Protein kinase C (PKC)- and protein kinase A (PKA)-mediated modulation of the transactivation potential of human aryl hydrocarbon receptor nuclear translocator (hARNT), a basic helix-loop-helix (bHLH)-PAS transcription factor, and the bHLH-ZIP transcription factors USF-1 (for upstream regulatory factor 1) and c-Myc were examined. An 81 nM dose of the PKA activator phorbol-12-myristate-13-acetate (PMA), shown here to specifically activate PKC in COS-1 cells, or a 1 nM dose of the PKA activator 8-bromoadenosine-3',5'-cyclic monophosphate (8-Br-cAMP) results in 2.6- and 1.9-fold enhancements, respectively, in hARNT-mediated transactivation of the class B, E-box-driven reporter pMyc3E1bLuc relative to identically transfected, carrier solvent-treated COS-1 cells. In contrast, 81 nM PMA and 1 nM 8-Br-cAMP did not enhance transactivation of pMyc3E1bLuc-driven by USF-1 and c-Myc expression relative to identically transfected, carrier-treated COS-1 cells. Co-transfection of pcDNA3/ARNT-474-Flag, expressing a hARNT carboxyl-terminal transactivation domain deletion, and pMyc3E1bLuc does not result in induction of reporter activity, suggesting PMA’s effects do not involve formation of unknown hARNT-protein heterodimers. Additionally, PMA had no effect on hARNT expression relative to Me2SO-treated cells. Metabolic 32P labeling of hARNT in cells treated with carrier solvent or 81 nM PMA demonstrates that PMA does not increase the overall phosphorylation level of hARNT. These results demonstrate, for the first time, that the transactivation potential of ARNT in a dimer context can be specifically modulated by PKC or PKA stimulation and that the bHLH-PAS and bHLH-ZIP transcription factors are differentially regulated by these pathways in COS-1 cells.

The ARNT protein is a member of the bHLH-PAS family of transcription factors and is the central dimerization partner (Fig. 1) for a variety of PAS family transcription factor dimers capable of binding different core DNA elements (1). Currently ARNT is known to be involved in such diverse cell signaling events as xenobiotic response (AhR-ARNT dimers), and hypoxic response (HIF1α-ARNT and EPAS-ARNT dimers), as well as pathways involving central midline and central nervous system development (Sim-ARNT) (2–4). Importantly, null ARNT allele mice are not viable past embryonic day 10.5, are defective in angiogenesis, and have impaired development (5), demonstrating that ARNT plays an essential role in normal cellular metabolism, growth, and development. Finally, the potential importance of regulating ARNT’s activity in these different bHLH-PAS dimer contexts is further underscored by the apparent involvement of one of ARNT’s dimerization partners, Sim, in mammalian central nervous system development and Down’s syndrome (6).

ARNT is a phosphoprotein that contains discreet, functionally defined domains. DNA binding and the formation of ARNT heterodimers involves the NH2-terminal half of this protein, which contains a basic region, a helix-loop-helix motif, and the PAS domain (7), with these latter two domains being required for optimal dimerization. The COOH-terminal 34 amino acids of ARNT can act as a TAD (8), but in the AhR-ARNT and HIF1α-ARNT heterodimer contexts the ARNT TAD is not absolutely required for transactivation of target genes (2, 9), but appears to be required for a maximal transcriptional response. These observations suggest that modulation of ARNT’s transactivation potential in the variety of different dimer contexts involving this bHLH-PAS protein may be an important mechanism contributing to the combinatorial transcriptional regulation of target genes.

c-Myc and USF-1 both belong to the bHLH-ZIP family of transcription factors and are capable of transactivating class B, E-box (5′-CACGTG-3′) regulated genes (10–13). c-Myc is involved in cellular proliferation, differentiation, neoplasia, and apoptosis (14, 15), while USF-1 is involved in regulation of the

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† To whom correspondence should be addressed: Center for Molecular Toxicology, Dept. of Veterinary Science, Pennsylvania State University, 115 Henning Bldg., University Park, PA 16802. Tel.: 814-865-0400; Fax: 814-863-6140; E-mail: ghp2@psu.edu.

§ The abbreviations used are: ARNT, aryl hydrocarbon receptor nuclear translocator protein; AhR, human aryl hydrocarbon receptor; α-MEM, α-minimal essential medium; ANOVA, analysis of variance; bHLH-PAS, basic helix-loop-helix-PAS protein; bHLH-ZIP, basic helix-loop-helix leucine zipper protein; BSA, bovine serum albumin; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; Che1, chelerythrine chloride; CYP1A1, cytochrome P450 1A1; CRE, dioxin-responsive element; 8-Br-cAMP, 8-bromoadenosine-3′,5′-cyclic monophosphate; FBS, fetal bovine serum; 4-O-Me-PMA, 4-O-methylphorbol-12-myristate-13-acetate; hAhR, human aryl hydrocarbon receptor; hARNT, human aryl hydrocarbon receptor nuclear translocator protein; HIF1α, hypoxia-inducible factor 1α; HRP, horseradish peroxidase; HSTD, high salt triple detergent lysis buffer; IGF, immunoprecipitation; mAb, monoclonal antibody; MAP, mitogen-activated protein; MOPS, 4-morpholinoethane-sulfonic acid; PAGE, polyacrylamide gel electrophoresis; PAS, PER/ARNT/SIM; PBS, phosphate-buffered saline; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; PVDF, polyvinylidene difluoride; RLU, relative light unit(s); Sim, single-minded; TAD, transcription activation domain; TCDD, 2,3,7,8-tetrachlorodibenzop-dioxin; Tricine, N-1-hydroxy-1,1-bis(hydroxymethyl)ethyglycine.

2 In this work, “transactivation potential” refers to the ability of a given, assembled, transcription factor complex to activate responsive genes.
viral Ad-ML (16), and human immunodeficiency virus-long terminal repeat promoters (17), as well as a variety of cellular genes, including murine p53 (18), human cyclin B1 (19), and human aldolase (20), among others. Importantly, the ability of c-Myc to transactivate class B, E-box-driven reporter constructs is modulated by the phosphorylation status of c-Myc (10, 21, 22), while USF-1 has recently been demonstrated to be a phosphoprotein in vivo (23). Additionally, MAP kinases can phosphorylate Ser-62 of c-Myc in vitro (21), and modulate c-Myc phosphorylation and transactivation potential in vivo (22). Together, these observations suggest that the serine/threonine kinases PKA and PKC may directly, or indirectly, modulate the ability of c-Myc and USF-1 to transactivate class B, E-box-responsive genes.

Recently it has been demonstrated that the bHLH-PAS protein ARNT is capable of forming homodimers that bind class B E-boxes and, like the bHLH-ZIP proteins c-Myc and USF-1, can transactivate class B, E-box-driven reporter constructs (1, 24, 25). Like c-Myc, hARNT is predominantly phosphorylated on serine and threonine residues, suggesting serine/threonine kinases such as PKC and PKA may also be involved, directly or indirectly, in the regulation of ARNT’s transactivation potential. A study of the potential regulatory role of the PKC and PKA serine/threonine kinase pathways, in the context of the hARNT homodimer, was undertaken to begin to understand the regulation of hARNT’s transactivation potential and ultimately the role of ARNT in the combinatorial control of such diverse biological phenomena as xenobiotic-induced toxicity, hypoxic response, central nervous system development, Down syndrome, and other, as yet, unexplored pathways.

An important benefit of examining the role of PKC- or PKA-mediated modulation of ARNT transactivation potential in the ARNT homodimer context is that this model is uncomplicated by the presence of ARNT’s heterologous dimerization partners. Additionally, the COS-1 model system described here allows the role of PKC and PKA in modulating the transactivation potential of the bHLH-ZIP transcription factors, c-Myc and USF-1, to be examined and compared with the ability of these kinase pathways to regulate the transactivation potential of the bHLH-PAS transcription factor hARNT.

In the COS-1 model system presented here, comparison of the consequences of 81 nM PMA and 1 nM 8-Br-cAMP treatments on hARNT-, c-Myc-, and USF-1-driven transactivation of the class B, E-box-driven, reporter construct pMyc3E1bLuc revealed that these compounds have differential effects on the transactivation potentials of these transcription factors. In contrast to the bHLH-ZIP proteins USF-1 and c-Myc, the transactivation potential of the bHLH-PAS protein hARNT is shown to be enhanced approximately 2-fold by both PMA and 8-Br-cAMP, presumably through stimulation of the PKC and PKA serine/threonine kinase pathways. Importantly, these results may indicate that the previously reported ability of PKC to enhance hAhR-mediated transactivation (26, 27) may, at least in part, be due to ARNT’s contribution to the transactivation potential of this heterodimer. Taken as a whole, these results demonstrate, for the first time, that the transactivation potential of ARNT in a dimer context can be specifically modulated by PKC or PKA stimulation and that the bHLH-PAS and bHLH-ZIP transcription factors are differentially regulated by these pathways in COS-1 cells.

EXPERIMENTAL PROCEDURES

Reagents—Glycerol, acrylamide, and bisacrylamide were purchased from Research Organics, Inc. (Cleveland, OH). Goat anti-mouse IgG (Fc-specific) antibodies, HRP-conjugated goat anti-mouse IgG (Fc-specific), and HRP-streptavidin were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). PVDF membrane was obtained from Millipore Corp. (Bedford, MA). Restriction enzymes were from New England Biolabs, Inc. (Beverly, MA). [32P]I-Labeled sheep anti-mouse IgG and [32P]PO4 (10 mCi/ml) was from Amersham Pharmacia Biotech. All other reagents, unless otherwise specified, were obtained from Sigma.

Plasmids—The pSV-β-galactosidase control vector (pBGAL) and pCI-neo plasmids were from Promega (Madison, WI). pSV-Sport1 and pcDNA3 were from Life Technologies, Inc. and Invitrogen (Carlsbad, CA), respectively. The pMycE1bLuc and pMyc3E1bLuc reporter constructs consist of zero and three E-boxes, respectively, located 5'-upstream of the luciferase reporter gene, and modulate c-Myc phosphorylation and transactivation potential in vivo (22). In the present study, the truncated peptide is FLAG-tagged, was generated by polymerase chain reaction from a phuARNT template, using the forward primer 5'-CCGCTGGAGCTCGACGCCGCTGTCATCGTCGTCCTTGTAGTCTGTAGGCCGTGGTT-9', and the reverse primer 5'-CCC-TTAGCTGCGTCGTCCTGTCATCGTCGTCCTTGTAGTCTGTAGGCCGTGGTT-9'. Subsequently, the fragment was cloned into the pCI-neo vector (gifts from R. Davis) as described (10). phuARNT, expressing full-length human ARNT (a gift from C. A. Bradfield), was constructed as described (28). pcDNA3/ARNT-47-Flag, in which hARNT’s carboxyl-terminal TAD has been deleted and the truncated peptide is FLAG-tagged, was generated by polymerase chain reaction from a phuARNT template, using the forward primer 5'-CCCT-CAGGTCGACCTGTCGTCCTGTCATCGTCGTCCTTGTAGTCTGTAGGCCGTGGTT-9' and the reverse primer 5'-CCCTCGAGGTCGACCTGTCGTCCTGTCATCGTCGTCCTTGTAGTCTGTAGGCCGTGGTT-9', followed by HindIII/XhoI digestion and subcloning of this fragment into the HindIII/XhoI sites of pcDNA3. pcUSF1, expressing full-length c-Myc (a gift from M. Sawadogo) was generated as described (13). pMK10-p53, expressing full-length c-Myc (a gift from D. M. Wojcikowski), was constructed by subcloning the murine c-Myc cDNA (provided by Dr. Ed Prochownik, Children’s Hospital, Pittsburgh, PA).
were then visualized using the Vector® VIP substrate kit (Vector Laboratories). Cell lysates were then prepared and assayed for luciferase and for 14 h in complete medium prior to treatment with compounds for 6 h. Transfected cells were allowed to recover to 80% confluence in tissue culture vessels and transfected with plasmids consisting of 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 0.02% NaN₃, 1 mM EDTA, 100 μg/ml phenylmethylsulfonyl fluoride, and 10 mM benzamide, and stored at −80 °C until needed. The membrane-containing pellet was resuspended in 0.5 ml of homogenization buffer consisting of 5% (v/v) Tween 20. Resuspended pellets were then incubated on ice and periodically vortexed to extract membrane-associated proteins. After 30 min, resuspended pellet mixtures were centrifuged at 100,000 × g for 45 min at 4 °C and the supernatant was collected, flash-frozen in an ethanol/dry ice bath, and stored at −80 °C until needed. The membrane-containing pellet was resuspended in 0.5 ml of homogenization buffer containing 0.5% Brij 58 by sonication on ice (three cycles of 5 s at 22 Hz of pulse duration of 15 s). Resuspended pellets were then incubated on ice and periodically vortexed to extract membrane-associated proteins. After 30 min, resuspended pellet mixtures were centrifuged at 100,000 × g for 45 min at 4 °C and the supernatant (membrane fraction protein extract) was collected. Protein content of cytosolic and membrane fraction extracts was determined using the BCA protein assay (Pierce).

Translocation Assay—Examination of α-PKC levels in the cytosolic and membrane protein-containing fractions was used to assess the ability of a given compound to activate α-PKC. Briefly, 1 μg of recombinant human α-PKC (PanVera Corp., Madison, WI), 100 μg of cytosolic protein, or 100 μg of membrane fraction protein from treated cells was subjected to Tricine SDS-PAGE and transferred to PVDF membrane as described earlier. Blots were then probed with an α-PKC antibody (2B10). Zytoimmunochemical labeling of PVDF membranes was performed as described (30) while the FLAG (M2)-specific mAb was generated as described (30) and affinity purified. Whole Cell Lysates—COS-1 cells were grown to 80% confluence, transfected in 60 × 15-mm tissue culture dishes, washed twice with PBS, and incubated in complete medium for 20 h. Cells were washed twice with PBS and then covered in 0.4 ml of HSTD lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 0.02% NaN₃, 1 mM EDTA, 100 μg/ml phenylmethylsulfonyl fluoride, and 10 μg/ml protease inhibitor mixture (Sigma). Cell debris was pelleted by centrifugation for 15 min at 17,350 × g, and protein content was determined with the BCA protein assay (Pierce).

Preparation of Whole Cell Lysates—COS-1 cells were grown to 80% confluence, transfected in 60 × 15-mm tissue culture dishes, washed twice with PBS, and incubated in complete medium for 20 h. Cells were washed twice with PBS and then covered in 0.4 ml of HSTD lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 0.02% NaN₃, 1 mM EDTA, 100 μg/ml phenylmethylsulfonyl fluoride, and 10 μg/ml protease inhibitor mixture (Sigma). Cell debris was pelleted by centrifugation for 15 min at 17,350 × g, and protein content was determined with the BCA protein assay (Pierce).

Preparation of Cytosolic and Membrane Protein Fractions for PKC Translocation Assays—COS-1 cells were grown to 90% confluence in 175-cm² tissue culture flasks and treated for 30 min with MEM/0.8% dialyzed FBS, or 30 μM MENGPI at 4 °C, followed by two washes of 0.6 ml each in MENGPI containing 0.5% (v/v) Tween 20. The ARNT-specific mAb 2B10 was generated as described (30) while the FLAG (M2)-specific mAb was generated as described (30) and affinity purified. Whole Cell Lysates—COS-1 cells were grown to 80% confluence, transfected in 60 × 15-mm tissue culture dishes, washed twice with PBS, and incubated in complete medium for 20 h. Cells were washed twice with PBS and then covered in 0.4 ml of HSTD lysis buffer consisting of 50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 0.02% NaN₃, 1 mM EDTA, 100 μg/ml phenylmethylsulfonyl fluoride, and 10 μg/ml protease inhibitor mixture (Sigma). Cell debris was pelleted by centrifugation for 15 min at 17,350 × g, and protein content was determined with the BCA protein assay (Pierce).

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PKC stimulation enhances hARNT-driven transactivation of pMyc3E1bLuc. COS-1 cells were grown, transiently transfected, and treated with compounds in the 24-well format as described under “Experimental Procedures.” Transfections consisted of 0.12 μg of pMyc0E1bLuc, 0.12 μg of pMyc3E1bLuc, 0.05 μg of phuARNT, and 0.06 μg of pBGAL plasmids per well as indicated. A, hARNT expression in COS-1 cells activates the class B, E-box-driven reporter construct pMyc0E1bLuc, whereas pMyc3E1bLuc transactivation in phuARNT-transfected COS-1 cells. Error bars represent standard deviations for a given measurement. Treatments with the same letter were not significantly different as determined with ANOVA and Tukey’s all-pairwise multiple comparisons test (α = 0.05).

RESULTS

PMA Enhances hARNT-driven Transactivation of pMyc3E1bLuc—Previously it had been reported that ARNT is capable of forming a homodimer that binds class B E-boxes and can transactivate class B, E-box-driven reporter constructs (1, 24, 25). COS-1 cells were transiently co-transfected with phuARNT and pMyc0E1bLuc or pMyc3E1bLuc reporter constructs, containing zero and three class B E-boxes 5′ to luc, respectively, alone or in the presence of phuARNT to confirm these observations. Fig. 2A demonstrates that transient expression of hARNT in pMyc0E1bLuc-transfected cells results in a dramatic induction of this class B, E-box-driven reporter construct relative to cells transiently co-transfected with phuARNT and pMyc0E1bLuc. These observations are consistent with those of others (24, 25) and demonstrate that the COS-1 transfection system is a valid model in which to examine the regulation of hARNT homodimer activity.

In order to examine the role of PKC in the regulation of hARNT homodimer transactivation activity, COS-1 cells were co-transfected with phuARNT and either pMyc0E1bLuc or pMyc3E1bLuc, followed by treatment with PMA, a PKC activator (31). Fig. 2B demonstrates that an 81 nM PMA treatment of phuARNT and pMyc3E1bLuc-transfected COS-1 cells results in a statistically significant 2.6-fold enhancement in hARNT-driven transactivation of the transfected reporter construct relative to identically transfected cells treated with Me2SO alone. Additionally, treatment with 3 μM Chel., a PKC inhibitor (32), attenuated this “PMA effect.” Together these results suggest that the transactivation potential of the class B E-box binding, hARNT homodimer can be regulated by the serine/threonine kinase PKC.

PMA Specifically Enhances hARNT-driven Transactivation of pMyc3E1bLuc and Activates α-PKC in COS-1 Cells—Control experiments utilizing 4-O-Me-PMA, a PKC structural analog that is a poor PKC activator (33), were performed to confirm that the PMA effect observed in Fig. 2B results specifically from the ability of PMA to activate PKCs in COS-1 cells. To demonstrate that PMA specifically enhances hARNT-driven transactivation, COS-1 cells were co-transfected with phuARNT and either pMyc0E1bLuc or pMyc3E1bLuc, followed by treatment with 81 nM 4-O-Me-PMA or 81 nM PMA. Fig. 3A demonstrates that, unlike the PKC activator PMA, treatment of phuARNT- and pMyc3E1bLuc-transfected COS-1 cells with 4-O-Me-PMA does not result in a statistically significant difference in hARNT-driven transactivation of the transfected
reporter construct relative to identically transfected cells treated with MeSO alone. Importantly, these results largely eliminate the possibility that the ability of PMA to enhance hARNT-driven transactivation of pMyc3E1bLuc in COS-1 cells is a nonspecific effect of PMA treatment.

Activation of PKC results in the translocation of this kinase from the cytosol to cell membranes (34); therefore, in order to demonstrate that PMA specifically activates PKCs in COS-1 cells, the ability of this compound and 4-O-Me-PMA to induce the translocation of α-PKC to membranes was examined. Fig. 3B demonstrates that PMA treatment of COS-1 cells results in a pronounced decrease in the cytosolic levels of this kinase relative to cytosolic α-PKC levels in MeSO-treated cells; importantly, cytosolic levels of α-PKC in 4-Me-O-PMA-treated COS-1 cells are essentially the same as those in MeSO-treated cells. Fig. 3C demonstrates that PMA specifically activates α-PKC translocation to the membrane relative to MeSO-treated COS-1 cells; 4-Me-O-PMA, however, does not activate α-PKC in COS-1 cells. Together, the results in Fig. 3 demonstrate that PMA can specifically activate PKCs in COS-1 cells and indicate that the ability of PMA to enhance hARNT-driven transactivation is a direct result of PKC activation.

USF-1-driven Transactivation of pMyc3E1bLuc Is Unaffected by PMA—USF-1 is also capable of forming a homodimer that binds class B E-boxes and can transactivate class B, E-box-driven, reporter constructs (12, 13). COS-1 cells were grown and transiently transfected with either the pMyc0E1bLuc or pMyc3E1bLuc reporter constructs, alone or in the presence of psvUSF1, to demonstrate that USF-1 is able to drive transactivation of pMyc3E1bLuc in the COS-1 model system. Fig. 4A demonstrates that transient transfection of COS-1 cells with psvUSF1 dramatically increases transactivation of pMyc3E1bLuc relative to cells transfected with pMyc3E1bLuc alone. It is important to note that a pronounced difference exists between the extent of luc' reporter induction in cells co-transfected with psvUSF1 and pMyc0E1bLuc relative to pMyc3E1bLuc- and psvUSF1-co-transfected COS-1 cells. These observations are consistent with those reported by others (12, 13) and demonstrate that the COS-1 transfection system is a valid model in which to examine the regulation of USF-1 homodimer activity.

COS-1 cells were co-transfected with psvUSF1 and either pMyc0E1bLuc or pMyc3E1bLuc to examine the effect of the PKC activator PMA (31) on the regulation of USF-1 homodimer-mediated transactivation. Fig. 4B demonstrates that an 81 nM PMA treatment of psvUSF1- and pMyc3E1bLuc-transfected COS-1 cells has no effect on transactivation of the reporter construct relative to identically transfected cells treated with MeSO alone. Furthermore, treatment with a 3 μM dose of Chel, has no effect on USF-1 homodimer-driven transactivation of pMyc3E1bLuc. These results suggest that the transactivation potential of the class B, E-box binding USF-1 homodimer is not regulated by the serine/threonine kinase PKC and that the PMA effect observed with the bHLH-PAS protein hARNT, in Fig. 2, is not due to artifactual activation of pMyc3E1bLuc.

c-Myc-driven Transactivation of pMyc3E1bLuc Is Unaffected by PMA—c-Myc has been demonstrated to drive transactivation of class B, E-box-driven reporter constructs such as pMyc3E1bLuc (10, 11). COS-1 cells were grown and transiently transfected with either the pMyc0E1bLuc or pMyc3E1bLuc reporter constructs alone or in the presence of pMK1059/Myc to confirm these observations in the COS-1 model system. Fig. 5A demonstrates that transient transfection of pMK1059/Myc increases transactivation of the class B, E-box-driven reporter construct pMyc3E1bLuc relative to cells transfected with pMK1059/Myc alone. Furthermore, transient co-transfection of pMK1059/Myc in pMyc3E1bLuc-transfected cells causes induction of this class B, E-box-driven reporter construct relative to cells transiently co-transfected with pMK1059/Myc and pMyc0E1bLuc (Fig. 5A). These observations are consistent with those reported previously (10, 11) and demonstrate that the COS-1 transfection system is a valid model in which to examine the regulation of c-Myc-mediated transactivation.

The transactivation potential of c-Myc has been demonstrated to be modulated by mutation of Thr-58 and Ser-62, both of which are phosphorylated in vitro (10). MAP kinases can phosphorylate c-Myc Ser-62 in vitro and are also capable of modulating c-Myc phosphorylation and transactivation potential in vivo (22). Importantly, the serine/threonine kinase or kinase pathway responsible for phosphorylation of c-Myc Thr-58 has yet to be delineated. In order to determine the possibility that the PKC pathway may modulate c-Myc-driven transactivation, COS-1 cells were co-transfected with pMK1059/Myc and either pMyc0E1bLuc or pMyc3E1bLuc to investigate the effect of the PKC activator PMA (31) on transactivation of these reporter constructs. Fig. 5B shows that an 81 nM PMA treatment of pMK1059/Myc and pMyc3E1bLuc-co-transfected COS-1 cells has no effect on transactivation of this reporter construct relative to identically transfected cells treated with MeSO alone. Additionally, a 3 μM dose of the PKC inhibitor Chel. has no effect on c-Myc-driven transactivation of pMyc3E1bLuc.

These results suggest that the transactivation potential of c-Myc is...
not regulated by the serine/threonine kinase PKC and that the PMA effect observed in Fig. 2, with the bHLH-PAS protein hARNT is not due to artifactual activation of pMyc3E1bLuc.

8-Br-cAMP Differentially Affects hARNT-, USF-1-, and c-Myc-driven Transactivation of pMyc3E1bLuc—hARNT and c-Myc are both phosphorylated on serine and threonine residues (10), while USF-1 has recently been demonstrated to be a phosphoprotein in vivo (23). In light of the observation that PMA only enhances hARNT-mediated transactivation of pMyc3E1bLuc, it appeared that the transactivation potential of c-Myc and USF-1 may be differentially regulated by various serine/threonine kinase pathways. To further examine this possibility, the PKA activator 8-Br-cAMP (35) was used to probe the ability of this serine/threonine kinase pathway to modulate hARNT-, c-Myc-, and USF-1-driven transactivation of pMyc3E1bLuc in vivo. Fig. 6A demonstrates that 1 nM 8-Br-cAMP treatment of phuARNT and pMyc3E1bLuc co-transfected COS-1 cells increases hARNT-driven transactivation of the luc" gene 1.9-fold relative to identically transfected, M_ε_2SO-treated cells. However, Fig. 6 (B and C) demonstrates that 1 nM 8-Br-cAMP treatment of COS-1 cells, in which transactivation of pMyc3E1bLuc is driven by psvUSF1 or pMK10_ε_/c-Myc, has no statistically significant effect on the transactivation of the luc" reporter gene relative to identically transfected, Me_2SO-treated cells. Together these results suggest that the PKA pathway is capable of modulating hARNT-driven transactivation of pMyc3E1bLuc, but that PKA does not modulate the transactivation potential of the bHLH-ZIP transcription factors c-Myc and USF-1.

The PMA Effect Requires the hARNT Transactivation Domain—One mechanism by which PMA may enhance the hARNT-driven transactivation of pMyc3E1bLuc is by inducing a second transcription factor capable of forming unknown hARNT-protein heterodimers or other dimer species that bind
COS-1 cells were grown, transiently transfected, and treated with compounds in the 24-well format as described under “Experimental Procedures.” Transfections consisted of 0.12 μg of pMyc0E1bLuc, 0.12 μg of pMyc3E1bLuc, 0.58 μg of phuARN, 0.58 μg of pcDNA3/ARNT-474-Flag, and 0.06 μg of pBGAL plasmids per well as indicated. Expression of hARNT (B) or ARNT-474-Flag (C) was confirmed by probing Western blots of lysates with mAb 2B10 or mAb M2, respectively. A, effect of 81 μM PMA on pMyc3E1bLuc and pMyc0E1bLuc transactivation in COS-1 cells transiently transfected with pcDNA3/ARNT-474-Flag (Δ) and phuARN (WT). B, expression of hARNT (WT) in COS-1 cells. C, expression of ARNT-474-Flag (Δ) in COS-1 cells. Error bars represent standard deviations for a given measurement. Treatments with the same letter (a, b, c, or d) were not significantly different as determined with ANOVA and Tukey’s all pairwise multiple comparisons test (α = 0.05).

Class B E-boxes and efficiently activate the luc+ reporter gene, although, at present, no group has presented evidence that might support the existence of such a mechanism. Nevertheless, the ability of pcDNA3/ARNT-474-Flag to drive transactivation of pMyc3E1bLuc and mediate the PMA effect was examined in order to address this possibility. pcDNA3/ARNT-474-Flag expresses a FLAG-tagged hARNT truncation mutant in which the COOH-terminal TAD has been deleted. Fig. 7A demonstrates that the hARNT TAD is required for hARNT to drive transactivation of pMyc3E1bLuc and consequently is required for the PMA effect to occur. Fig. 7C demonstrates that the inability of pcDNA3/ARNT-474-Flag to drive transactivation of pMyc3E1bLuc is not due to a failure of this construct to express properly or at a high level in COS-1 cells. Furthermore, panel B demonstrates that transfection of phuARN into COS-1 cells results in proper expression of full-length hARNT.

The current paradigm for ARNT’s role in a variety of dimer contexts is that this transcription factor is a central dimerization partner (Fig. 1), which provides half-site specificity and makes a non-dominant contribution to the ability of various ARNT protein heterodimers to efficiently transactivate responsive genes. This paradigm holds for both the AhR:ARNT heterodimer (2) and has recently been demonstrated to hold for the HIF1α:ARNT heterodimer (9); in both these heterodimer contexts, ARNT’s TAD is not essential for the transactivation of dioxin and hypoxia responsive genes. The results in Fig. 7 suggest that a hARNT homodimer is responsible both for driving transactivation of pMyc3E1bLuc and for the occurrence of the PMA effect in the COS-1 model system. Finally, the results in Fig. 7 strongly suggest that the PMA effect does not involve formation of unknown hARNT-protein heterodimer species capable of binding class B E-boxes and driving transactivation of pMyc3E1bLuc. Instead these results indicate that the transactivation potential of hARNT in a hARNT: hARNT homodimer context is being modulated by the PKC activator PMA.

PMA Treatments Do Not Alter Cellular hARNT Levels—One candidate mechanism by which PMA may stimulate hARNT homodimer-driven transactivation of pMyc3E1bLuc is simply by increasing cellular hARNT levels. To address this possibility, COS-1 cells were transiently transfected with phuARN, treated with Me2SO or 81 μM PMA, and hARNT levels in these whole cell lysates were quantified (Fig. 8). These analyses demonstrate that cellular hARNT levels in phuARN-transfected cells are unchanged by PMA treatments (Fig. 8), relative to identically transfected, Me2SO-treated cells. Additionally, the observation that mock-transfected COS-1 cells express ARNT at very low levels (Fig. 8) means that endogenous ARNT levels in the COS-1 model system are minimal, making this model well suited for examining the in vivo regulation of ARNT. Importantly, these data demonstrate that the enhanced transactivation effect induced by the PKC activator, PMA, does not occur through a mechanism in which PMA induces increased expression of hARNT in phuARN-transfected cells.

PMA Treatments Do Not Increase hARNT Phosphorylation—PMA may stimulate hARNT homodimer-driven transactivation of pMyc3E1bLuc by a direct mechanism involving increased, PKC-mediated, phosphorylation of the hARNT protein. COS-1 cells transiently transfected with phuARN...
were treated with Me2SO or 81 nM PMA and hARNT was simultaneously metabolically labeled with 32P-O4-. hARNT's transactivation potential makes an important contribution to the transcriptional regulation of target genes. Reisz-Porszasz et al. (7) previously generated a plasmid construct encoding the bHLHAB ARNT protein in which the bHLH, PAS A, and PAS B functional domains of ARNT are intact, but the carboxyl-terminal ARNT TAD has been deleted and a small deletion has been made at the amino terminus. Experiments performed by Reisz-Porszasz et al. (7) in the ARNT-deficient Hepa-1-c4 (B13Nbil) cell line demonstrated that this peptide is essentially unimpaired in its ability to dimerize with the AhR and bind DREs. However, AhR/bHLHAB ARNT heterodimers were only capable of activating a DRE-driven chloramphenicol acetyltransferase reporter with approximately 50% of the efficiency of the wild-type AhR/ARNT heterodimer (7) implying that ARNT's TAD contributes to the transactivation potential (2) of the AhR/ARNT heterodimer context supports the idea that ARNT's transactivation potential makes an important contribution to the transcriptional regulation of target genes. Moreover, Ko et al. (2) found that the ability of GAL4DBD-AhR/ARNT C to transactivate the GAL4-responsive reporter pGECAT in response to TCDD was impaired by roughly 50% relative to the ability of GAL4DBD-AhR/ARNT to transactivate of this report in response to TCDD. Additionally, Ko et al. (2) examined CYP1A1 mRNA levels in ARNT-deficient BP'C cells transiently expressing the ARNT C peptide, in which the ARNT TAD has been deleted. Although Ko et al. did not quantify these Northern blots, it appears that a substantial decrease in CYP1A1 transcription occurs in BP'C cells when an AhR/ARNT TAD dimer is formed instead of a AhR/ARNT heterodimer (2). Importantly, these apparent decreases in AhR/ARNT C heterodimer-mediated transcription (2) are similar to the observations of Reisz-Porszasz et al. (7). Together these observations lend credence to the notion that modulation of ARNT's transactivation potential may provide an important, addi-
The observation that PMA can enhance the transcription potential of bHLH-PAS proteins is not without precedent, as PMA has previously been reported to enhance AhR-mediated transactivation in mammalian cell lines stably transfected with a DRE-driven luciferase reporter construct. Importantly, this effect was shown not to be artifactual, appeared to be cell line-independent, and did not involve any of the following parameters: alteration of cytosolic AhR or hARNT levels, TCDD-induced down-regulation of the AhR, alteration of nuclear levels of the hAhR, or hAhR/hARNT heterodimer DRE binding activity (26, 27). Significantly, the observation that hARNT levels in phuARNT-transfected COS-1 are essentially unaltered by 81 nM PMA treatment (Fig. 8) is consistent with these previous observations (26). While it is possible that PMA's effects on the hARNT-driven transactivation of pMyc3E1bLuc may involve increased homodimerization of this bHLH-PAS transcription factor, the observation that PMA does not increase hAhR/hARNT dimer activity (26, 27) suggests PMA's effects on bHLH-PAS transcription factor-driven transactivation do not occur through this mechanism. Importantly, it was concluded that one mechanism by which PMA may enhance hAhR/hARNT heterodimer-mediated transactivation is by altering the ability of the hAhR/hARNT complex to form a fully functional transcriptional complex, perhaps by altering the recruitment or the ability of the hAhR, or hAhR/hARNT complex to form a fully functional transcriptional complex, perhaps by altering the recruitment or the ability of a specific co-activator associated with these bHLH-PAS transcription factors.

The co-activator CBP/p300 has been shown to interact specifically with ARNT's TAD (36). Interestingly, infection of cells with a DRE-driven luciferase reporter construct. The PMA-induced, enhanced hARNT homodimer-mediated transactivation of pMyc3E1bLuc reported here does not involve increased, direct, PKC-mediated phosphorylation of hARNT or alteration of cellular hARNT expression. These observations suggest that recruitment of a co-activator specific to the hARNT homodimer may be one possible mechanism by which the PMA effect occurs; alternatively, the PMA effect could be due to increased hARNT homodimer formation. Finally, we postulate that modulation of ARNT's transactivation potential may provide an important, additional level of control for the transcriptional regulation of target genes responsive to various ARNT-containing heterodimers, a conclusion that is consistent, and not incompatible, with the conclusions and findings of others (2, 7, 36).

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