration of 5 mg/ml immediately before addition of compounds. Cells were incubated for 4 hr with compounds at final concentrations of 1 nM TCDD, 81 nM PMA, 81 nM phorbol, or 81 nM 4-O-methyl-PMA as indicated and assayed for luciferase activity as described in Materials and Methods. All compounds were solubilized in DMSO. *Error bars*, standard deviations for a given measurement.

Effect of TCDD and PMA on hAhR-mediated *trans*-activation in the P2D12 and P1B12 cell lines. Before proceeding with a detailed examination of the PMA effect, it was necessary to confirm that the effect was not a clonal artifact unique to the P5A11 cell line. Cells from the independently derived, stably transfected cell lines P2D12 and P1B12 were treated with DMSO, 1 nM TCDD, or 81 nM PMA as indicated (Fig. 2). The P2D12 and P1B12 cell lines are nonresponsive to DMSO and 81 nM PMA alone (Fig. 2); in addition, the cells respond as expected to 1 nM TCDD. Importantly, treatment of these cell lines simultaneously with 1 nM TCDD and 81 nM PMA (Fig. 2) results in an enhanced *trans*-activation effect like that observed in similar experiments performed using P5A11 cells (Fig. 1). These results demonstrate that the PMA effect is not a clonal artifact unique to the P5A11 cell line and that further investigation of this effect in the P5A11 cell line was warranted.

Transient transfection of HeLa cells with the pGL3-Control vector. HeLa cells, the parent cell line of the P5A11 line, were transfected transiently with the pGL3-Control vec-

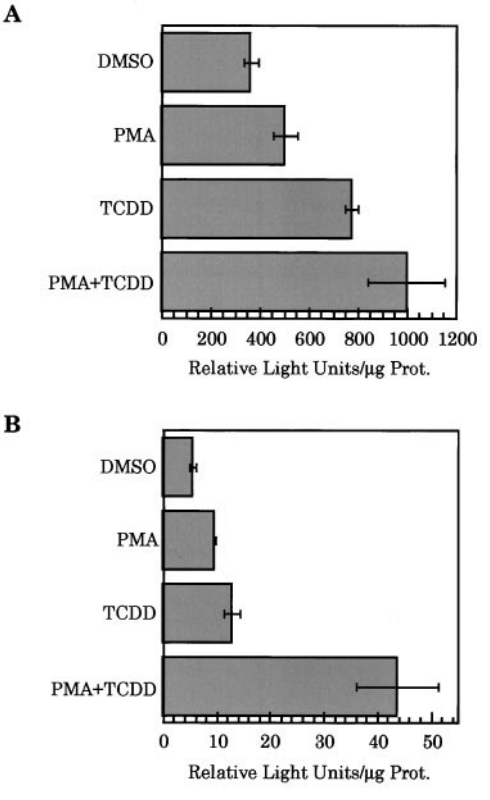

Fig. 2. Effects of TCDD and PMA on DRE/luciferase reporter *trans*-activation in the P2D12 and P1B12 cell lines. Cells were grown to 80% confluency and serum fasted in α- MEM for 14 hr before treatment, and BSA was added to a final concentration of 5 mg/ml immediately before the addition of compounds. P2D12 (A) or P1B12 (B) cells were incubated for 4 hr with compounds at final concentrations of 1 nM TCDD or 81 nM PMA as indicated and assayed for luciferase activity as described in Materials and Methods. All compounds were solubilized in DMSO. *Error bars*, standard deviations for a given measurement.

tor to address the possibilities that the PMA effect is due to fortuitous, PMA-responsive TREs in the pGL3 vector series or that the effect occurs through a luciferase message/gene product stabilization mechanism (Reifel-Miller *et al*., 1996). The pGL3-Control vector contains SV40 promoter and enhancer sequences and constitutively expresses luciferase. HeLa cells were grown; transfected with the pGL3-Control vector; and treated with DMSO, 81 nM PMA, 1 nM TCDD, or both compounds as indicated (Fig. 3),and cell extracts were assayed for luciferase. No differences in luciferase activities were observed between these treatments (Fig. 3), suggesting that the PMA effect is not due to a luciferase message/gene product stabilization mechanism. In addition, no PMA-responsive TRE elements seem to be present in the pGL3 vector series. Last, these results suggest the PMA effect does not involve the basal transcription machinery.

Effects of PKC inhibitors on AhR-mediated *trans*-activation in P5A11 cells. The effects of the specific PKC inhibitors Chel and Bis I on *trans*-activation of the pGUDLUC6.1 reporter construct were examined in the P5A11 cell line to confirm further that the PMA effect is due to stimulation of PKC activity (Herbert *et al*., 1990; Toullec *et al*., 1991). Chel and Bis I doses used in this experiment were from dose-response experiments in which P5A11 cells were pretreated for 15 min with increasing doses of Chel or Bis I followed by cotreatment with 1 nM TCDD and 81 nM PMA for 4 hr, at which time luciferase assays were performed (Long WP, unpublished observations). These dose-response experiments demonstrated that the PMA effect could be abolished in a dose-dependent manner by both Chel and Bis I and that the minimal inhibitor doses required to abolish the PMA effect are 3 and 4 μM for Chel and Bis I, respectively. P5A11 cells were treated with DMSO, 1 nM TCDD, 81 nM PMA, 3 μM Chel, or 4 μM Bis I as indicated (Fig. 4) and assayed for luciferase. Fig. 4 clearly shows that the PKC inhibitors Chel and Bis I specifically abolish the PMA effect. Importantly, treatment of P5A11 cells with these PKC-specific inhibitors and 1 nM TCDD eliminated AhR-mediated *trans*-activation of the reporter construct (Fig. 4). These results demonstrate that PMA is specifically activating PKC to generate the PMA effect and indicate that a PKC-mediated phosphorylation event is required for the AhR signaling pathway to function.

Effects of TCDD, PMA, PMA analogs, and PKC inhibitors on hAhR-mediated *trans*-activation in HG40/6 cells. HG40/6 cells, a HepG2-derived cell line, were treated with TCDD, PMA, PMA structural analogs, and PKC inhibitors to address the possibility that the PMA effect is cell line dependent. HG40/6 cells were treated with 5 nM TCDD, 81 nM PMA, 81 nM phorbol, 81 nM 4-O-methyl-PMA, 4.6 μM Chel, or 4.0 μM Bis I as indicated (Fig. 5); doses were optimized by conducting dose-response experiments (Tsai J, unpublished observations). Treatment of HG40/6 cells with 5 nM TCDD (Fig. 5A) results in a 39.2-fold increase in *trans*-activation of the stably transfected pGUDLUC6.1 reporter construct relative to cells treated with DMSO alone. However, cotreatment of HG40/6 cells with 1 nM TCDD and 81 nM PMA (Fig. 5A) results in a 6.5-fold increase in *trans*-activation of the reporter construct relative to cells treated with 1 nM TCDD alone. Simultaneous treatment of HG40/6 cells with 1 nM TCDD and 81 nM phorbol (Fig. 5A) failed to generate any increase in *trans*-activation of the reporter construct relative to cells treated with 1 nM TCDD alone. Importantly, treatment of cells with 81 nM PMA, phorbol, or 4-O-methyl-PMA alone (Fig. 5A) does not stimulate *trans*-activation of the reporter construct relative to cells treated with DMSO alone. In addition, the PKC inhibitors Chel and Bis I abolished the PMA effect and impaired TCDD-induced *trans*-activation of the reporter construct in HG40/6 cells (Fig. 5B). These data demonstrate that the PMA effect is not unique to HeLa-derived cell lines, such as the P5A11 line, but instead also occurs in the HepG2-derived HG40/6 cell line and thus seems to be cell line independent. Taken together,

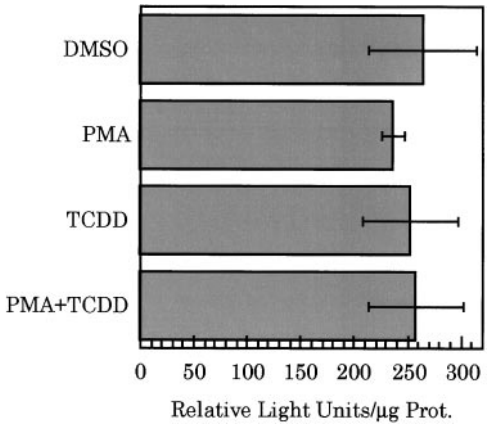

Fig. 3. Effects of TCDD and PMA treatment on HeLa cells transiently transfected with the pGL3-Control vector. HeLa cells were grown to 80% confluency and transfected with 3 μg of the pGL3-Control vector/dish using LipofectAMINE as described in Materials and Methods. Cells were incubated with DNA/liposome complexes in OPTI-MEM (Life Technologies) for 5 hr, after which an equal volume of OPTI-MEM containing 20% FBS was added to each transfected dish. At 10 hr later, the cells were washed twice with PBS and serum fasted for 14 hr in α-MEM. Immediately before treatment, BSA was added to a final concentration of 5 mg/ml. Cells were incubated for 4 hr with compounds at final concentrations of 1 nM TCDD or 81 nM PMA as indicated and assayed for luciferase activity as described in Materials and Methods. All compounds were solubilized in DMSO. *Error bars*, standard deviations for a given measurement.

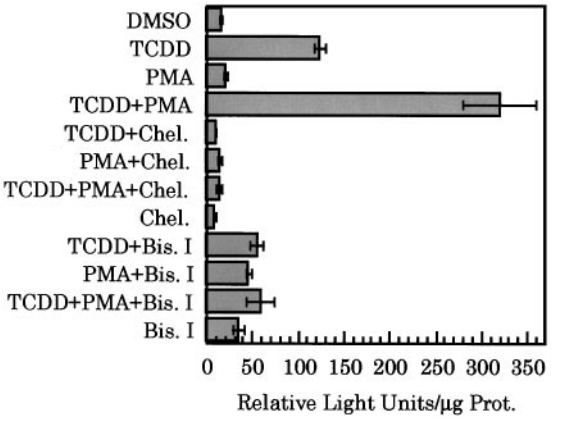

Fig. 4. Effects of PKC inhibitors on DRE/luciferase reporter *trans*-activation in P5A11 cells. P5A11 cells were grown to 80% confluency and serum fasted in α-MEM for 14 hr before treatment, and BSA was added to a final concentration of 5 mg/ml immediately before the addition of compounds. Cells treated with 3 μM Bis I or 4 μM Chel were preincubated with these compounds for 15 min before the addition of other compounds. TCDD and PMA were added to final concentrations of 1 and 81 nM, respectively. Cells subsequently were incubated for 4 hr as indicated and assayed for luciferase activity as described in Materials and Methods. All compounds were solubilized in DMSO. *Error bars*, standard deviations for a given measurement.

these data further suggest a PKC-mediated event is required for AhR-mediated *trans*-activation.

Examination of cytosolic hAhR and hARNT levels in treated P5A11 cells. Quantification of cytosolic hAhR and hARNT levels was performed to examine the possibility that Chel and PMA treatments generate their effects by altering cytosolic levels of these proteins. Quantitative autoradiography of Western blots probed with the mAbs RPT-1 and 2B10 was performed as indicated (Figs. 6 and 7) to quantify hAhR and hARNT levels in cytosols from treated P5A11 cells. These data (Figs. 6 and 7) demonstrate that Chel does not abolish the enhanced *trans*-activation effect by promoting down-regulation of the hAhR or hARNT. Furthermore, Chel and PMA treatments do not drastically alter cytosolic levels of the hAhR relative to P5A11 cells treated with DMSO alone (Fig. 6.). In addition, PMA and Chel treatments (Fig. 6) do not prevent TCDD-induced down-regulation of the hAhR (Reick *et al.*, 1994). Together, these data demonstrate that the PMA effect does not occur as a result of an increase in hAhR or hARNT levels or by some mechanism that impairs TCDD-induced down-regulation of the hAhR.

hAhR levels and hAhR/hARNT heterodimer DRE-binding activities in nuclear extracts from treated P5A11 cells. Alteration of nuclear hAhR levels or hAhR/hARNT heterodimer DRE-binding activity in the nuclear compartment are two possible mechanisms by which the PMA effect may occur. To begin to address these possibilities, a control experiment was performed in which P5A11 cells were treated as indicated in Fig. 8 and assayed for luciferase (Long WP, unpublished observations). This control experiment confirms that the PMA effect occurs when nuclear hAhR levels in TCDD-treated HeLa cells are maximal (Singh *et al.*, 1996); in addition, the PKC inhibitor Chel abolishes the PMA effect and TCDD-induced *trans*-activation of the reporter when P5A11 cells are treated as indicated in Fig. 8. After these control experiments, nuclear extracts were prepared from P5A11 cells treated with 10 nM TCDD, 81 nM

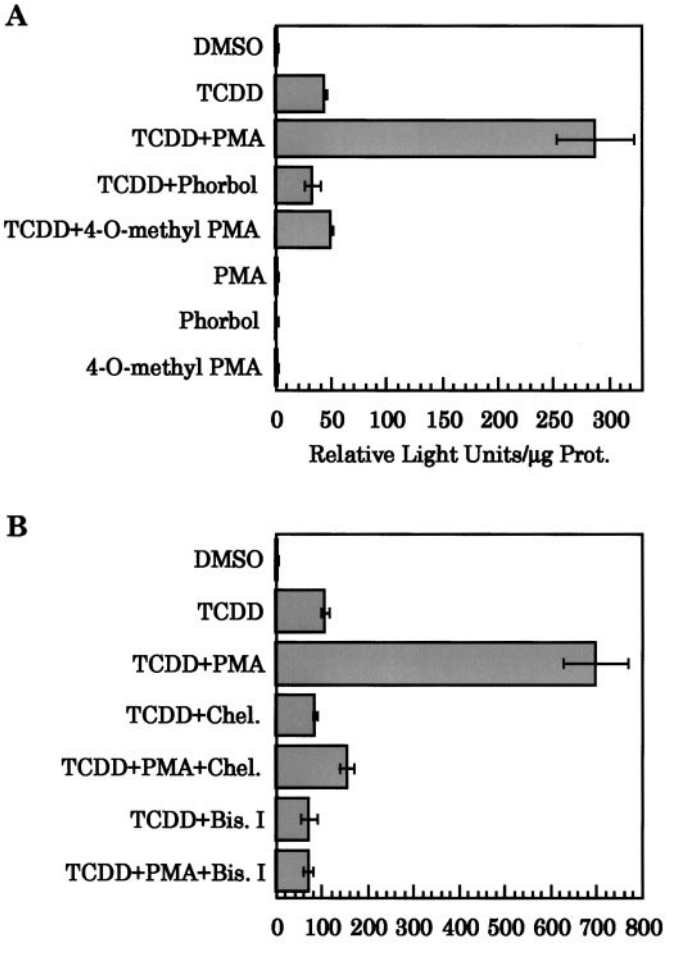

Fig. 5. Effects of PMA structural analogs and PKC inhibitors on DRE/luciferase reporter *trans*-activation in HG40/6 cells. HG40/6 cells were grown to 80% confluency and serum fasted in α-MEM for 14 hr before treatment, and BSA was added to a final concentration of 5 mg/ml immediately before addition of compounds. A, Cells were incubated for 4 hr with compounds at final concentrations of 5 nM TCDD, 81 nM PMA, 81 nM phorbol, or 81 nM 4-O-methyl-PMA as indicated and assayed for luciferase activity as described in Materials and Methods. B, Cells treated with 4 μM Bis I or 4.6 μM Chel were preincubated with these compounds for 15 min before the addition of other compounds. TCDD and PMA were added to final concentrations of 5 and 81 nM, respectively. Cells were incubated with compounds for 4 hr as indicated and assayed for luciferase activity as described in Materials and Methods. All compounds were solubilized in DMSO. *Error bars*, standard deviations for a given measurement.

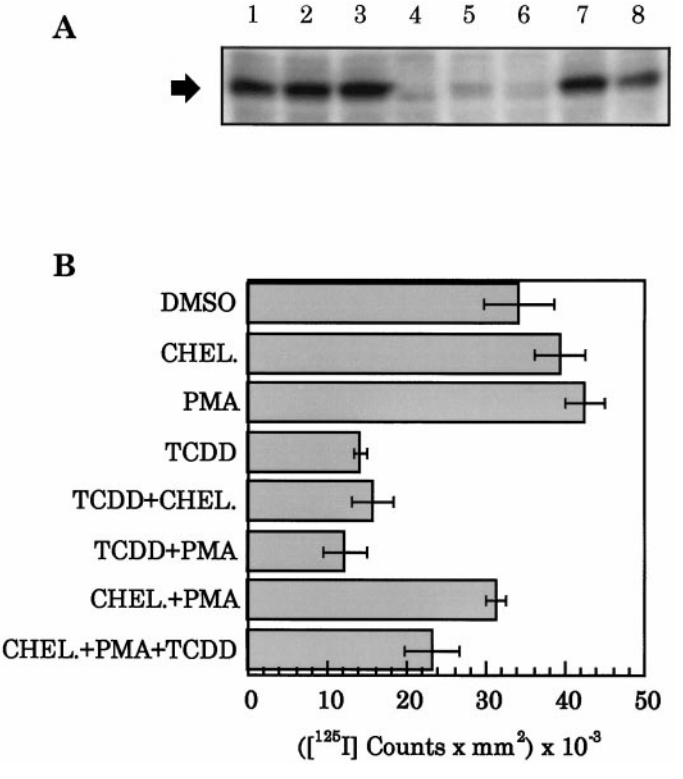

Fig. 6. Cytosolic hAhR levels in treated P5A11 cells. P5A11 cells were grown to 80% confluency and serum fasted in α-MEM for 14 hr before treatment, and BSA was added to a final concentration of 5 mg/ml immediately before addition of compounds. Cells treated with 4 μM Chel were preincubated for 15 min before the addition of other compounds. TCDD and PMA were added to final concentrations of 1 and 81 nM, respectively, and cells were then treated as indicated for 4 hr and assayed for luciferase activity as described in Materials and Methods. All compounds were solubilized in DMSO. A, Western blot analysis of cytosolic hAhR levels in treated P5A11 cells. Blots were prepared, probed with mAb Rpt 1 followed by ^{125}I-labeled GAM IgG, and visualized as described in Materials and Methods. *Lane 1*, 100 μg of cytosolic protein from DMSO. *Lane 2*, CHEL. *Lane 3*, PMA. *Lane 4*, TCDD. *Lane 5*, TCDD + CHEL. *Lane 6*, TCDD + PMA. *Lane 7*, PMA + CHEL. *Lane 8*, TCDD + PMA + CHEL. B, Quantification of cytosolic hAhR levels in treated P5A11 cells. hAhR levels were quantified as described in Materials and Methods. *Error bars*, standard deviations for a given measurement.

PMA, or 4 μM Chel as indicated (Fig. 8), and quantitative autoradiography (Fig. 8A) of Western blots probed with the mAb RPT-1 was performed. These quantitative Western blots (Fig. 8) indicate that the PMA effect is not due to a drastic increase in nuclear hAhR levels and that Chel does not abolish TCDD-induced *trans*-activation of the reporter construct by preventing the hAhR from translocating into the nucleus.

Examination of DRE-binding activity in nuclear extracts was performed to address the possibility that the PMA effect is due to a mechanism in which the hAhR/hARNT heterodimer DRE-binding activity in the nuclear compartment is enhanced relative to cells treated with TCDD alone. Nuclear extracts were prepared from P5A11 cells treated with 10 nM TCDD, 81 nM PMA, or 4 μM Chel as indicated (Fig. 9), and DRE-specific EMSAs were performed and quantified (Fig. 9B). These data (Fig. 9A) demonstrate that Chel does not impair TCDD-mediated signal transduction by reducing the DRE-binding activity of the hAhR/hARNT heterodimer or by abolishing nuclear translocation of the AhR. Furthermore, these data (Fig. 9) indicate that the PMA effect is not due to an increase in the DRE-binding activity of the nuclear hAhR/hARNT heterodimer.

Discussion

Currently, the mechanism by which PKC modulates AhR-mediated signal transduction is poorly understood. However, studies conducted by Carrier *et al*. (1992) and Chen and Tukey (1996) demonstrated that a PKC-mediated signaling event is required for AhR-mediated signal transduction. We generated HeLa- and HepG2-derived cell lines stably transfected with the minimal DRE/luciferase reporter construct pGUDLUC6.1 as a tool with which to build on the findings of these earlier studies regarding the role of PKC in AhR-mediated signal transduction. Previous reports addressed this issue by using the endogenous CYP1A1 gene as a reporter or used reporter constructs in which large portions of the 5′-regulatory region of the CYP1A1 gene were linked to CAT or luciferase reporter genes (Okino *et al*., 1992; Reiners *et al*., 1992; Berghard *et al*., 1993; Moore *et al*., 1993; Chen and Tukey, 1996). One limitation of such approaches is that

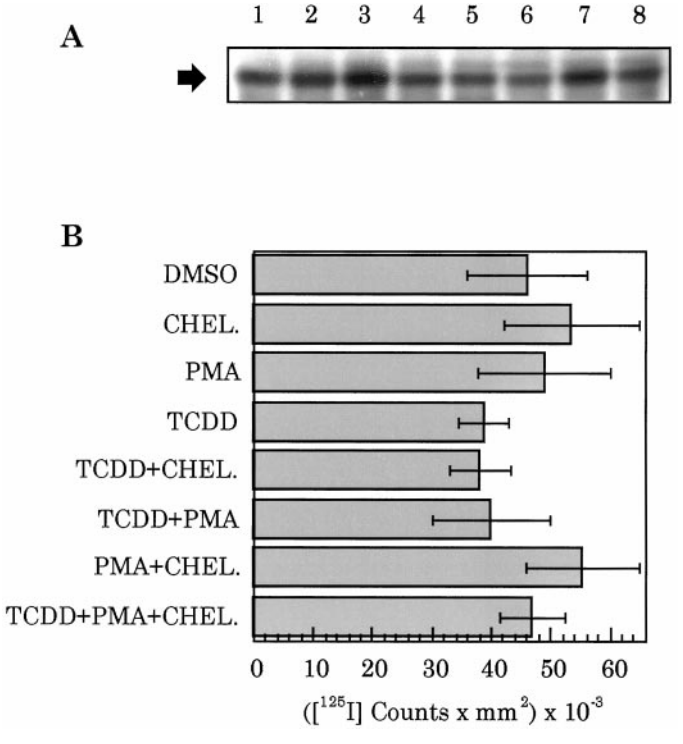

Fig. 7. Cytosolic hARNT levels in treated P5A11 cells. P5A11 cells were grown to 80% confluency and serum fasted in α-MEM for 14 hr before treatment, and BSA was added to a final concentration of 5 mg/ml immediately before the addition of compounds. Cells treated with 4 μM Chel were preincubated with this compound for 15 min before the addition of other compounds. TCDD and PMA were added to final concentrations of 1 and 81 nM, respectively. Cells were incubated with compounds for 4 hr as indicated and assayed for luciferase activity as described in Materials and Methods. All compounds were solubilized in DMSO. A, Western blot analysis of cytosolic hARNT levels in treated P5A11 cells. Blots were prepared, probed with mAb Rpt 1 followed by biotinylated-GAM IgG/[^{125}I]streptavidin, and visualized as described in Materials and Methods. *Lane 1*, 100 μg of cytosolic protein from DMSO. *Lane 2*, CHEL. *Lane 3*, PMA. *Lane 4*, TCDD. *Lane 5*, TCDD + CHEL. *Lane 6*, TCDD + PMA. *Lane 7*, PMA + CHEL. *Lane 8*, TCDD + PMA + CHEL. B, Quantification of cytosolic hARNT levels in treated P5A11 cells. hARNT levels were quantified as described in Materials and Methods. *Error bars*, standard deviations for a given measurement.

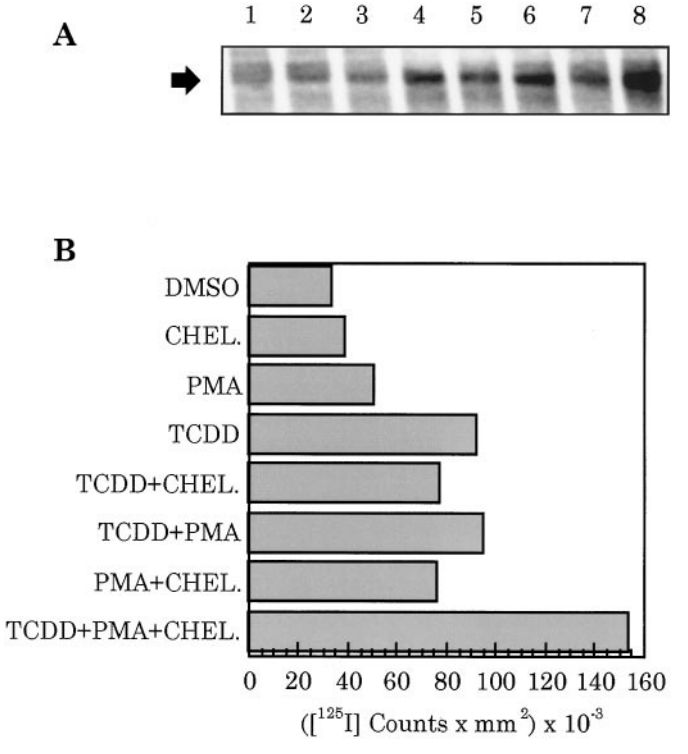

Fig. 8. Nuclear hAhR levels in treated P5A11 cells. P5A11 cells were grown to 80% confluency and serum fasted in α-MEM for 14 hr before treatment, and BSA was added to a final concentration of 5 mg/ml immediately before the addition of compounds. Cells treated with 4 μM Chel were preincubated with this compound for 15 min before the addition of other compounds. TCDD and PMA were added to final concentrations of 10 and 81 nM, respectively. Cells were incubated with compounds for 1.5 hr as indicated and assayed for luciferase activity as described in Materials and Methods. All compounds were solubilized in DMSO. A, Western blot analysis of nuclear hAhR levels in treated P5A11 cells. Blots were prepared, probed with mAb Rpt 1 followed by biotinylated-GAM IgG/[^{125}I]streptavidin, and visualized as described in Materials and Methods. *Lane 1*, 100 μg of nuclear protein from DMSO. *Lane 2*, CHEL. *Lane 3*, PMA. *Lane 4*, TCDD. *Lane 5*, TCDD + CHEL. *Lane 6*, TCDD + PMA. *Lane 7*, PMA + CHEL.. *Lane 8*, TCDD + PMA + CHEL. B, Quantification of nuclear hAhR levels in treated P5A11 cells. hAhR levels were quantified as described in Materials and Methods. hAhR levels are representative of the results of two independent experiments.

CYP1A1 is an endogenous gene in mammalian cells and its activity and expression in the cell likely are regulated by mechanisms occurring at the levels of transcription initiation, transcript stability, translational, or enzymatic activity levels like other cytochrome P450s (Kloepper-Sams and Stegeman, 1989; Ko *et al.*, 1996). In contrast to the stably transfected reporter construct used by Chen and Tukey, the pGUDLUC6.1 reporter construct, used in this study, contains four DREs and no other know regulatory elements that make this minimal DRE/luciferase reporter construct ideal for the examination of the role of PKC in AhR-mediated transcriptional regulation.

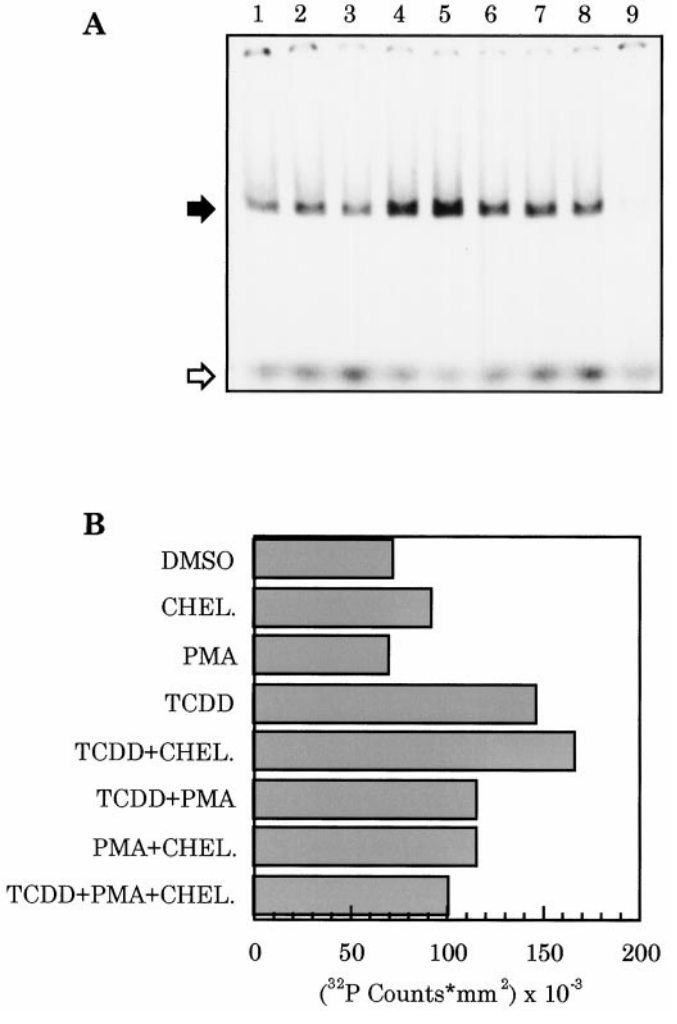

Fig. 9. DRE-specific EMSA of nuclear extracts from treated P5A11 cells. P5A11 cells were grown to 80% confluency and serum fasted in α-MEM for 14 hr before treatment, and BSA was added to a final concentration of 5 mg/ml immediately before the addition of compounds. Cells treated with 4 μM Chel were preincubated for 15 min before the addition of other compounds. TCDD and PMA were added to final concentrations of 10 and 81 nM, respectively. Cells were incubated with compounds for 1.5 hr as indicated and assayed for luciferase activity as described in Materials and Methods. Nuclear extract preparations of DRE-specific EMSAs are described in Materials and Methods. All compounds were solubilized in DMSO. A, *Filled arrow*, shifted complex. *Open arrow*, free ^{32}P-labeled DRE. *Lane 1*, nuclear extract from DMSO. *Lane 2*, CHEL. *Lane 3*, PMA. *Lane 4*, TCDD. *Lane 5*, TCDD + CHEL. *Lane 6*, TCDD + PMA. *Lane 7*, PMA + CHEL. *Lane 8*, TCDD + PMA + CHEL. *Lane 9*, 100-fold molar excess of unlabeled, wild-type DRE and nuclear extract from TCDD-treated cells. B, Quantification of the shifted, ^{32}P-labeled DRE complex in EMSA of nuclear extracts from treated P5A11 cells. The ^{32}P-labeled DRE complex levels were quantified as described in Materials and Methods.

Consistent with the observations of others, stimulation of PKC activity with PMA, a potent activator of DAG-binding PKC isoforms (Nishizuka, 1995), and the AhR pathway with TCDD in P5A11 and HG40/6 cells results in a several-fold enhancement in *trans*-activation of the stably transfected pGUDLUC6.1 reporter construct relative to cells treated with 1 nM TCDD alone. Importantly, this study demonstrates that the PMA effect in P5A11 cells is not a clonal artifact (Fig. 2) and was not unique to HeLa-derived cell lines (Fig. 5). Previously, Chen and Tukey (1996) suggested a possible mechanism by which PKC stimulation by PMA promotes the formation of DNA-binding AP-1 complexes, which then act in conjunction with the DRE-associated AhR/ARNT heterodimer to enhance transcription of AhR-responsive genes. This candidate mechanism is unlikely to contribute to the PMA effect in the P5A11 and HG40/6 cell lines because the stably transfected pGUDLUC6.1 reporter construct has no known AP-1 sites in the promoter region located 5′ to the luciferase reporter (Angel and Karin, 1991). Treatments of P5A11 and HG40/6 cells with PMA structural analogs (Figs. 1 and 5A) implicate the 12-myristate and 13-acetate moieties of PMA, which mimics the conformation of the acyl side chains in *sn*-1,2-diacylglycerol a compound required *in vivo* for stimulation of DAG-binding PKC isoforms, as required for the PMA effect. In addition, the PMA effect was abolished by the specific PKC inhibitors Chel and Bis I, which also abolished AhR-mediated *trans*-activation, but not the PKA activator 8-bromo-cAMP (Pray-Grant M, unpublished observations). These data are consistent with previous findings, thus further demonstrating that the PMA effect is due to stimulation of a DAG-binding PKC activity and that a PKC-mediated event is required for AhR-mediated *trans*-activation (Chen and Tukey, 1996). Importantly, our data demonstrate that the PMA effect is not a clonal artifact or unique to HeLa- or HepG2-derived cell lines.

The mechanism by which PMA-induced PKC stimulation results in the PMA effect may be through alteration of cellular levels of the hAhR or hARNT (Puga *et al.*, 1992), TCDD induced down-regulation of the hAhR, hAhR/hARNT heterodimer DRE-binding activity, the activity of coactivators, or the basal transcription machinery itself. As demonstrated quantitatively in Fig. 6, TCDD-induced down-regulation of the AhR is unaffected by PMA or Chel treatments, demonstrating that this mechanism is not responsible for the PMA effect in P5A11 cells. Furthermore, the quantitative analyses in Figs. 6 and 7 demonstrate that 81 nM PMA alone does not alter AhR or ARNT levels in the cell sufficiently to account for the fold enhancement in *trans*-activation of the reporter construct, relative to TCDD-treated cells, which occurs during the PMA effect in P5A11 cells. The observation that Chel (Figs. 6 and 7) does not decrease AhR or ARNT levels in the cytosolic fraction of P5A11 cells relative to DMSO-treated cells demonstrates that this PKC inhibitor does not abolish the PMA effect or TCDD-induced AhR signal transduction by decreasing AhR or ARNT levels in the cell. Quantitative examination of nuclear AhR levels (Fig. 8), under conditions in which nuclear hAhR levels are maximal in HeLa cells (Singh *et al.*, 1996), revealed that the PMA effect is not the result of increased nuclear translocation of the AhR, which is consistent with EMSA data generated previously (Chen and Tukey, 1996). This experiment (Fig. 8) also revealed that Chel does not abolish the PMA effect or TCDD-mediated AhR

signal transduction by preventing the hAhR from translocating into the nucleus. EMSAs performed using nuclear extracts from treated P5A11 cells (Fig. 9) demonstrate that the PMA effect is not due to an increase in nuclear hAhR/hARNT heterodimer DRE-binding activity relative to cells treated with 1 nM TCDD alone, which also is consistent with EMSA data generated previously (Chen and Tukey, 1996). Quantification of the shifted, ^{32}P-labeled DRE complex (Fig. 9B) suggests that PMA treatments cause a decrease in DRE-binding activity, although EMSA is not an ideal method for obtaining quantitative data. In addition, the mechanism by which the inhibitor Chel abolishes the PMA effect and TCDD-mediated signal transduction does not seem to involve a reduction in nuclear hAhR/hARNT heterodimer DRE-binding activity (Fig. 9). Fig. 3 demonstrates that the PMA effect is not due to a luciferase gene/message stabilization mechanism or a fortuitous, PMA-responsive TRE in the pGL3-vector series (Reifel-Miller *et al*., 1996). Importantly, Fig. 3 also demonstrates that PMA stimulation of PKC does not seem to affect the basal transcription machinery, which previously had been suggested as an explanation for the PMA effect (Chen and Tukey, 1996). Furthermore, it seems that the PMA effect may be due to an event-mediated directly by PKC, because the effect occurs as early as 1.5 hr after PMA treatment (Long WP, unpublished observations). Based on these data, and consistent with the studies of others, the most likely mechanism by which the PMA effect occurs may be through a PKC-mediated event that alters the ability of the AhR/ARNT complex to recruit coactivators or through a direct effect on a specific coactivator involved in AhR/ARNT directed assembly of transcription complexes.

A related explanation for the PMA effect is that stimulation of PKC by PMA alters cellular phosphorylation patterns and results in alterations in the activities of proteins involved in the basal transcription machinery and chromatin structure (Chen and Tukey, 1996). Although possible, this seems unlikely because in transient transfections of HeLa cells with the pGL3-Control vector (Fig. 3), the basal expression levels of luciferase are unaffected by PMA treatments. In addition, PMA has no effect by itself (Fig. 1) on basal expression levels of luciferase in P5A11 cells, which constitutively have detectable nuclear levels of the hAhR and hAhR/hARNT heterodimer (Figs. 8 and 9; Singh *et al*., 1996). Although not entirely eliminating the possibility that the PMA effect is due to a more "open" chromatin structure, these experiments make this alternative explanation seem unlikely.

In summary, we generated HeLa- and HepG2-derived cell lines, the P5A11 and HG40/6 cell lines, stably transfected with the minimal DRE/luciferase reporter construct pGUDLUC6.1, that are suitable for screening the effects of biologically active compounds on AhR-mediated signal transduction. Data generated using these model systems indicate that PKC activity is required for the AhR to direct assembly of a fully functional transcription complex, a finding consistent with the studies of others (Carrier *et al*., 1992; Schafer *et al*., 1993; Chen and Tukey, 1996; Li and Dougherty, 1997). The exact PKC isoforms and classes involved in this process, however, are unresolved because PMA activates both conventional isoform category PKCs and novel isoform category PKCs. Furthermore, the specific PKC inhibitors Chel and Bis I, used here, have not been shown to inhibit preferentially the activity of a particular PKC isoform or isoform class. It also should be noted that in light of our data and those of others, the activation requirements and differential expression of PKC isoforms in specific cell types and tissues may make an important contribution to the cell-specific modulation of AhR activity in different tissues and during development.

Acknowledgments

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