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# Regulation of Cytochrome P4501B1 (CYP1B1) in Mouse Embryo Fibroblast (C3H10T1/2) Cells by Protein Kinase C (PKC)

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**ABSTRACT.** The effects of co-treatment of C3H10T1/2 (10T1/2) cells with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on the expression of the novel cytochrome P4501B1 (CYP1B1) were investigated. As monitored by CYP1B1-catalyzed 7,12-dimethylbenzanthracene (DMBA) metabolism, TPA suppressed basal and TCDD-induced DMBA metabolism in a concentration-dependent manner, with a maximum inhibitory concentration of 100 nM. The suppression of CYP1B1 catalytic activity occurred at two time points during which protein kinase C (PKC) was activated and down-regulated in these cells as judged by analyses of cellular PKC content and PKC-inhibitor (chelerythrine chloride)-influenced suppression of CYP1B1 catalytic activity. Experiments in which TCDD and benzanthracene (BA)-induced DMBA metabolism were monitored in PKCβ1-overexpressing 10T1/2 cells revealed that the suppression of CYP1B1 activity is a consequence of cellular PKC elevation. This suppression phenomenon could be accounted for by PKC-mediated suppression of TCDD-induced CYP1B1 mRNA and apoprotein and of nuclear translocation of the Ah-receptor. In contrast, the mitogen-activated protein kinase (MAPK) proteins ERKs 1 and 2 were stimulated by TCDD under conditions in which PKC was activated. Collectively, our results suggest that PKC participates in the regulation of CYP1B1 in 10T1/2 cells, positively by directly suppressing the Ah-receptor signaling pathway, followed by an indirect or negative activation of the MAPK signaling pathway. BIOCHEM PHARMACOL **57**;6:619–630, 1999. © 1999 Elsevier Science Inc.

**KEY WORDS.** TCDD; BA; CYP1B1; protein kinase C; AhR; MAPK

TCDD† is the most toxic of a large chemical class of environmental contaminants, the PAHs, which include polychlorinated dibenzo-*p*-dioxins, dibenzofurans, polychlorinated biphenyls, and polybrominated biphenyls [1]. The toxic effects of TCDD in domestic animals, wildlife, and humans [2, 3] include cancer (tumor promotion), teratogenesis, immune suppression, and death [3]. Various studies have demonstrated that the untoward effects of TCDD emanate from alterations in cell growth and differentiation [2, 4, 5]. TCDD also modulates the expression of several enzymes that catalyze the biotransformation of endo- and xenobiotics to reactive intermediates that can cause DNA damage by adducting to DNA and initiating the mutagenic events responsible for tumor initiation [6].

Extensive research has established that the pleiotropic biologic effects of TCDD are mediated through high-affinity binding to a cytosolic protein called the AhR, a member of the basic helix-loop-helix (bHLH) family of DNA-binding proteins [7]. TCDD and related ligands bind initially to the inactive cytosolic complex composed of the AhR and the heat shock proteins HSP90 and HSP40 [8, 9]. Upon binding TCDD, the heat shock proteins dissociate from the AhR (transformation), and the latter translocates to the nucleus, where it heterodimerizes with the Arnt. This AhR:Arnt complex subsequently binds to specific genomic promoter sequences, called dioxin or xenobiotic responsive elements (DREs or XREs), resulting in the transcriptional activation of genes that contain the appropriately located XREs [10, 11].

Thus, signal transduction for the induction of specific genes by TCDD and related PAHs is mediated by the AhR. These genes (Ah-battery) include those encoding the enzymes cytochromes P4501A1, 1A2, and 1B1, as well as glutathione *S*-transferase, Ya subunit, quinone oxidoreductase, UGT (UDP-glucuronosyltransferase), and aldehyde dehydrogenase 3C, which participate in the metabolism of the PAHs themselves [12]. However, thus far only the induction of CYP1A1 has been studied extensively, making it a model for studies of AhR-mediated and TCDD-dependent modulation of the expression of genes inducing

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† *Abbreviations*: AhR, aryl hydrocarbon receptor; Arnt, aryl hydrocarbon nuclear translocator; BA, benz[*a*]anthracene; BCA, bicinchoninic acid; CC, chelerythrine chloride; CYP1A1, CYP1A2, and CYP1B1, cytochrome P4501A1, P4501A2, and P4501B1; DMBA, 7,12-dimethylbenzanthracene; ECL, enhanced chemiluminescence; ERK, extracellular signal-regulated protein kinase; FBS, fetal bovine serum; MAPK, mitogen-activated protein kinase; PAH, polycyclic aromatic hydrocarbon; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; SSPE, 0.75 M NaCl, 0.05 M $NaH_2PO_4$, 0.005 M EDTA, pH 7.4; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; and TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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the enzymes that activate procarcinogens and promutagens [13]. These studies include the modulation by PKC of the activity and expression of CYP1A1 in many cell types (MCF-7, Hepa-1, and keratinocytes) and tissues (liver, kidney, and mouse skin) [14–20]. The results from these studies reveal that PKC activation effects a suppression of TCDD-induced CYP1A1 expression due to a reduced concentration of nuclear AhR. The effects of PKC on CYP1A1 induction may, however, be cell-specific. For instance, even though PKC activation by TPA provoked a suppression of TCDD-induced CYP1A1 expression in MCF-7 and Hepa 1c1c7 cell lines, only MCF-7 cells recovered from this suppression after a prolonged time interval [15]. Furthermore, the interaction between TPA and TCDD with respect to CYP1A1 expression was not as pronounced in T47D breast cancer cell lines as in MCF-7 or Hepa 1c1c7 cell lines. Other studies have reported differences in the induction of CYP1A1 and CYP1A2 between tissues following co-administration of TCDD and TPA to C57BL/6J mice [18, 19]. Thus, AhR-mediated and PKC-directed signal transduction mechanisms for the induction of *CYP1A1* and *1A2* genes are different from cell to cell and from tissue to tissue [16, 18, 20]. This difference presumably is due to an alteration in the expression of dioxin-responsive genes, as well as the importance of such an alteration to the cells.

One such gene is the *CYP1B1* gene, which encodes CYP1B1 and has been cloned recently in this laboratory from mouse embryo fibroblasts (C3H10T1/2 cells) and rat adrenal cortex [21, 22]. *CYP1B1* also has been cloned by other laboratories from rat liver and kidney as well as from human keratinocytes [23, 24]. *CYP1B1* is an AhR-battery gene, that is, its induction is stimulated by PAHs including TCDD, via the Ah receptor [21]. Relative to CYP1A1, CYP1B1 is expressed preferentially in steroidogenic tissues (adrenals, testis, ovary) and in stromal fibroblasts of steroid-sensitive tissues such as breast, prostate, testis, and embryonic cells [21, 25–27]. Unlike CYP1A1, CYP1B1 is expressed constitutively as well as after PAH induction in normal breast ductal epithelial cells and in human breast carcinoma cells [26, 27]. A more recent extensive investigation has revealed that of cytochromes 1A1, 1A2, and 1B1, 1B1 possesses the highest catalytic activity for the activation of several carcinogenic hydrocarbons, heterocyclics, and arylamines in extrahepatic tissues [28].

In view of this finding, coupled with the evidence for the tissue and cellular distribution of CYP1B1, CYP1B1 may play some role in the metabolism of chemicals, including carcinogen activation in cells and tissues of endocrine origin. Therefore, studies on the signal transduction mechanisms whereby CYP1B1 is regulated in cells and tissues that express this cytochrome P450 are clearly justified. Even though the uncontrolled, proliferative aspects of cancers at the molecular and cellular levels are closely tied up with the signaling pathways that control cell division [29], information on the cellular signaling mechanisms that are mediated by the complete carcinogen TCDD in particular [30], and by the PAHs in general, is still scanty [31]. In the present study, we investigated, with modulators of PKC, the role that this enzyme (PKC), which is central to cellular signal transduction mechanisms [32], plays in regulating the induction of the activity and expression of CYP1B1. In addition, we inquired whether the MAPK signaling pathway is also responsive to the effects of PKC on CYP1B1 regulation. Mouse embryo fibroblasts (10T1/2 cells), which preferentially express CYP1B1 as virtually their sole cytochrome P450 [33, 34], were used in this investigation. To our knowledge, no studies on the regulation of any cytochrome P450 gene have been conducted on this cell type.

We show that short- or long-term exposure of these cells to TPA, which corresponds to activation and down-regulation of PKC, respectively, inhibited CYP1B1 expression. CYP1B1 suppression was also observed following inhibition of PKC by a PKC-specific inhibitor. Our findings were fortified by observations of CYP1B1 down-regulation in mouse embryo fibroblasts, which contain high levels of PKC due to overexpression of the PKCβ1 isoform by these fibroblasts. The results of our experiments additionally suggest an interaction between CYP1B1 suppression and MAPK activation by PKC in 10T1/2 cells.

## MATERIALS AND METHODS

### *Materials*

TPA and CC were bought from L. C. Services Inc.; TCDD was obtained from Accustandard, Inc. [$\alpha$-$^{32}$P]dCTP and [$\gamma$-$^{32}$P]ATP were purchased from New England Nuclear. Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 (DMEM/F12) and RPMI 1640 medium were obtained from GIBCO. FBS was bought from Gemini. Rabbit polyclonal antibody to CYP1B1 was generated in this laboratory. Rabbit antibodies to AhR and Arnt were gifts from Dr. Edward Glover of the McArdle Laboratory. Anti-active MAPK polyclonal antibody and horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody were bought from the Promega Corp. Solvents for HPLC as well as tissue culture plates were bought from Fisher Scientific. DMSO, PMSF, glucose-6-phosphate, NADP, DMBA, penicillin/streptomycin, and protease inhibitor mixture were bought from the Sigma Chemical Co. The chemicals for SDS–PAGE were obtained from Bio-Rad, Inc. ECL film (Hyperfilm), ECL reagents, nitrocellulose, and nylon membranes were purchased from Amersham. A BCA protein kit was obtained from the Pierce Chemical Co. Mouse embryo fibroblasts infected with the pMV7 vector containing a PKCβ1 cDNA insert (P9/PKC), as well as fibroblasts infected with the PMV7 vector alone (P9), were gifts from Dr. I. Bernard Weinstein of Columbia University.

### *Preparation and Treatment of Cell Culture*

Mouse embryo fibroblasts (C3H10T1/2 cells) were maintained routinely in monolayer culture in DMEM/F12 medium supplemented with 10% FBS, 2 mM L-glutamine

(Sigma), and 10 μg/mL of penicillin/streptomycin (Sigma) and kept in a humidified incubator at 37° in an atmosphere of 95% air and 5% $CO_2$. The cells were usually grown to confluence, but prior to each experiment the cells were rendered quiescent by replacing the medium with DMEM/F12 devoid of 10% FBS for 16–18 hr. For some of the experiments, cells were grown in either 150- or 100-mm dishes (Corning), while for others, the cells were grown in 6- or 12-well dishes (Falcon). Generally, the cells were treated with the effector compounds, TCDD, TPA, CC, or DMBA, which had been dissolved in DMSO. Control cells received DMSO alone to a final concentration that did not exceed 0.2% in every case. The concentrations of the effectors used, as well as the times of treatments, are indicated in the respective figure legends.

### *Assay of PKC Activity*

PKC activity was assayed in whole cell extracts, which were prepared by a modification of a previously described method [20]. Briefly, cells were harvested into a lysis buffer of 20 mM Tris–HCl, pH 7.5, 5 mM EGTA, 2 mM EDTA, 250 mM sucrose, 1 mM PMSF, 15% (v/v) glycerol, 0.02% leupeptin (Sigma), and 50 KIU of aprotinin (Sigma). However, the cells were incubated for 1 hr at 4° before being sonicated for 5 sec followed by centrifugation at 14,000 rpm and 4° in a microcentrifuge. The precipitate obtained was suspended in the above buffer containing 1.0% Triton X-100. After a 1-hr incubation at 4°, the suspension was recentrifuged at 14,000 rpm to obtain the Triton X-soluble fraction. PKC activity was determined in this fraction by measuring the incorporation of $^{32}P$ from [$\gamma$-$^{32}P$]ATP (New England Nuclear, No. NEG 035) to the threonine residue of a PKC-specific peptide substrate using a PKC assay kit supplied by Amersham. The reaction was performed for 20 min at 30° and thereafter stopped on ice. Protein in the sample was precipitated with trichloroacetic acid to a final concentration of 10% with the addition of 50 μL of a solution of 10 mg/mL of BSA as a carrier protein [35]. The protein precipitate was collected on 2.4 cm Whatman GF/C glass fiber filters on a Millipore vacuum manifold, washed three times with 5% trichloroacetic acid, and after air-drying the filter fibers, the isotope content was measured by scintillation counting. Specific activity of PKC was expressed as picomoles per minute per milligram of protein.

### *Cell Fractionation*

CYP1B1 protein in treated and control cells was analyzed in lysates prepared with a modified RIPA buffer [36]. Briefly, the cells were washed two times in PBS and then scraped into RIPA buffer, which contained 50 mM Tris–HCl, pH 7.4; 150 mM NaCl; 1.0 mM EDTA; 1.0 mM EGTA; 1.0 mM $Na_3VO_4$; 40 mM NaF; 10 mM $Na_2MoO_4$; 1.0 mM PMSF; 0.05% SDS; 0.25% deoxycholate; 1.0% NP-40 detergent; and 100 μL of a protease inhibitor mixture containing 4-(2-aminoethyl)benzyl sulfonyl fluoride (AEBSF); pepstatin A; transepoxysuccinyl-*l*-leucylamido(4-guanidino)butane (E-64); bestatin; leupeptin; and aprotinin. The cells were then homogenized at 4° by passing the resulting viscous solution 5–10 times through a 23-gauge needle. The supernatant from a 14,000 rpm microcentrifugation was used for analysis.

AhR and Arnt proteins were identified in treated and control cells, which were fractionated into the cytosolic and nuclear components according to a previously described method [37]. In brief, the media in treated and control cells were aspirated off completely and the cells washed briefly with a trypsin/EDTA solution (0.05% trypsin/0.5 mM EDTA). After adding trypsin/EDTA solution to completely cover the cell monolayer, the cells were left for 5 min in the humidified cell culture incubator to detach from the plates. The cells, which were collected from a 10,000 rpm centrifugation, were washed twice with PBS and suspended in a 2× lysis buffer made up of 50 mM HEPES, pH 7.4, 2 mM dithiothreitol (DTT), 40 mM sodium molybdate, 10 mM EGTA, 6 mM $MgCl_2$, 20% glycerol, 0.1 mg/mL of PMSF, and 2% NP-40. All subsequent procedures were as previously described [37]. Dually phosphorylated, activated forms of MAPK (ERKs 1 and 2) were identified in a 97,000 *g* supernatant prepared according to a procedure described previously [38, 39]. Briefly, cell lysates were generated in a buffer consisting of 20 mM Tris–HCl, pH 7.5, 20 mM *p*-nitrophenyl phosphate, 1 mM EGTA, 50 mM sodium orthovanadate, and 5 mM benzamidine before being homogenized with 30–50 strokes in a Dounce homogenizer at 4°. The 97,000 *g* supernatant was obtained from an initial supernatant derived from an initial 4000 *g* centrifugation of the cell lysate.

### *Immunoblot (Western) Analysis*

Aliquots containing known amounts of protein from the cell fractions above were separated by SDS–PAGE according to the method of Laemmli [40]. For the immunodetection of the AhR and Arnt proteins, 7.5% polyacrylamide gel was used to fractionate proteins; a 12% gel was employed for MAPK proteins. Thereafter, fractionated proteins were transferred electrophoretically at room temperature for 2 hr onto a nitrocellulose membrane, using the method of Towbin *et al.* [41]. Transfer efficiency was monitored with Ponceau S stain. Non-specific binding sites were blocked overnight at 4° with 5% fat-free dried milk in TBST solution (20 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.2% Tween 20), and the membrane was incubated subsequently for 1 hr at room temperature with each of the primary antibodies indicated in the appropriate figure legends. The washed membrane was incubated further at room temperature for 1 hr in horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody. After washing the membrane with TBST, the immunoreactive proteins were visualized using ECL detection reagents (Amersham) according to the manufacturer's protocol. The

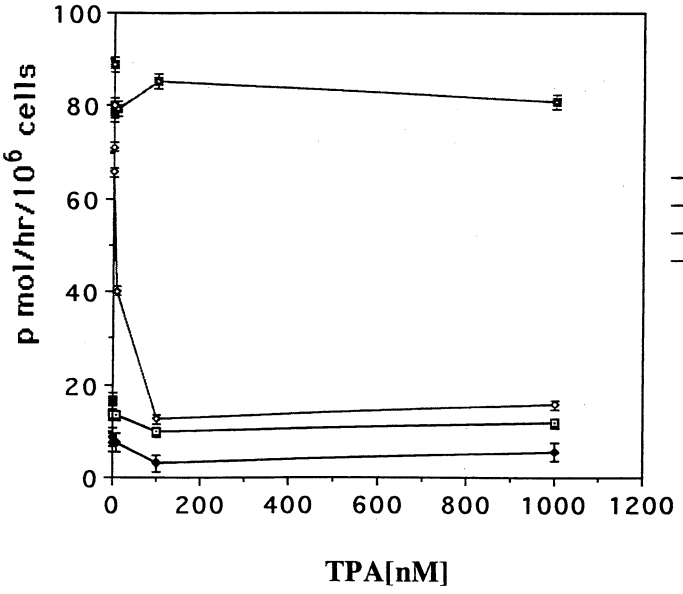


**FIG. 1. Effects of TPA on basal and TCDD-induced CYP1B1 functional activity in 10T1/2 cells. 10T1/2 cells were treated in 6-well culture dishes with 10 nM TCDD and the indicated concentrations of TPA. The cells were washed with PBS at the end of 6 hr and refed with fresh media containing 10 mM DMBA. At the end of 90 min, DMBA metabolites in the respective media were analyzed as described in Materials and Methods. The cells were counted in a hemocytometer. Each point on the graph represents the mean of two independent experiments ± range as indicated by the error bars.**

intensities of the protein bands were quantified by laser densitometry.

### *RNA Extraction and Northern Blot Analysis*

Total RNA from treated and control cells was extracted by the acidic phenol extraction method of Chomczynski and Sacchi [42] using the Trizol reagent. RNA was resolved on a 1% agarose, formaldehyde-denaturing gel. Ethidium bromide (5.0 μL of a 10 mg/mL stock solution) was added to the gel in order to ensure that the integrity of the RNA was maintained after electrophoresis prior to transfer. Fractionated RNA was capillary-transferred with 20× SSPE and cross-linked in a UV Stratalinker, 1800 (Stratagene) onto nylon membrane using an earlier procedure [22]. A *Sma*I fragment of clone 11 of the mouse CYP1B1 cDNA (175 base pairs of 5′-noncoding and 853 base pairs of coding regions) was labeled with [α-$^{32}$P]dCTP (3000 Ci/mmol) using a 'ready-to-go' DNA labeling reaction mixture bead supplied by Pharmacia Biotech Inc. The membrane was prehybridized at 65° for 30 min with Express Hyb solution formulated by Clontech. Hybridization in the same solution was performed at 65° for 1 hr with the labeled probe. Prehybridization and hybridization procedures were done in a rotisserie hybridization oven/shaker (Amersham Life Sciences). After hybridization, the membrane was initially washed at room temperature with four changes of 2× SSPE, 0.05% SDS solution, and finally at 50° with two changes of 0.1× SSPE, 0.1% SDS solution. The membrane was subsequently exposed to X-ray film, and the hybridized CYP1B1 cDNA to CYP1B1 complementary RNA was identified by autoradiography. A similarly labeled β-actin cDNA was used to check loading efficiency and to quantify CYP1B1 mRNA.

### *Assay of DMBA Metabolism*

The metabolism of the carcinogen DMBA by control and treated cells was assessed according to earlier methods [43]. Thus, DMBA metabolites were released from the intact cells by β-glucuronidase at pH 5.0 and 37° and quantified by HPLC. DMBA metabolites were also monitored in metabolic studies with microsomes derived from control and treated cells according to previously described procedures [25]. DMBA metabolism in the intact cells was expressed as picomoles of metabolites per hour per $10^6$ cells, while microsomal DMBA metabolism was expressed as picomoles of metabolites per hour per milligram of microsomal protein.

### *Other Procedures*

Protein content was routinely assayed with the BCA kit. Protein assay was performed according to the manufacturer's directions. The trypan blue exclusion method was always used to monitor the viability of the cells. By this method, cell suspension was mixed with 0.5 mL of a solution of 1% trypan blue dye solution in 0.9% saline before being counted under the microscope in a hemocytometer. Dead cells were clearly stained with the dye solution. Cells from which data were obtained were 98–100% viable. Western and northern blots were scanned and quantified in a soft laser densitometer (Zeineh model SL-504-XL).

## RESULTS

Other studies have demonstrated that CYP1A1 activity is down-regulated in mouse kidney [18], epidermis [19], and human keratinocytes [20] following phorbol ester (TPA) treatment. We therefore investigated if the constitutive

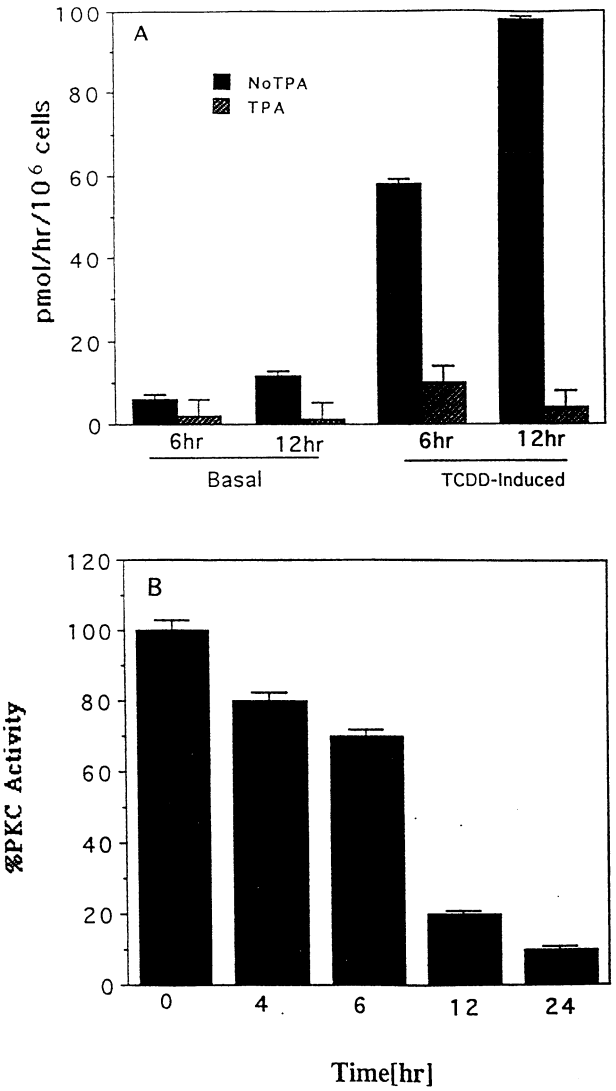


**FIG. 2. Effects of TPA on basal and TCDD-induced CYP1B1 activity (A) and on PKC activity (B). 10T1/2 cells were treated with DMSO ± 100 nM TPA (basal) and also with 10 nM TCDD ± 100 nM TPA (TCDD-induced) for the indicated time intervals. The cells were processed thereafter according to the legend to Fig. 1, followed by analysis of DMBA metabolites. Each of the plotted values in (A) represents the mean of two independent experiments ± range as indicated by the error bars. (B) Total PKC activity in cell membrane extracts prepared from control (DMSO) plus 100 nM TPA-treated 10T1/2 cells at the indicated time points. Cell membrane extracts and PKC activity were obtained as described in Materials and Methods. Each histogram represents the mean of three independent determinations ± SEM. Control PKC activities were subtracted from TPA-induced activity at each time point. The mean PKC activity at 0 hr was 1856 ± 6.2 pmol/min/mg protein and was taken as 100% activity.**

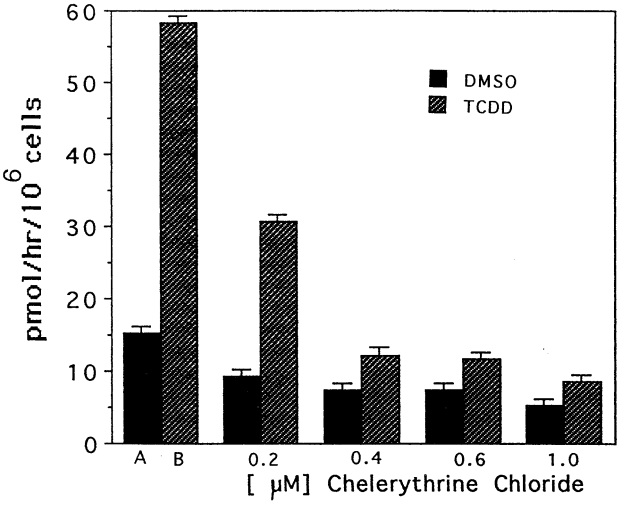


**FIG. 3. Inhibition of CYP1B1 activity by the PKC-specific inhibitor CC. Quiescent 10T1/2 cells were pretreated with the indicated concentrations of CC or with an equal volume of DMSO (control) at 37° for 2 hr before being treated with 10 nM TCDD or vehicle control for a further 6-hr period. Then the cells were analyzed for DMBA metabolism as in the legend to Fig. 1. Each histogram represents the mean of two independent determinations ± range as shown by the error bars. The histograms labeled A and B represent the values for basal (DMSO) and TCDD-induced DMBA metabolism in cells that did not receive CC.**

and/or TCDD-induced activity of the novel CYP1B1 was affected by TPA treatment in the same manner. The assessment of CYP1B1-catalyzed DMBA metabolism in 10T1/2 cells has been established in this laboratory as a convenient measure of the functional expression of the CYP1B1 protein, which is the only form of cytochrome P450 expressed by the 10T1/2 cell system [33, 34]. In the intact 10T1/2 cells, 10 nM TCDD provoked a dramatic 9-fold increase in CYP1B1 activity when compared with DMSO-treated cells (Fig. 1). This increase was suppressed by TPA in a concentration-dependent manner. The strongest suppression (85%) occurred at $10^{-7}$ M (100 nM), after which no more inhibition was noticeable. A 50% suppression of basal CYP1B1 activity (DMSO) also occurred with this TPA concentration. Basal and induced CYP1B1 activities were each suppressed by TPA (100 nM) in a time-dependent fashion (Fig. 2A) and in a manner that paralleled the inhibition of PKC activity in TPA-treated cells (Fig. 2B), in which the 30% decrease in PKC activity at 6 hr was decreased further by 55% at 12 hr.

To further examine the role of PKC in the induction of CYP1B1 activity, we pretreated quiescent 10T1/2 cells for 2 hr with a PKC-specific inhibitor, CC [44], at a concentration of 0.4 μM before the addition of either TCDD, TPA, or a combination of TCDD and TPA. The cells were subsequently analyzed for DMBA metabolism (see Materials and Methods). The concentration of CC used suppressed basal and TCDD-induced CYP1B1 activity by 50 and 80%, respectively, over a 2-hr period without inflicting cell death (Fig. 3). Higher CC concentrations resulted in some loss of cell viability.

To determine if intrinsic high PKC activity would provoke a suppression of CYP1B1 activity, DMBA metabolic experiments were conducted with mouse embryo fibroblasts, which overexpress the PKC isoform PKCβ1 (P9/PKC) and exhibit a 3-fold increase in PKC activity

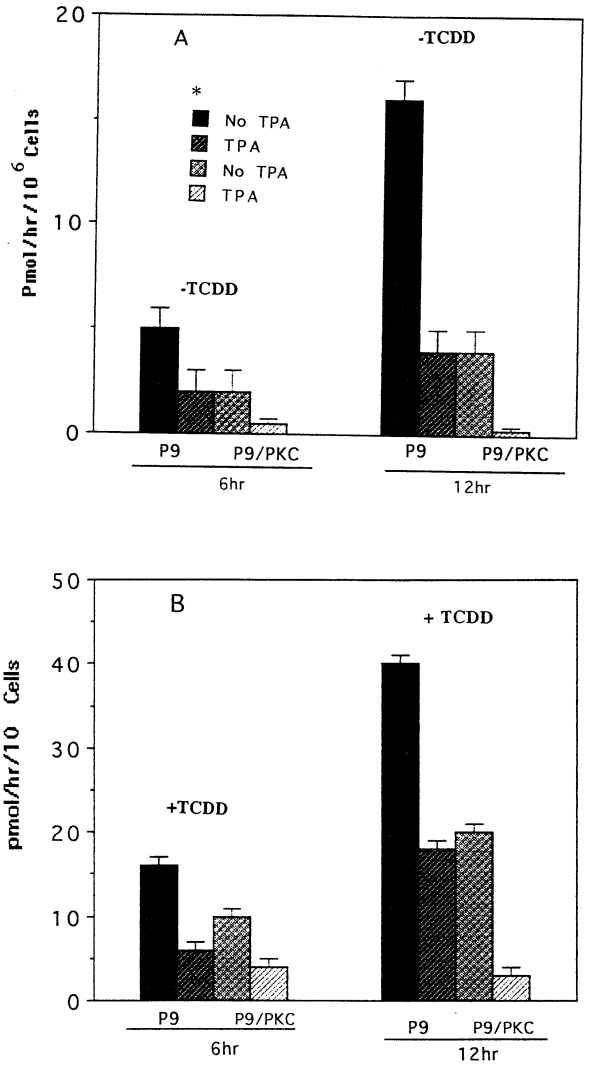


**FIG. 4. Effect of elevated PKC level due to PKC β1 overexpression in 10T1/2 cells on basal (A) and TCDD-induced (B) CYP1B1 activity in 10T1/2 cells. P9 (10T1/2 cells + PMV7 vector and no PKC cDNA) and P9/PKC (10T1/2 cells + PMV7 + PKC β1 cDNA) were treated with and without 100 nM TPA for the indicated time intervals. Thereafter, DMBA metabolism was monitored as described in the legend to Fig. 1 and in Materials and Methods. Each histogram represents the mean from two independent determinations ± range designated by the error bars. (A) and (B) are not represented on the same scale. *Also applicable to (B).**

when compared with the control cells infected with the empty viral vector (P9) [45]. Elevation of PKC due to an overexpression of PKCβ1 (P9/PKC) isoform suppressed basal (Fig. 4A) and TCDD-induced (Fig. 4B) CYP1B1 activity when compared with the control cells. Basal CYP1B1 activity was enhanced in P9 and P9/PKC cells in a time-dependent fashion (Fig. 4A). In P9 cells, a 40% increase in CYP1B1 activity occurred at 12 hr over the activity at 6 hr. In contrast, only a 15% increase was obvious in P9/PKC cells. Apparently the elevated PKC activity in P9/PKC cells suppressed the time-dependent increase in CYP1B1 activity. The elevated PKC level in P9/PKC cells also may have accounted for an additional 55% (6 hr) and 95% (12 hr) suppression of CYP1B1 activity by 100 nM TPA, in contrast with P9 cells, in which CYP1B1 activity was suppressed by 45% at 6 hr and 60% at 12 hr. TCDD induced CYP1B1 activity in P9 and P9/PKC cells also in a time-dependent fashion (Fig. 4B). A 65 and 50% increase in CYP1B1 activity occurred in P9 and P9/PKC cells, respectively, over a 6-hr period. Generally, TCDD induction of CYP1B1 activity in P9/PKC cells was less than in P9 cells, apparently due to intrinsically high PKC activity in P9/PKC cells. Endogenously elevated PKC activity also may have accounted for the time-dependent increased suppression of CYP1B1 activity (50% at 6 hr and 90% at 12 hr) in P9/PKC cells.

Evidence that elevation of PKC also suppressed BA-induced CYP1B1 activity induction is shown by the results in Table 1. The various regioselective DMBA metabolites were HPLC-quantified from a DMBA metabolic study performed with microsomes derived from uninduced and BA-induced P9 and P9/PKC cells. Clearly, in both uninduced and BA-induced cells, CYP1B1 activity was suppressed by the elevated PKC activity in P9/PKC cells as reflected by the 3-fold decrease in nearly all the individual as well as the total DMBA metabolites.

To investigate whether PKC activation and inhibition affect the induction of the *CYP1B1* gene in 10T1/2 cells, we employed northern blot hybridization (see Materials and Methods) to analyze the total RNA isolated from control and treated cells. After a 6-hr exposure, CYP1B1 mRNA accumulation was induced 7-fold by 10 nM TCDD, while 100 nM TPA suppressed gene expression to 60% below the control (DMSO) level (Fig. 5). A 2-hr pre-exposure to CC suppressed basal (DMSO) gene expression by 10% and provoked a 12-fold suppression of TCDD-induced *CYP1B1* gene expression. When compared with the control (DMSO), there was a 2.5-fold induction of CYP1B1 mRNA in cells that were treated with TCDD + TPA. This may be due to the residual inductive effect of TCDD. However, CC pretreatment dramatically suppressed this value well below the control (DMSO) level. Taken together, our results indicate that activation or down-regulation of PKC activity in 10T1/2 cells suppresses TCDD-induced *CYP1B1* gene induction in a manner that suggests that PKC is required for *CYP1B1* gene expression in 10T1/2 cells. Basal gene expression was not suppressed as dramatically.

To ascertain whether cellular CYP1B1 protein expression is as susceptible to the suppressive effects of PKC as *CYP1B1* gene expression, we employed immunoblot analysis to examine CYP1B1 expression in whole cell lysates from 10T1/2 cells that had been pretreated with CC or DMSO control as described in Materials and Methods. As depicted in panels A and B of Fig. 6, the 35-fold stimulation of CYP1B1 protein by TCDD was suppressed by 50% in response to PKC inhibition by CC. In contrast, PKC activation by TPA provoked a 96% suppression of CYP1B1 protein, which remained suppressed at this level in the presence of CC. Apparently, the inductive effect of TCDD

**TABLE 1. Suppression of basal and BA-induced CYP1B1 activity in P9/PKC cells that overexpress PKCβ1**

| C3H10T1/2 Treatment* | DMBA metabolite (pmol/mg/hr) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Dihydrodiols | | | | Phenols† | | |
| | 5,6− | 8,9− | 10,11− | 3,4− | A | B | Total |
| UT/P9 | 5.8 | 25 | 72 | 95 | 172 | 35 | 405 |
| UT/P9/PKC | 2.0 | 17 | 25 | 32 | 36 | 3 | 115 |
| BA/P9 | 13 | 88 | 178 | 257 | 448 | 66 | 1050 |
| BA/P9/PKC | 5.5 | 24 | 56 | 83 | 160 | 25 | 354 |

Values in the table are representative of at least three independent experiments with similar result patterns.

*UT denotes 10T1/2 cells that were not treated with BA (basal CYP1B1 activity). P9 = 10T1/2 cells infected with the empty vector. P9/PKC = 10T1/2 cells infected with pMV7 viral vector containing a PKCβ1 cDNA insert. BA denotes 10T1/2 cells that were treated with 10 μM BA.

†Total identifiable DMBA phenols consisting of 2- and 3-OH DMBA (phenol A) and 4-OH DMBA (phenol B).

accounted for the 16-fold increase in CYP1B1 protein over DMSO control in TCDD + TPA-treated cells. This increase was diminished by 60% following CC pretreatment. CC had no effect on DMSO-treated cells, which typically reflect basal CYP1B1 expression.

The suppression of CYP1B1 protein expression was reproduced in the microsomes of uninduced and BA-induced P9 and P9/PKC 10T1/2 cells (Fig. 6C) in which 2 and 5 μg microsomal protein derived from P9 and P9/PKC cells, respectively, were immunoblotted. Due to the overexpression of the PKCβ1 isoform in P9/PKC cells, CYP1B1 protein level was suppressed 4-fold even in the absence of BA stimulation. BA treatment provoked 9-fold suppression of microsomal CYP1B1 in P9/PKC cells. Together with the findings in Table 1, these observations indicate that overexpression of PKCβ1 suppresses CYP1B1 expression in these cells.

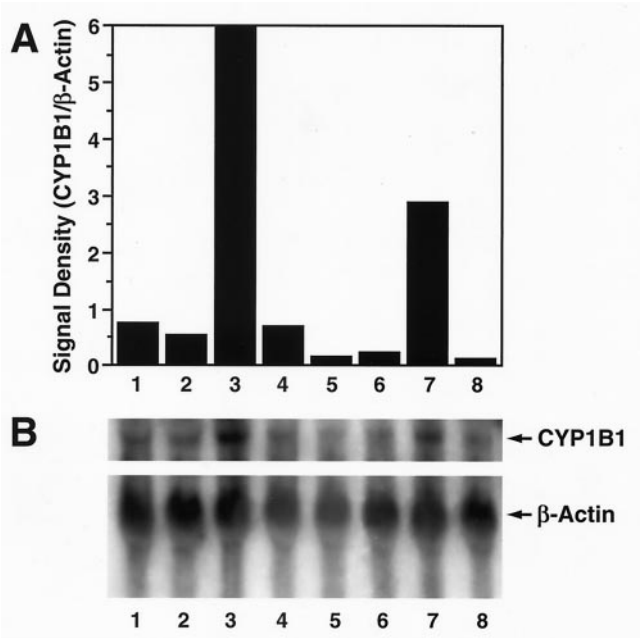


**FIG. 5. Effects of PKC activation by TPA and inhibition by CC on CYP1B1 mRNA accumulation in 10T1/2 cells. Quiescent normal 10T1/2 cells cultured in 150-mm culture flasks were pretreated with 0.4 μM CC or DMSO for 2 hr, at the end of which time 10 nM TCDD, 100 nM TPA, or TCDD + TPA were added to each flask. After 6 hr, total RNA was prepared from each of the flasks, and 20 μg of RNA was fractionated on a 1% agarose–formaldehyde gel. Fractionated RNA was analyzed for CYP1B1 mRNA by the northern blot hybridization method described in Materials and Methods. The blot shown is a representative of two runs with similar results. 1, DMSO; 2, DMSO + CC; 3, TCDD; 4, TCDD + CC; 5, TPA; 6, TPA + CC; 7, TPA + TCDD; 8, TPA+ TCDD + CC. Panel (A) shows the laser densitometer scans of the images on the blot (B).**

To determine whether the suppression of CYP1B1 expression by PKC results in a concomitant suppression of AhR translocation into the nucleus, we examined the effect of CC pretreatment on the translocation of the AhR protein into the nuclear fraction of treated cells. We initially determined that following TCDD exposure, maximal translocation of the AhR into the nuclear fraction of 10T1/2 cells occurred at 20 min, after which a down-regulation of AhR translocation was obvious with no changes in Arnt levels (data not shown). Following a 2-hr pretreatment with CC and subsequent treatment with TCDD, the quantity of AhR translocated from the cytosol (Fig. 7A, lane 3) into the nuclear fraction of 10T1/2 cells was decreased by 80% (Fig. 7B, lane 3). Since PKC inhibition by CC suppresses the quantity of nuclear AhR (lane 3), these results support the notion that PKC activation is required to effect a total TCDD-induced translocation of AhR from the cytosol (Fig. 7A, lane 4) to the nucleus (Fig. 7B, lane 4) in 10T1/2 cells.

To determine whether suppression of CYP1B1 and AhR nuclear translocation due to PKC activation would result in the activation of MAPK, we monitored, by immunoblot analysis, the expression of the dually phosphorylated active forms of MAPK proteins (ERKs 1 and 2) in 10T1/2 cells pretreated with and without CC in order to obtain an accurate measurement of activation of the ERK proteins in response to PKC activation and inhibition (Fig. 8). When compared with the DMSO control (lane 2), TCDD (lane 4), TPA (lane 6), or a combination of TCDD and TPA (lane 8), respectively, provoked an 8-, 16-, and 22-fold activation of ERK1 protein and a 10-, 38-, and 50-fold activation of ERK2 protein. In each case, this stimulation was antagonized by the PKC inhibitor CC as evidenced by the inhibition of ERK 1 and 2 proteins in CC-pretreated

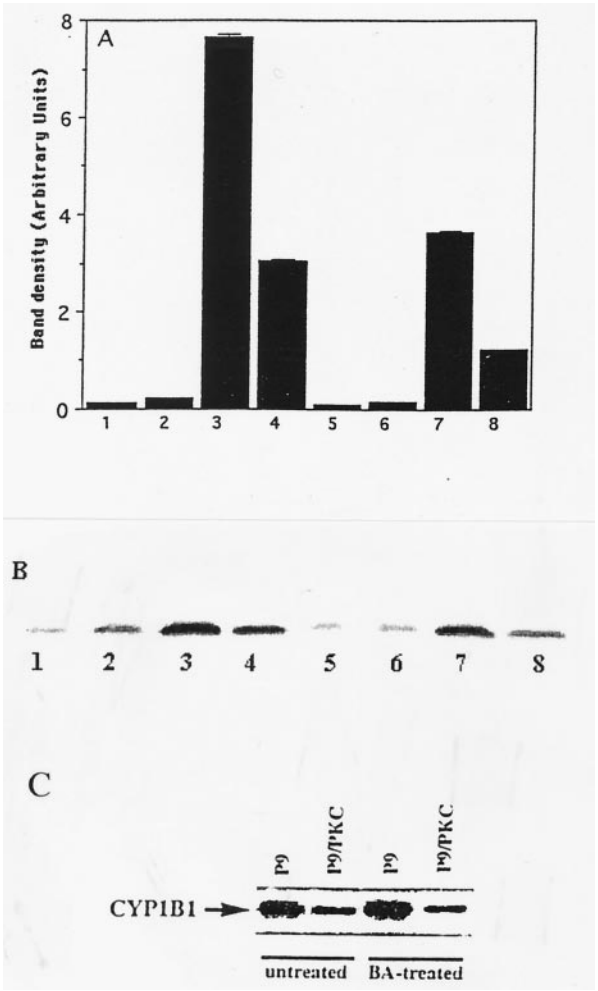


**FIG. 6. Effects of PKC activation and inhibition (A, B) or intrinsic elevation due to PKC β1 overexpression (C) on CYP1B1 protein expression in 10T1/2 cells. Quiescent normal 10T1/2 cells were treated with CC, TCDD, TPA (A, B) or BA (C) as stated in the legends to Fig. 5 and Table 1. Cell lysates (B) and microsomes (C) were prepared from the cells after harvest. Thirty micrograms of protein of cell lysates, or 2 and 5 μg microsomal protein from P9 and P9/PKC cells, respectively, were fractionated by SDS–PAGE gel electrophoresis. CYP1B1 protein was detected by western immunoblot (see Materials and Methods). The immunoreactive CYP1B1 protein was visualized by ECL using a kit supplied by Amersham. The upper panel (A) shows the densitometric scans of the immunoblot (B). Each bar represents the mean of the scans of three independent blots ± SEM. 1, DMSO; 2, DMSO + CC; 3, TCDD; 4, TCDD + CC; 5, TPA; 6, TPA + CC; 7, TPA + TCDD; 8, TPA + TCDD + CC.**

cells (lanes 1, 3, 5, and 7). Surprisingly, however, in the presence of TPA, CC increased the induction level of ERKs 1 and 2 when compared with the DMSO control. This unusual sensitivity of 10T1/2 cells to TPA with respect to MAPK activation requires further investigation.

## DISCUSSION

The present study provides evidence for the possible participation of PKC in the regulation of the novel CYP1B1 in

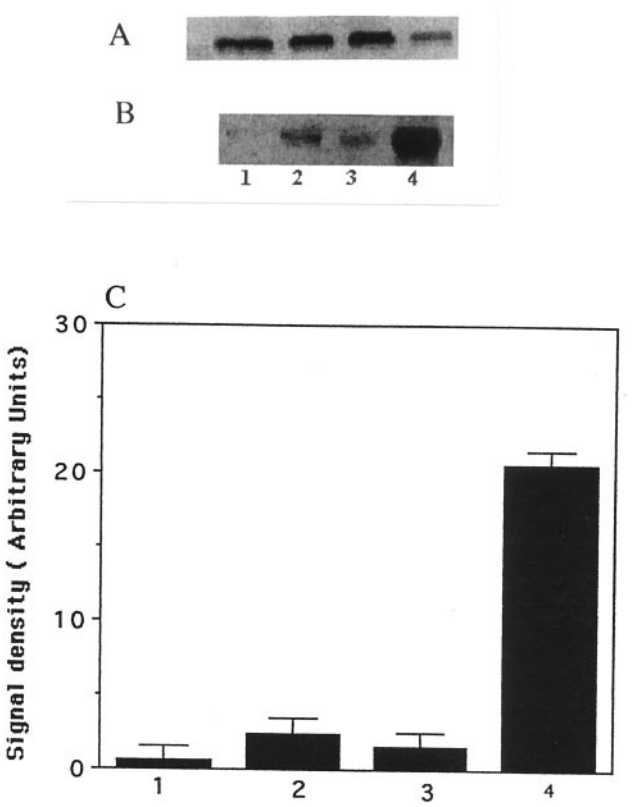


**FIG. 7. Effect of inhibition of PKC by CC on nuclear translocation of AhR. Quiescent normal 10T1/2 cells were pretreated for 2 hr with 0.4 μM CC or DMSO. The cells were treated thereafter with 10 nM TCDD, harvested after 20 min, and analyzed for AhR expression in the cytosolic (A) and nuclear (B) fractions as described in Materials and Methods. Twenty micrograms of each fraction was resolved on SDS–PAGE gel. (C) shows the laser densitometric scans of a representative nuclear AhR blot (B). Each bar represents the mean of three different blots ± SEM. 1, DMSO + CC; 2, DMSO; 3, TCDD + CC; 4, TCDD.**

10T1/2 cells. Our studies reveal that in TPA-exposed cells a considerable amount of PKC activity remained at 6 hr but was diminished dramatically almost to basal level at 12 hr. This diminished PKC activity paralleled CYP1B1 activity in cells that had been exposed for 2 hr to the PKC specific inhibitor CC.

Our observation that activation or inhibition of PKC suppresses basal and TCDD-induced CYP1B1 activity as judged by the measurement of CYP1B1-catalyzed DMBA metabolism in 10T1/2 cells is reminiscent of the results from previous studies [15, 19], which demonstrated a suppression of TCDD-induced CYP1A1-catalyzed EROD activity in murine hepatoma 1c1c7 cells and MCF-7 cells. In those studies, cells were pretreated with TPA prior to exposure to TCDD. In the present study, however, a simultaneous treatment protocol with TPA and TCDD, which also has been used in other studies [15], was adopted. The similar suppressive effect of CYP1B1 (DMBA metabolism) and CYP1A1 (EROD) activities in this and previous studies emphasizes that the suppression of cytochrome P450 activity is one of the target effects of PKC activation or

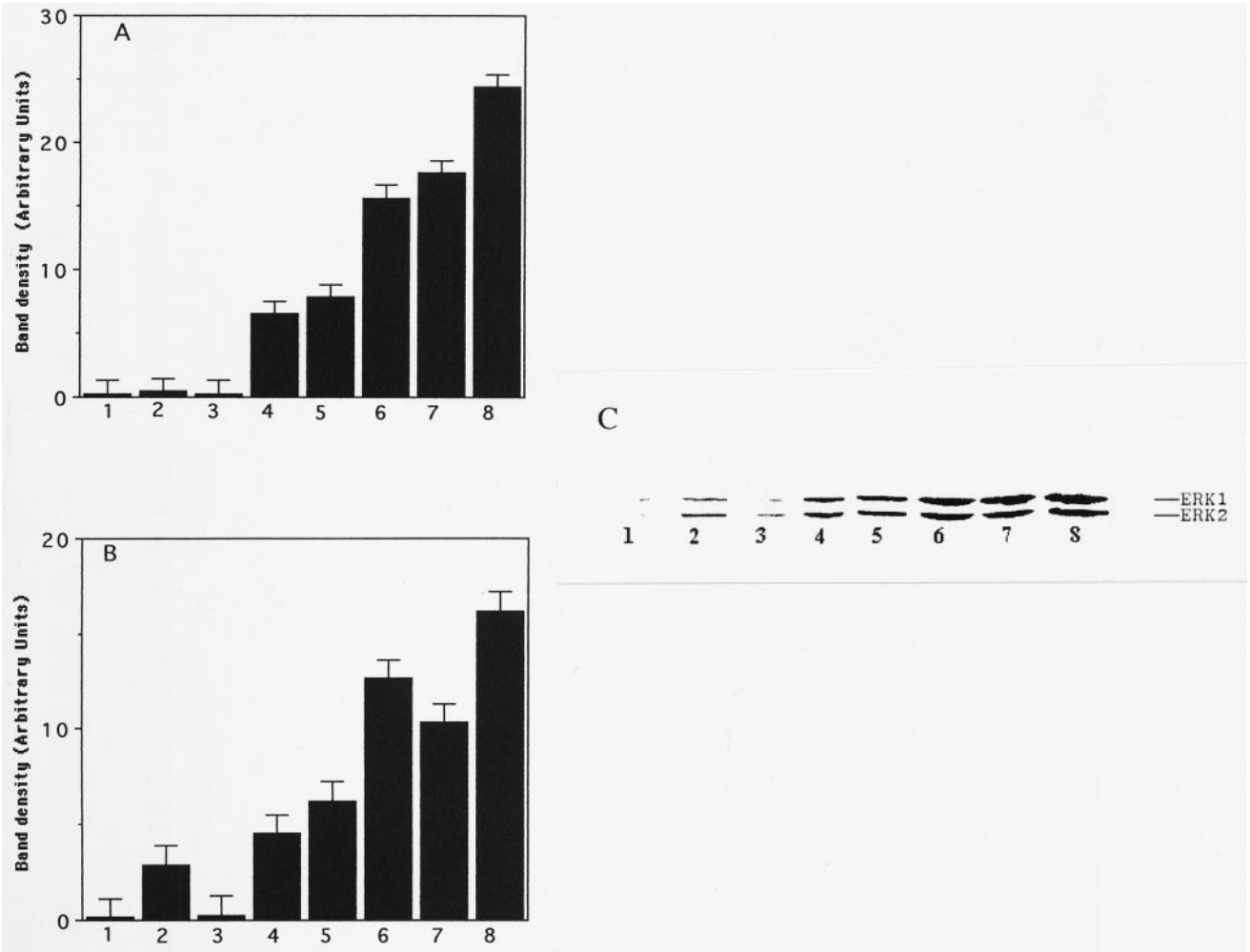


**FIG. 8. Effects of PKC activation and inhibition on the expression of the dually phosphorylated MAPK (ERKs 1 and 2) proteins in 10T1/2 cells. Quiescent normal 10T1/2 cells were pretreated with either 0.4 μM CC or DMSO for 2 hr. Cells were harvested, and 20 μg protein was processed by western analysis for MAPK (ERK 1 and 2 proteins) (see Materials and Methods). Each value on the histogram is the mean ± SEM of three independent densitometric scans of ERK1 (A) or ERK2 (B) proteins shown in the representative blot (C). Lanes 1, 3, 5, and 7 contain DMSO + CC; TCDD + CC; TPA + CC; and TPA + TCDD + CC, respectively. Lanes 2, 4, 6, and 8 contain DMSO; TCDD; TPA; and TPA + TCDD, respectively.**

inhibition. The finding that the inhibition of PKC activity concomitantly inhibits CYP1B1 activity suggests a requirement of PKC for both basal and TCDD-induced CYP1B1 activity. Apparently, PKC may still be required to maintain the constitutive as well as induced levels of CYP1B1 in 10T1/2 cells.

Cell lines that overexpress the various isoforms of PKC have been employed by several investigators to establish the roles of specific PKC isotypes in various cellular processes that are influenced by the PKC system. Such investigations are based on the premise that the different PKC isotypes are differentially expressed and regulated and subserve different functions in different cell types [46]. We observed that in the presence or absence of TPA, basal or TCDD-induced CYP1B1 activity was suppressed in 10T1/2 cells, which have an elevated PKC level due to the overexpression of the PKCβ1 isoform. Since overexpression of this PKC isotype in 10T1/2 cells has been shown previously to promote disordered growth as well as a malignant phenotype [45], it is tempting to suggest that PKCβ1-mediated suppression of CYP1B1 in 10T1/2 cells observed in the current study may play a signaling role in the expression of the malignant phenotype in 10T1/2 cells. In view of the differential expression, regulation, and functions of the various PKC isotypes, studies are currently underway to examine the role of different PKC isotypes in basal and PAH-induced CYP1B1 activity.

The requirement of PKC for CYP1B1 suppression seems to be more pronounced at the transcriptional level than at the translational level of the *CYP1B1* gene. While in every case PKC activation by TPA and inhibition by CC each suppressed CYP1B1 mRNA accumulation to control or below control levels, only in one case (TPA + CC) did such a trend occur with respect to CYP1B1 apoprotein. In fact, CYP1B1 protein was considerably visible in TPA + TCDD-treated cells. Thus, residual apoprotein level may be a consequence of a rapid turnover rate of CYP1B1 protein from the transcribed *CYP1B1* gene and may support the

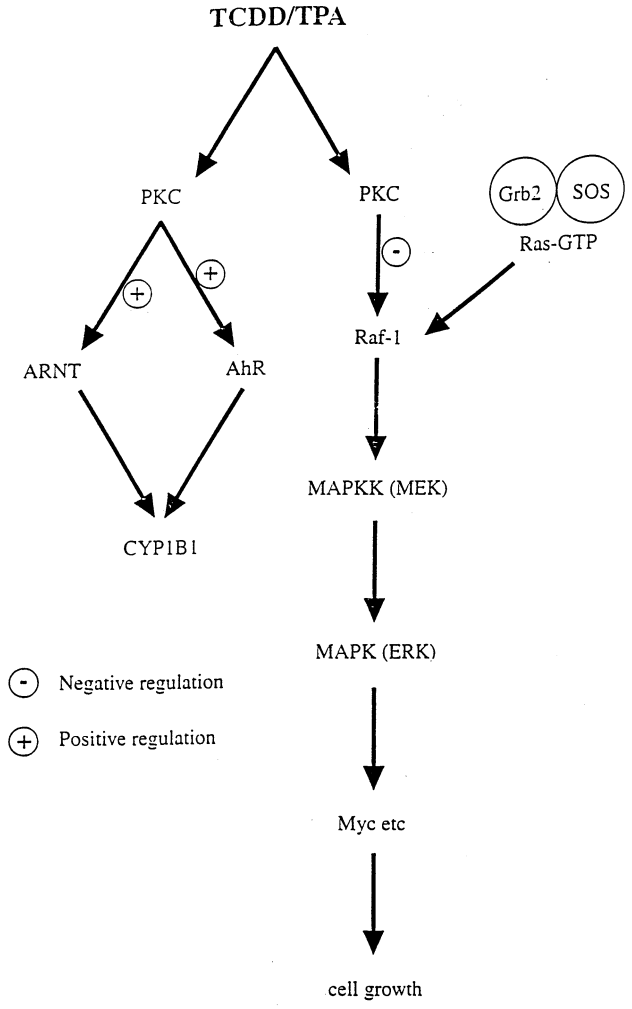


**FIG. 9. Proposed model for the regulation of CYP1B1 by PKC in C3H10T1/2 mouse embryo fibroblasts. TPA and TCDD each activate PKC. Activated PKC directly (positively) phosphorylates AhR and Arnt, and suppresses AhR translocation into the nucleus. MAPK is activated en route to the control of cell growth and proliferation via the activation of the immediate early genes c-*myc* and c-*fos*.**

notion that a certain threshold level of PKC may be required for basal and TCDD-induced *CYP1B1* gene expression in 10T1/2 cells. Possibly the inhibition of TCDD-induced CYP1B1 activity by PKC is due to an inhibition of TCDD-inducible gene products. These results point to a role for PKC-dependent phosphorylation in the processes, which in 10T1/2 cells, culminate in *CYP1B1* gene expression.

We employed two Ah receptor agonists (TCDD and BA) to demonstrate that the suppression phenomenon is Ah receptor-dependent. Our results, which show that nuclear translocated AhR is diminished but not totally precluded by CC treatment (Fig. 7B, lanes 3 and 4), suggest that in agreement with previous reports on PKC-influenced suppression of CYP1A1 [15, 17, 18], PKC activation affects AhR nuclear translocation only in a quantitative manner. Consequently, PKC may be necessary for total AhR nuclear translocation in 10T1/2 cells. It is possible that this PKC-mediated suppression of AhR nuclear translocation in 10T1/2 cells would modify either the AhR protein *per se*, or the signal transduction pathway that it modulates. Reports on the requirement of PKC for AhR nuclear transformation and functions (DNA binding) are contradictory, however. While the pretreatment of 1c1c7 cells with staurosporine reduced the amount of liganded AhR that bound to a DRE as monitored by gel shift assays [17], this was not the case in the hepatic cytosol of guinea pigs [47]. These contradictory findings suggest cell-specific effects of PKC on AhR nuclear translocation and functions and underscore the need for investigation of these parameters in different cell systems including 10T1/2 cells.

Taken together, our studies, which show that CYP1B1 expression was reproducibly suppressed on inhibition of PKC by CC, suggest that in 10T1/2 cells, PKC plays a positive or permissive role in regulating CYP1B1 activity, *CYP1B1* gene induction, CYP1B1 apoprotein expression, and AhR nuclear translocation. However, our observations that the intrinsic elevation of PKC also provokes suppressive effects on CYP1B1 suggest that PKC also may play a negative role in CYP1B1 regulation. The suppression, as in normal 10T1/2 cells, of basal or TCDD- or BA-induced CYP1B1 activity by PKC-overexpressing 10T1/2 cells supports this notion. By and large, the suppression of CYP1B1 expression appears to be a common pathway for the modulation of CYP1B1 by PKC in 10T1/2 cells.

PKC is known to induce and activate the MAPK signaling pathway in various cell types, presumably through the activation of Ras and Raf-1 cytoplasmic proteins [48–52]. This activation is characterized by the translocation of activated MAPK proteins (extracellular regulated kinases, ERK1 and ERK2) into the nucleus where they induce the immediate early genes (c-*fos*, c-*jun*, *jun*-B), increase AP-1 transcription factor, and regulate cell proliferation and differentiation [53–55]. We obtained results demonstrating that in TCDD and TPA-treated 10T1/2 cells, the dually phosphorylated active forms of ERKs 1 and 2 MAPKs are stimulated (Fig. 8). The antagonism of this stimulation by CC suggests a requirement of PKC for MAPK activation in response to these treatments. These data, therefore, highlight the possibility that the suppression of CYP1B1 expression, which, as currently observed, also involves a suppression of AhR nuclear translocation, may be accompanied by an activation of the MAPK proteins and presumably the MAPK signaling pathway that controls cell proliferation, differentiation, and development in many cell types [52, 53, 56]. From the results of the present study, we propose that PKC participates in the regulation of CYP1B1 expression in TCDD-stimulated 10T1/2 cells positively by a direct phosphorylation of the AhR and negatively by the activation of the MAPK signaling pathway. Whether PKC-influenced CYP1B1 suppression constitutes a primary signal for the pleiotropic effects of TCDD as a tumor promoter is presently unknown. Studies are currently underway to more precisely dissect the

components of this proposed signaling model (Fig. 9) for CYP1B1 regulation.

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