SYNERGISTIC ACTIVATION OF AH RECEPTOR-DEPENDENT GENE EXPRESSION BY ACTIVATORS OF PROTEIN KINASE C

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Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor known to mediate a diverse range of toxic and biological effects in a variety of species and tissues as a result of exposure to halogenated aromatic hydrocarbons (HAHs) and non-halogenated polycyclic aromatic hydrocarbons (PAHs). Many of the chemicals within these groups are ubiquitous, persistent and highly toxic environmental contaminants to which humans and animals are chronically exposed. Exposure to various HAHs, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin), the prototypical and most potent HAH, produces such effects as tumor promotion, teratogenicity, lethality, wasting, immuno-, hepato- and dermal toxicity as well as alterations in the expression of a battery of genes. TCDD and other AhR agonists bind to the AhR in the cytosol, wherein the receptor dissociates from its associated subunits, translocates into the nucleus and dimerizes with the Ah receptor nuclear translocator (ARNT) protein, a resident nuclear protein factor. Formation of the AhR:ARNT heterodimer converts the AhR complex into its high affinity DNA binding form, which binds to its specific DNA recognition sequence, the dioxin responsive element, stimulating the expression of assorted downstream genes. Several recent studies have shown that there is cross-talk between the AhR pathway and other signal transduction pathways, most notably that of protein kinase C (PKC). The PKC activator, phorbol-12-myristate-13-acetate (PMA) has been shown to synergistically increase the ability of AhR ligands (TCDD and β-naphthoflavone) to induce AhR-dependent gene expression. Using AhR ligand and DNA binding assays, we have identified several classes of bioactive lipids which can bind to and transform the AhR into its DNA binding form in vitro, albeit weakly. However, exposure of cell lines to some of these chemicals results in induction of AhR-dependent reporter gene expression to levels equivalent to or several-fold greater than that produced by a maximally inducing dose of TCDD. The known ability of some of these compounds to activate protein kinase signaling pathways suggests that the synergistic increase in gene expression is due to the concomitant activation of the AhR and second messenger signaling pathways. Our results are consistent with the hypothesis that cross-talk between AhR and second messenger signaling pathways can synergistically enhance AhR-dependent gene expression and suggests a scenario in which AhR-dependent signaling by weak ligands, either endogenous or exogenous, can be dramatically enhanced.

Materials and Methods

Cell Culture and Luciferase Assays. The recombinant mouse hepatoma cell line, H1L1.1c2, was derived from the Hepa1c1c7 line and grown and maintained as described in detail[1]. These cells contain a stably integrated DRE-driven firefly luciferase reporter gene plasmid, pGudLuc1.1, whose transcriptional activation occurs in a time-, dose-, ligand-, and AhR-dependent manner[1]. For chemical treatment, H1L1.1c2 cells were grown to 80% confluency in 6-well tissue culture

plates and incubated with the indicated chemical(s) in triplicate for 4 hours at 37°C. Where genistein (GEN) was used, it was added 2 hours prior to addition of TCDD and where chelerythrine chloride (CHEL) was used, this chemical was added 15 minutes prior to the addition of the other chemical(s) indicated. After incubation the cells were washed twice with 1X PBS and lysed. Luciferase activity was determined using a Dynatech ML3000 Microplate Luminometer with automated injection of Promega stabilized luciferase substrate as previously described[1]. Luciferase activity was normalized to sample protein concentration, determined using the fluorescamine protein assay[2] and bovine serum albumin as the standard and was calculated as relative light units (RLU) per mg of protein.

Results and Discussion

Treatment of the recombinant mouse hepatoma cell line, H1L1.1c2, indicates that the protein kinase A activator, 8-bromo-cAMP, and the tyrosine kinase inhibitor, genistein, do not significantly affect TCDD-induced reporter gene expression (Fig. 1). Although luciferase activity increases when the cells are co-treated with genistein and TCDD over treatment with TCDD alone, the effect appears to be additive rather than synergistic and suggests that activation by genistein is mechanistically different. The PKC activator PMA synergistically enhances TCDD-induced reporter activity two- to three-fold greater than TCDD treatment alone, as previously observed[3]. In addition, pre-treatment of the same cell line with the PKC inhibitor, chelerythrine chloride, inhibits both TCDD-induced reporter activity as well as the enhancement of this activity with PMA treatment, indicating the specific involvement of the PKC pathway in AhR-dependent gene expression (Fig. 2). A dose response for TCDD and β-naphthoflavone, a relatively weaker AhR ligand, in the presence and absence of PMA indicates that enhancement of AhR-dependent gene expression by PMA occurs for weak as well as high affinity ligands, at or near physiological concentrations (Fig. 3).

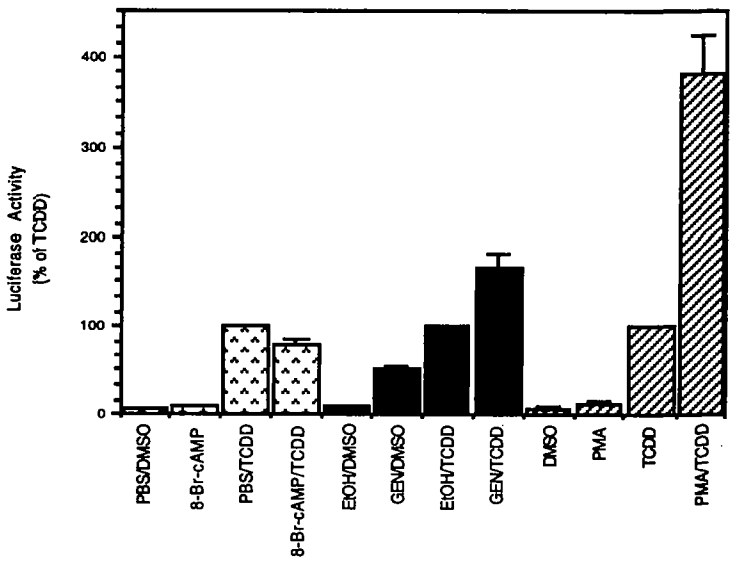

Figure 1. Effects of 1 mM 8-Br-cAMP, 5 μM genistein, and 81 nM PMA on TCDD-induced expression of a DRE-driven reporter gene.

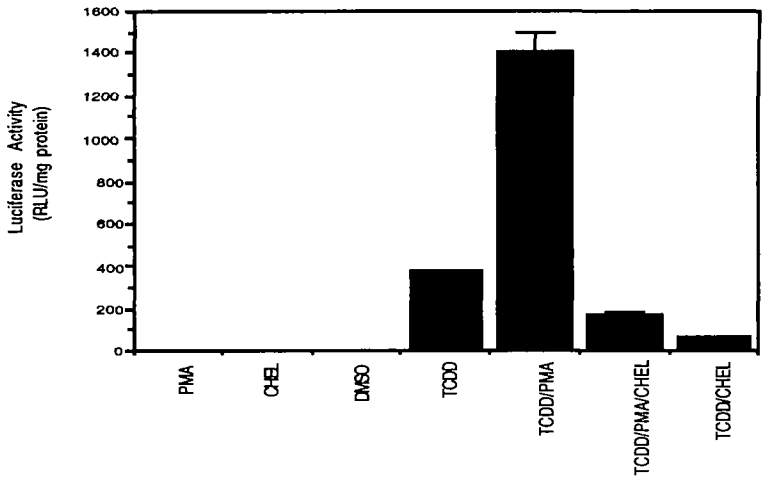

Figure 2. Effect of 81 nM PMA and 4 μM chelerythrine chloride on TCDD-induced expression of a DRE-driven reporter gene.

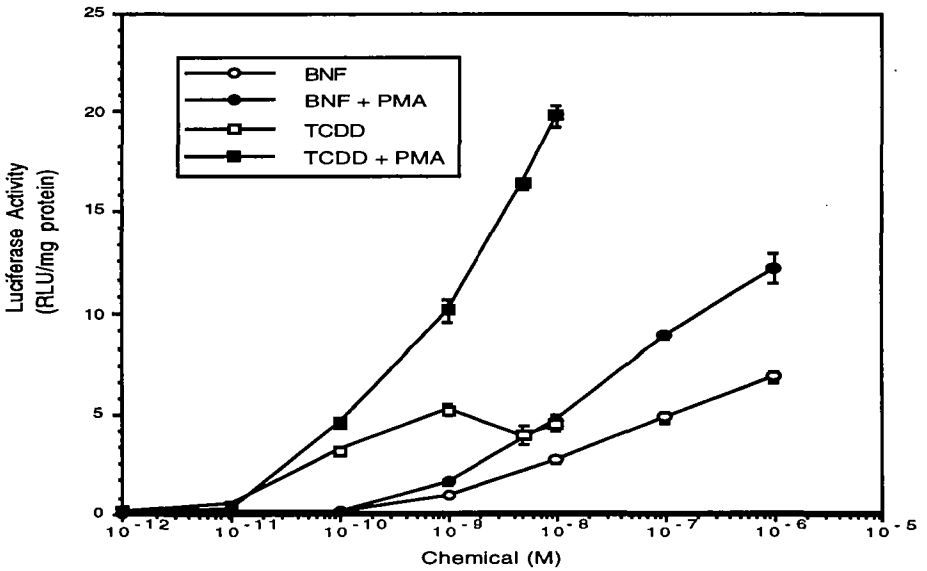

Figure 3. Effects of 81 nM PMA on TCDD and β-naphthoflavone dose response.

Historically, studies of the AhR and its ligands have been focused on planar, hydrophobic HAHs and PAHs. But recent studies in our laboratory and others suggest that the AhR can bind and be activated by a diverse range of compounds whose structural and physiochemical properties are distinct from those of "classical" AhR ligands. Compared with HAHs and PAHs, these novel compounds are relatively weak AhR ligands and activators that encompass a variety of chemical *classes, including* methylenedioxybenzenes, imidazoles, pyridines, indoles, carotinoids, heterocyclic amines and other structurally unrelated chemicals[4]. In spite of the number of exogenous AhR ligands that have been identified so far, studies involving AhR knockout mice

and continuous cell lines provide evidence for the existence of an endogenous ligand(s) in animals. AhR knockout mice exhibit a spectrum of hepatic defects as well as slight variations in immune function and effects in other tissues, suggesting that activation of the AhR by an endogenous ligand(s) is necessary in one or more critical stages in development[5,6] though no high affinity endogenous ligand for the AhR has been identified.

The ability of the AhR to bind chemicals of such diverse structural and size differences suggests that it contains a promiscuous ligand binding site capable of binding multiple endogenous activators. It is possible that the endogenous AhR ligand(s) has remained elusive due to its relatively weak affinity for the AhR, compared to TCDD, and perhaps rapid degradation by coordinately induced detoxification enzymes has made induction by this ligand(s) transient and difficult to detect. With respect to HAHs and PAHs, the synergistic effects of second messenger signaling pathways may have an impact on the potency or toxicity of these chemicals under the proper conditions. Recently, several classes of naturally occurring chemicals, including some dietary plant compounds and endogenous water-soluble metabolites of tryptophan as well as the heme degradation products, bilirubin and biliverdin, have been found to bind and activate and/or induce AhR-dependent gene expression[7,8]. These chemicals are relatively weak ligands and inducers, compared to TCDD, and thus they have been studied at concentrations significantly above those that exist physiologically. The studies here provide an avenue for examining the AhR-dependent effects of these chemicals at or near physiological concentrations when other second messenger pathways are concurrently activated. Further characterization of the effects of cross-talk between the AhR and second messenger signaling pathways on the transactivation potential of the AhR will be insightful in the identification of possible endogenous AhR ligands and in elucidating the role of the AhR under physiological circumstances.

Acknowledgments

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