Phorbol ester-modulation of estrogenic genomic effects triggered by the environmental contaminant benzanthracene

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Aryl hydrocarbon receptor-dependent genomic effects of environmental polycyclic aromatic hydrocarbons (PAHs) have been shown to be modulated by non-genomic protein kinase C (PKC)-related pathways. The present study was designed to determine whether PKC activation may also impair estrogenic genomic response triggered by PAHs. Treatment by the PKC activator phorbol 12-myristate 13-acetate (PMA) was found to markedly and differentially impair the up-regulation of estrogenic markers triggered by the estrogenic PAH benzanthracene (BZA) in cultured human mammary cells; BZA-mediated mRNA up-regulation of pS2 and amphiregulin was thus increased, whereas that of progesterone receptor and CXCL12 was repressed. BZA/PMA cotreatment however failed to alter BZA-mediated increase of activity of a luciferase gene reporter construct driven by an estrogen response element, thus discarding any global effect of PMA toward BZA-triggered estrogen receptor activation. Various chemicals inhibiting PKCs or extracellular signal-regulated kinase (ERK) as well as the knock-down of PKCα expression counteracted the PMA-mediated increase of pS2 mRNA up-regulation triggered by BZA, demonstrating that it was dependent on PKCs, including PKCα isoform, and ERKs. This non-genomic modulation of estrogenic effects of PAHs by PKC activation may have to be considered when considering the deleterious effects of these environmental contaminants towards the endocrine system.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widely-distributed environmental contaminants, to which humans are commonly exposed (Schell et al., 2010). They originate from incomplete combustion of organic materials and are notably found in diet, cigarette smoke, diesel exhaust particles and some occupational atmospheres (Phillips, 1999; Zedeck, 1980). They exert various deleterious effects towards human health, especially carcinogenic, immunosuppressive, atherogenic and inflammatory effects (Curfs et al., 2005; Irigaray and Belpomme, 2010; Podechard et al., 2008; Wojdani and Alfred, 1984). They are also considered as endocrine disruptors and some PAHs such as benzanthracene (BZA) and 3-methylcholanthrene have notably been shown to display estrogenic effects (Kummer et al., 2008; Pliskova et al., 2005; Shipley and Waxman, 2006; Swedenborg et al., 2008).

Most of the toxic effects of PAHs have been linked to activation of the aryl hydrocarbon receptor (AhR), a ligand-activated basic-helix-loop-helix transcription factor (Hankinson, 1995). PAHs bind to cytoplasmic AhR, thereby triggering its translocation into the nucleus, association with the AhR nuclear translocator (ARNT), and interaction with xenobiotic responsive element (XRE) found in the 5′-flanking regions of PAH-regulated genes, including cytochromes P450 (CYP) 1A1 and 1B1, which in turn bioactivate PAHs into mutagenic metabolites (Nebert et al., 2004). In addition to AhR, some PAHs also directly bind to, and activate, the estrogen receptor (ER) α, which has been directly involved in their estrogenic effects (Abdelrahim et al., 2006; Fertuck et al., 2001a).

Besides activation of receptors acting as transcription factors, non-genomic pathways have been incriminated as effectors or modulators of the toxic effects of PAHs (Archuleta et al., 1993; Asare et al., 2009; Mayati et al., 2011; Tannheimer et al., 1997). Among these non-genomic signaling ways, those related to protein kinases C (PKCs) are notably presumed to affect the AhR signaling cascade (Chen and Tukey, 1996; Long et al., 1998; Machemer and Tukey, 2005). Phorbol 12-myristate 13-acetate (PMA), a phorbol ester known as a potent activator of PKCs (Goel et al., 2007), has been consequently shown to alter regulation of AhR-responsive
genes such as CYP1A1 or interleukin (IL)-6 by PAHs (Hollingshead et al., 2008; Reiners et al., 1993). Whether PKC activation may also affect estrogenic response to PAHs remains by contrast unknown, but may be suspected because PKCs have been reported to modulate ER-α transcriptional activity (Cho and Katzenellenbogen, 1993; Martin et al., 1995). To get insights about this point in the present study, we have analyzed the effects of PMA on regulation of estrogenic targets by BZA in cultured human mammary cells. Our data indicate that PMA differentially regulates estrogenic target genes in a PKC-dependent manner. Such a non-genomic modulation of estrogenic effects of PAHs by PKC activation may have to be considered when considering the deleterious effects of these environmental contaminants towards the endocrine system and estrogen-related carcinogenesis.

2. Materials and methods

2.1. Chemicals and reagents

BZA, 3-methylcholanthrene, benzo(a)pyrene, estradiol, PMA, ICI 182,780 and G418 were purchased from Sigma–Aldrich (St. Louis, MO), whereas 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was obtained from Cambridge Isotope Laboratories (Cambridge, MA). The PKC inhibitors GF 109203X (also known as bisindolylmaleimide I or Gö 6850), Gö 6983, Gö 6976 and rottlerin and the extracellular signal-regulated kinase (ERK) inhibitors U0126 and PD98059 were provided by Calbiochem (La Jolla, CA). Trizol reagent was obtained from Invitrogen/Lifes Technologies (Paisley, United Kingdom). Other reagents were commercial products of the highest purity available.
2.2. Cell culture

MELN and MXLN cells were derived from ERα- and AhR-positive MCF-7 cells by stable transfection with plasmids carrying the firefly luciferase gene under the control of either the estrogen response element (ERE) or the XRE, respectively, using the β-globin promoter, as previously described (Balaguer et al., 2001). They were routinely cultured in DMEM/F12, Glutamax™ medium (Gibco/Lifes Technologies), containing 17.51 mM glucose and supplemented with 20 IU/mL penicillin, 20 μg/mL streptomycin, 1 mg/mL G418 and 5% (vol/vol) fetal bovine serum. Cells were starved of estrogens before xenobiotic or estradiol treatments through a 96 h period culture in red phenol-free DMEM/F12 medium supplemented with 20 IU/mL penicillin, 20 μg/mL streptomycin, 1 mg/mL G418 and 2.5% (vol/vol) charcoal/dextran-treated fetal bovine serum (Hyclone/ThermoScientific, Logan, UT).

2.3. Small interfering RNA (siRNA) transfection

Cells were transfected with 80 nM siRNAs targeting PKCδ (siPKCδ: 5'-GUUGAUGUCUGUCAGUAUtt-3') or with 80 nM nontargeting siRNAs (siNT: Mission® siRNA Universal negative control 1), provided by Sigma-Aldrich. Transfection was performed using DharmaFECT 1 Transfection reagent (Dharmacon Chicago, IL), as recommended by the supplier. The next day following transfection, cells were starved of estrogens, and exposed to xenobiotics 96 h later.

2.4. RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNAs were extracted using the TRIzol method, and then reverse-transcribed into cDNAs using the RT kit from Applied Biosystems (Foster City, CA). qPCR assays were next performed using the fluorescent dye SYBR Green methodology and an ABI Prism 7300 detector (Applied Biosystems), as previously described (Sparfél et al., 2010). The gene specific primers used are indicated in Supplementary Table S1. The specificity of each gene amplification was verified at the end of qPCR reactions through analysis of dissociation curves of the PCR products. Amplification curves were next analysed with ABI Prism 7000 SDS software using the comparative cycle threshold method. Relative quantification of the steady-state

Fig. 2. PMA-mediated modulation of AhR-related response to BZA. MXLN cells were either untreated (control) or exposed to 10 μM BZA in the absence or presence of 80 nM PMA for 8 h (A–C). (A) mRNA expression of CYP1A1, CYP1B1 and IL-6 was then determined by RT-qPCR. Data are expressed relatively to expression level found in cells treated by BZA in the absence of PMA, arbitrarily set at 100%; they are the means ± SEM of at least three independent assays. (B) IL-6 secretion in culture supernatant was determined by ELISA. Data are expressed as pg/ml and are the means ± SEM of three independent assays. (C) XRE-related luciferase activity was determined as described in Section 2. Data are expressed relatively to XRE-related luciferase activity found in control cells not exposed to PMA, arbitrarily set at 1 unit, and are the means ± SEM of three independent assays. (A, B, C) *p < 0.05 (when compared to counterparts not exposed to PMA).
target mRNA levels was calculated after normalization of the total amount of cDNA tested to a 18S mRNA endogenous reference.

2.5. Luciferase reporter gene assay

After washing with phosphate-buffered saline, cells were lysed in buffer containing 25 mM tris phosphate, pH 7.8, 2 mM EDTA, 10% glycerol and 1% triton X-100. Lysates were transferred next in 96-well white opaque tissue culture plates (Greiner Bio-One, Courtaboeuf, France). 50 μL of Bright-Glo™ Luciferase assay reagent (Promega, Madison, WS) was applied per well, and luminescence was immediately measured for 5 s using a Centro XS2 LB 960 microplate luminometer (Berthold Technologies, Bad Wilbad, Germany). Luminescence data were routinely expressed

![Graphs showing mRNA expression and Western-blotting results](image)

**Fig. 3.** PMA-mediated modulation of estrogenic genomic effects triggered by BZA. MELN cells were either untreated (control) or exposed to 10 μM BZA in the absence or presence of 80 nM PMA for 8 h. (A) mRNA expression of pS2, PGR, CXCL12 and AREG was then determined by RT-qPCR. Data are expressed relatively to expression level found in control cells not exposed to PMA, arbitrarily set at 1 unit; they are the means ± SEM of at least three independent assays. (B) pS2 protein expression was determined by Western-blotting. A representative blot is shown on the top; HSC70 expression was determined in parallel as a gel loading control. The means ± SEM of values from densitometric analysis of three independent assays are indicated on the bottom; data were expressed as% of pS2 found in control cells, arbitrarily set at 100%. *p < 0.05 (when compared to control cells). (C) ERE-related luciferase activity was determined as described in Section 2. Data are expressed relatively to ERE-related luciferase activity found in control cells not exposed to PMA, arbitrarily set at 1 unit, and are the means ± SEM of four independent assays. (A, B) *p < 0.05 (when compared to counterparts not exposed to PMA).
relatively to values found in control cells, arbitrarily set at 1 unit.

2.6. IL-6 measurement

Level of the cytokine IL-6 in the supernatants of MELN cell cultures was quantified by ELISA using a dedicated kit (R&D Systems, Abington, UK), according to the manufacturer's instructions.

2.7. Western-blot analysis

Total cellular extracts were prepared as previously described (Lecureur et al., 2005). Protein lysates (20 μg) were then separated on polyacrylamide gels and electrophoretically transferred onto nitrocellulose membranes (Bio-Rad, Marne la Coquette, France). After blocking with tris-buffered saline containing 3% bovine serum albumin and 0.1% tween 20 for 1 h at room temperature, membranes were incubated overnight at 4 °C with primary antibodies against pS2 (Abgent, Oxfordshire, United Kingdom), phospho-ERK, total ERK (Cell Signaling, Danvers, MA), PKCδ or HSC70 (Santa Cruz Biotechnology, Santa Cruz, CA). After washing, membranes were next re-incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Dako A/S, Glostrup, Denmark). Immunolabeled proteins were finally visualized by chemiluminescence. The intensities of stained bands were measured by densitometry using the ImageJ 1.40 g software (National Institute of Health, Bethesda, MA).

2.8. Statistical analysis

Data were routinely expressed as means ± SEM of values from at least three independent experiments, knowing that each individual experiment was performed in duplicate (ELISA and qPCR assays) or in quadruplicate (luciferase reporter gene assays). Statistical analysis was performed using ANOVA followed by a Tukey post hoc test for multiple comparisons or using Student’s t test; the criterion of significance was p < 0.05. Effective concentration 50 (EC50) values were determined using GraphPad Prism software (GraphPad Software, La Jolla, CA) or Sigmaplot software (Systat Software, Chicago, IL).

3. Results

Because estrogenicity of environmental PAHs has been questioned (Arcaro et al., 1999; Fertuck et al., 2001b), we first carefully verified that PAHs can effectively trigger an estrogenic response in the human mammary MCF-7/MELN cell line. As indicated in Fig. 1A, various PAHs such as BZA, 3-methylcholanthrene and benzo(a)pyrene, used at 10 μM for 8 h, induced ERE luciferase activity. They concomitantly induced XRE luciferase activity in MCF-7/MLXN cells (Fig. 1B). The AhR-related activity of the three PAHs did not statistically differ from that triggered by the potent AhR agonist TCDD used at 10 nM (Fig 1B). By contrast, whereas the estrogenic effect of BZA was similar to that caused by 10 nM estradiol, those of 3-methylcholanthrene and benzo(a)pyrene were significantly lower (Fig 1A). BZA was therefore retained for the following parts of the study. Time-course analysis of the effects of BZA next revealed that exposure times shorter than 8 h failed to enhance ERE luciferase activity, whereas a longer exposure (48 h) did not result in a greater induction (Fig 1C). The inducing effects of BZA towards ERE-luciferase activity were concentration-dependent (EC50 = 2.3 μM) (Fig 1D); BZA similarly enhanced XRE-luciferase activity in a concentration-dependent manner (EC50 = 1.3 μM) (Fig 1D); BZA-mediated induction of ERE luciferase activity was further found to be counteracted by the non-selective ER antagonist ICI 182,780, thus underlining that it involves ER activation (Fig 1E); ICI 182,780 similarly decreased ERE luciferase activity in estradiol-treated cells but also in untreated control cells (Fig 1E), thus suggesting a basal residual ER activity in these control cells. In addition to increase ERE-luciferase activity, BZA was found to robustly increase mRNA expressions of several ERα-referent targets such as pS2 (also known as trefoil factor 1/TFI), progesterone receptor (PGR), the chimiokine CXCL12 and amphiregulin (AREG), even if induction levels were somewhat lower than those triggered by estradiol (Fig 1F).

Before investigating the effects of the PKC activator PMA on estrogenic response to BZA, we checked that PMA affects BZA-dependent regulation of AhR target genes. The time treatment retained for PMA was relatively short (8 h), in order to discard any secondary down-regulation of PKC activity or alteration of ERα or AhR expression, which may occur for longer exposure to phorbol esters (Johnson et al., 1995; Martin et al., 1995; Spink et al., 1998). Cotreatment by 80 nM PMA and 10 μM BZA was found to reduce BZA-mediated up-regulation of CYP1A1 mRNA levels (Fig 2A). By contrast, it increased BZA-triggered induction of IL-6 mRNA expression; it however failed to act on CYP1B1 mRNA level increase caused by the PAH (Fig 2A). PMA also increased secretion of IL-6 and XRE-luciferase activity triggered by BZA (Fig 2B and C).

Fig. 4. Concentration-dependence of the modulating effects of PMA towards pS2, PGR and CXCL12 up-regulation triggered by BZA. MELN cells were exposed to 10 μM BZA in the presence of various concentrations of PMA for 8 h. mRNA expression of pS2, PGR and CXCL12 was then determined by RT-qPCR. Data are expressed relatively to gene expression levels found in cells exposed to BZA in the absence of PMA, arbitrarily set at 100%. They are the means ± SEM of at least three independent assays. EC50 values for PMA effect on each gene expression are indicated on the right of the graphs.
Exposure to PMA increased BZA-mediated mRNA up-regulation of the estrogenic targets pS2 and AREG whereas, by contrast, it counteracted BZA-mediated induction of PGR and CXCL12 (Fig. 3A). PMA by itself was found to significantly induce expression of pS2 and especially AREG, without however altering basal PGR and CXCL12 mRNA expression (Fig. 3A). Interestingly, BZA/PMA cotreatment also resulted in a marked induction of pS2 protein expression, as demonstrated by Western-blotting (Fig. 3B). By contrast, PMA failed to alter BZA-mediated activation of ERE-mediated luciferase activity (Fig. 3C). The differential modulating effects of PMA towards BZA-mediated up-regulation of pS2, PGR and CXCL12 were found to be concentration-dependent, with EC50 values around 5 nM for all the three estrogenic target genes (Fig. 4). PMA also altered estradiol-mediated up-regulation of pS2, PGR and CXCL12 in a similar concentration-dependent manner (Supplementary Fig. S1).

We further mainly focussed on PMA-mediated modulation of BZA-triggered up-regulation of pS2 expression, which led to a marked induction of pS2 mRNA and protein expression when compared to cells exposed to BZA or PMA alone (Fig. 3A and B). As shown in Fig. 5, cotreatment by the ER antagonist ICI 182,780 prevented BZA/PMA-mediated increase of pS2 expression; ICI 182,780 also counteracted basal induction of pS2 mRNA expression by BZA, but failed to modulate basal induction of pS2 by PMA (Fig. 5).

To verify that the effects of PMA towards BZA-mediated regulation of pS2 were due to PKC activation, chemical inhibitors of PKC were then used. Cotreatment by GF 109203X or Gö 6983, which are pan-PKC inhibitors blocking conventional PKCs (α, β1, β2 and γ isoforms) and novel PKCs (δ and ε isoforms) (Peterman et al., 2004; Toullec et al., 1991), was found to markedly inhibit the effects of BZA/PMA cotreatment towards up-regulation of pS2 mRNAs (Fig. 6A and B) and protein (data not shown); GF 109203X similarly counteracted PKC inhibitor targeting only conventional PKCs (Martiny-Baron et al., 1993), reduced PMA-mediated increase of BZA-related up-regulation of pS2, but only partially (Fig. 6C), suggesting that novel PKCs may also be implicated in PMA effects towards BZA-mediated up-regulation of pS2. To test this hypothesis, we next analyzed the effects of the PKCδ inhibitor rottlerin (Gschwendt et al., 1994), which was found to counteract the effects of PMA towards BZA-mediated induction of pS2 mRNA levels (Fig. 6D).

**Fig. 5.** Effect of the antiestrogenic compound ICI 182,780 on pS2 mRNA up-regulation triggered by coexposure to BZA and PMA. MELN cells were either untreated (control), exposed to 10 μM BZA or 80 nM PMA or coexposed to BZA and PMA, in the absence or presence of ICI 182,780 for 8 h. pS2 mRNA expression was then determined by RT-qPCR. Data are expressed relatively to expression level found in control cells not exposed to ICI 182,780, arbitrarily set at 1 unit; they are the means ± SEM of three independent assays. *p < 0.05 (when compared to counterparts not exposed to ICI 182,780).

**Fig. 6.** Effect of various PKC inhibitors on pS2 mRNA up-regulation triggered by coexposure to BZA and PMA. MELN cells were either untreated (control), exposed to 10 μM BZA or 80 nM PMA or coexposed to BZA and PMA, in the absence or presence of the PKC inhibitors GF 109203X (GFX) (2 μM) (A), Gö 6983 (5 μM) (B), Gö 6976 (5 μM) (C) or rottlerin (10 μM) (D) for 8 h. pS2 mRNA expression was then determined by RT-qPCR. Data are expressed relatively to expression level found in control cells not exposed to PKC inhibitors, arbitrarily set at 1 unit; they are the means ± SEM of three independent assays. *p < 0.05 (when compared to counterparts not exposed to PKC inhibitors).
Moreover, the transfection of siRNAs targeting PKCδ (siPKCδ), which markedly reduced PKCδ expression in MELN cells (Fig. 7A), concomitantly counteracted the effects of BZA/PMA cotreatment towards pS2 mRNA levels (Fig. 7B).

Downstream PKCs, various signaling pathways have been shown to be activated by PMA, including those related to the mitogen-activated protein kinase ERK or to NF-κb (Alblas et al., 1998; Baeuerele and Baltimore, 1988). Consistent with this assertion, exposure to PMA as well as BZA/PMA cotreatment, were found to activate/phosphorylate ERK in MELN cells in a PKC-dependent manner, i.e., PMA and BZA/PMA-mediated activations of ERK were inhibited by the PKC inhibitors GF 109203X and Gö 6983 (Fig. 8A); exposure to BZA alone however failed to activate ERK, as previously reported (Vondracek et al., 2002). The ERK inhibitors U0126 or PD98059 counteracted pS2 mRNA increase due to BZA/PMA cotreatment (Fig. 8B). By contrast, the NF-κb inhibitor iKIK inhibitor VII failed to modify pS2 mRNA increase in response to BZA/PMA coexposure (data not shown), thus discarding a major role for NF-κb in this effect.

4. Discussion

Activation of non-genomic ways has been shown to modulate activation of AhR-related target genes by environmental PAHs (Machemer and Tukey, 2005). The results reported in the present study extended this modulation to regulation of estrogenic targets of PAHs. Indeed, the phorbol ester PMA, which is a potent activator of PKC and which was notably found to alter BZA-mediated regulation of the AhR gene targets CYP1A1 and IL-6 and of XRE-related luciferase activity, in agreement with previous studies (Chen and Tukey, 1996; Hollingshead et al., 2008; Reiners et al., 1993), also concomitantly modulated BZA-mediated regulation of several estrogenic target genes such as pS2, PGR, AREG and CXCL12 in estrogen-responsive mammary MCF-7/MELN cells. It is noteworthy that the nature of this modulation depends on the target, i.e., BZA-related up-regulation of pS2 and AREG was increased by coexposure to PMA and BZA, whereas other BZA-induced targets were down-regulated. Such data argue against a global uniform effect of PMA towards PAH estrogenic effects and more likely suggest modulating effects depending on the nature of the target. The fact that PMA failed to alter BZA-mediated increase of ERE-luciferase reporter activity fully supports this hypothesis, through likely discarding a global repressing or inducing effect of PMA towards BZA-related activation of ERx-mediated transcription. In the same way, PMA differentially alters BZA-mediated activation of AhR gene targets since it decreased CYP1A1 induction, but concomitantly enhanced that of IL-6 and of an XRE-luciferase reporter construct, without however affecting CYP1B1 up-regulation. BZA-related gene activation dependent of the AhR cascade, like BZA-mediated gene activation related to the ERx cascade, is therefore not altered in a uniform way by PMA.

The differential effects of PMA toward regulation of estrogen-related target genes were not restricted to the use of BZA as an estrogenic compound, because similar effects were also observed when estradiol was used as a referent estrogenic agent (Supplementary Fig. S1). Interestingly, phorbol ester treatment has previously shown to decrease response to estradiol in MCF-7 cells through down-regulation of ERx gene expression and promotion of ERx degradation (Saceda et al., 1991). However, such effects occurred for long-term treatment by phorbol ester and shorter exposures failed to reduce estradiol-triggered ERx activity (Martin et al., 1995); in the same way we have found that the short exposure (8 h) to PMA used in the present study failed to repress ER-related luciferase reporter activity triggered by BZA (Fig 3C) or estradiol (data not shown).

The exact molecular mechanisms by which PMA may differentially modulate the response of estrogenic target genes to BZA or estradiol remain to be understood. Since a uniform action on ERx activity is unlikely, it is tempting to speculate that PMA may affect molecular factors, including transcription factors, that act independently of ERx or in indirect association with it. In this context, it is noteworthy that the two genes whose expression is increased by coexposure to PMA and BZA, i.e., pS2 and AREG, are also up-regulated by PMA alone. This up-regulation by the phorbol ester, putatively not directly related to ERx, is therefore likely to combine with that linked to BZA-triggered activation of ERx. The fact that a specific element of response to PMA, distinct from the ERE sequence, has been reported in the promoter region of pS2 (Barthhem et al., 2002) and that estradiol and phorbol ester regulate AREG expression by separate mechanisms (Martinez-Lacaci et al., 1995), may be consistent with this hypothesis. In addition, the lack of effect of the ER antagonist ICI 182,780 on induction of pS2 mRNA expression in response to PMA alone, whereas ICI 182,780 abolished BZA-mediated induction of pS2, also supports distinct effects of PMA and BZA towards this target gene.

The modulating effects of PMA towards up-regulation of estrogenic genomic targets triggered by BZA likely involved a common initial step. Indeed, PMA EC50 values were similar for modulations of BZA-related up-regulation of the estrogenic targets pS2, PGR and CXCL12, which is consistent with a primary effect of PMA on the same target, irrespective of the final estrogenic targets. In addition, this initial event may also be shared for PMA-mediated modulations of estradiol-related up-regulation of pS2, PGR and CXCL12,
because PMA EC50 values for modulation of estradiol-related genomic response were similar than those for modulation of BZA effects (Supplementary Fig. S1). Such a common early event may probably correspond to activation of PKCs. Indeed, PKCs are well-known as the primary targets of phorbol esters and the use of the pan-PKC inhibitor GF 109203X resulted in a marked inhibition of the modulating effects of PMA towards estrogenic genomic response to BZA in MCF-7/MELN cells. The conventional PKCα and the novel PKCδ, PKCε and PKCγ are the main PKC isoforms expressed by MCF-7 cells (Toricelli et al., 2011). Among these PKC isoforms, PKCα as well as PKCδ are likely to be involved in PMA modulating effects toward BZA-mediated up-regulation of pS2; indeed, chemical inhibition of these PKC isoforms and siRNA-mediated inhibition of PKCδ expression was found to counteract the effects of BZA/PMA cotreatment towards pS2 mRNA levels. Similar involvements of these PKC isoforms in various phenotypic effects triggered by phorbol ester exposure in mammary MCF-7 cells have previously been reported (Lin et al., 2008; Sengupta et al., 2006). Interestingly, PKC mobilization in BZA/PMA-coexposed MELN cells was found to trigger ERK activation, whose inhibition using chemical inhibitors counteracted pS2 up-regulation. Activation of a PKC/ERK signaling way is therefore likely required to increase pS2 expression in MELN cells coexposed to BZA/PMA.

Putative consequences of the modulation of estrogenic genomic response to PAHs by PKC activation in terms of PAH toxicity remain to be precised. In this context, it is first important to note that the estrogenic effects of PAHs have been questioned, owing notably to the fact that some reports have rather described antiestrogenic effects of PAHs (Ueng et al., 2004), knowing that such antiestrogenic effects are moreover well-established for related AhR agonists such as TCDD (Ohtake et al., 2003; Swedenborg and Pongratz, 2010). The results of the present study nevertheless unambiguously indicated that some PAHs such as BZA or 3-methylcholanthrene can exert reproducible estrogenic effects in human mammary cells, in agreement with previous studies (Kummer et al., 2008; Pliskova et al., 2005; Shiple and Waxman, 2006; Swedenborg et al., 2008). BZA is however rather less active than estradiol for inducing mRNA expression of estrogen target genes (Fig. 1F), whereas ERE luciferase activity is induced to the same extent by the PAH and estradiol. Such a discrepancy, occurring even in a much more accentuated manner, i.e., an induction of ERE luciferase activity by PAHs without concomitant changes in mRNA levels of estrogen target genes, has been previously reported (Gozgit et al., 2004). It could be linked to the fact that PAH-activated ER may be less efficient than estradiol-activated ER for triggering transcription of endogenous targets, which requires activation of ERE sequences in a physiological genomic context, potentially implicating recruitment of coactivator and adaptor proteins and changes in chromatin architecture. By contrast, PAH-activated ER may be as efficient as estradiol-activated ER for stimulating ERE sequences in the artificial architecture. By contrast, PAH-activated ER may be as efficient as estradiol-activated ER for stimulating ERE sequences in the artificial context corresponding to a luciferase reporter gene assay. Otherwise, a CYP-dependent metabolic activation of PAHs into monohydroxylated derivatives has been postulated to be required for getting estrogenic effects (Swedenborg et al., 2008; Fertuck et al., 2001b). BZA may be therefore hypothesized to have to be metabolically activated in MELN cells for up-regulating estrogenic targets; the fact that expression of the major PAHs-bioactivating enzymes CYP1A1 and CYP1B1 is induced by BZA in these cells (Fig. 2A) is
consistent with this hypothesis. In addition, the estrogenic effects occur for relatively low concentrations of PAHs (EC50 = 2.3 μM for BZA), closed to those activating the AhR cascade (Fig. 1D), thus suggesting that environmentally-relevant concentrations of PAHs may trigger estrogenic response. With respect to PMA modulating effects, the increase of PAH-mediated up-regulation of some estrogenic targets, such as pS2 and AREG, whereas others such as PGR and CXCL12 are down-regulated, may likely alter estrogenicity of environmental PAHs. In particular, physiopathological processes in which these estrogen target genes are known or suspected to be involved such as, for example, carcinogenesis for pS2 and AREG (Buser et al., 2011; Perry et al., 2008), may be impacted. In addition, the fact that humans are commonly exposed to mixtures of organic and inorganic contaminants, that may contain PAHs and also other chemicals activating or interacting with PKCs such as TCDD (DePetrillo and Kurl, 1993), various chlorinated pesticides like chlorodane, lindane, heptachlor, aldrin, endrin, dichlorodiphenyltrichloroethane (DDT) or toxaphene (Bagchi et al., 1997; Gauthier et al., 2001; Moser and Smart, 1989), the endocrine disruptor bisphenol A (Kuo et al., 2011), the surfactants octylphenol and nonylphenol (Li et al., 2006) or transition metal salts such as cadmium chloride or sodium dichromate (Bagchi et al., 1997), suggests that PKC-mediated modulation of PAH estrogenicity may occur in realistic environmental exposure conditions.

In summary, the phorbol ester PMA was shown to differentially modulate estrogenic genomic response triggered by the PAH BZA, in a PKC-dependent manner. Such a non-genomic modulation of estrogenic effects of PAHs by PKC activation may have to be considered when considering the deleterious effects of these environmental contaminants towards the endocrine system.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2012.05.006.

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In summary, the phorbol ester PMA was shown to differentially modulate estrogenic genomic response triggered by the PAH BZA, in a PKC-dependent manner. Such a non-genomic modulation of estrogenic effects of PAHs by PKC activation may have to be considered when considering the deleterious effects of these environmental contaminants towards the endocrine system.

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Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2012.05.006.

References


Arcaro, K.F., O’Keefe, P.W., Yang, Y., Clayton, W., Gierthy, J.F., 1999. Antiestrogenicity of octylphenol and nonylphenol (Li et al., 2006) or transition metal salts such as cadmium chloride or sodium dichromate (Bagchi et al., 1997), suggests that PKC-mediated modulation of PAH estrogenicity may occur in realistic environmental exposure conditions.

In summary, the phorbol ester PMA was shown to differentially modulate estrogenic genomic response triggered by the PAH BZA, in a PKC-dependent manner. Such a non-genomic modulation of estrogenic effects of PAHs by PKC activation may have to be considered when considering the deleterious effects of these environmental contaminants towards the endocrine system.


