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Dioxin-mediated up-regulation of aryl hydrocarbon receptor target genes is dependent on the calcium/calmodulin/CaMKIα pathway *

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Running title: AhR activity depends on Ca^{2+}/CaM/CaMKIα

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The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; AhR, Arylhydrocarbon Receptor; AhRR , AhR repressor ; ARNT, AhR Nuclear Translocator ; CYP, cytochrome P450 ; TCDD, 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin ; 2-APB, 2-aminoethoxydiphenylborate; XRE, xenobiotic responsive elements. Fura-2-AM, Fura-2acetoxymethylester; UNT, untreated ; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl) ester ; EROD , ethoxyresorufin ; XAP2 , X-associated protein 2, CaM , calmodulin ; CaMK , Ca^{2+}/CaM-dependent protein kinase ; ITG , integrin.

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ABSTRACT

Regulation of genes targeted by the ligand-activated aryl hydrocarbon receptor (AhR) has been shown to be controlled by calcium (Ca^{2+}) changes induced by AhR agonists such as the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The present study was designed to characterize this link between Ca^{2+} and AhR pathway. We report that fast elevation of intracellular Ca^{2+} in TCDD-exposed mammary MCF-7 cells was associated with transient enhanced activity of the Ca^{2+}/calmodulin(CaM)-dependent protein kinase (CaMK) pathway. Chemical inhibition of this pathway using the CaM antagonist W7 or the CaMK inhibitor KN-93 strongly reduced TCDD-mediated induction of the AhR target gene cytochrome P-450 1A1 (CYP1A1). SiRNAs-mediated knock-down expression of CaMKIα, one of the CaMK isoforms, similarly prevented CYP1A1 up-regulation. Both KN-93 and siRNA targeting CaMKIα were found to abolish TCDD-mediated activation of CYP1A1 promoter and TCDD-triggered nuclear import of AhR, a crucial step of the AhR signaling pathway. TCDD-mediated inductions of various AhR targets such as the drug metabolizing CYP1B1, the cytokine interleukin-1β, the chemokines interleukin-8 and CCL1, the adhesion molecule β7 integrin and the AhR repressor, were also prevented by KN-93 in human macrophages. Taken together, these data identified the Ca^{2+}/CaM/CaMKIα pathway as an important contributing factor to AhR-mediated genomic response.

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INTRODUCTION

The aryl hydrocarbon receptor (AhR) is an helix-loop-helix transcription factor activated by endogenous ligands and xenobiotics such as the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Barouki et al., 2007). In the absence of ligand, AhR, associated with heat shock protein-90 (HSP90), p23 and X-associated protein 2 (XAP2), is primarily located in the cytosol (Hankinson, 1995). Following ligand binding, AhR moves to the nucleus, dissociates from the chaperone complex, and forms a heterodimer with the AhR Nuclear Translocator (ARNT). This heterodimer binds to specific xenobiotic responsive elements (XRE) (core sequence: CACGCN(A/T)), found in the promoter of target genes, and subsequently regulates their transcription (Swanson, 2002). By this way, TCDD, and other AhR agonists such as carcinogenic environmental polycyclic aromatic hydrocarbons (PAHs), markedly induce expression of the drug metabolizing enzyme CYP1A1, known to detoxify, but also to bio-activate carcinogens, including PAHs, and commonly considered as a paradigm of AhR gene targets (Barouki et al., 2007).

In addition to activation of AhR, TCDD as well as PAHs have been shown to increase intracellular concentration of Ca^{2+} ($[Ca^{2+}]i$) (Archuleta et al., 1993; Burchiel et al., 1991; Davila et al., 1995; Le Ferrec et al., 2002; Tannheimer et al., 1997). Interestingly, blocking these $[Ca^{2+}]i$ changes through the use of intracellular Ca^{2+} chelator or store-operated channel blockers prevents induction of CYP1A1 (N'Diaye et al., 2006), thus suggesting cross-talks between Ca^{2+} variations and AhR-dependent regulatory pathways. Such interactions remain however to be characterized. For this purpose, it may be suitable to focus on key mediators of signaling pathways activated by Ca^{2+}. Among these, the CaMKs represent a major one (Braun and Schulman, 1995). CaMKs correspond to a family of structurally related serine/threonine protein kinases, which play important role in proliferation (Rodriguez-Mora et al., 2005) and differentiation (Zayzafoon, 2006). Interestingly, such cellular processes are also known to be affected upon AhR activation (Barouki et al., 2007). This has led us in the present study to analyze the putative contribution of CaMKs to AhR-dependent genomic response. Using mainly mammary MCF-7 cells, we report that activity of CaMKIα, one of the CaMK isoforms, is required for TCDD-triggered nuclear translocation of AhR and subsequent up-regulation of AhR

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target genes, especially of CYP1A1. Such data therefore highlight the Ca^{2+}/CaM/CaMKIα pathway as an important contributing factor to AhR-mediated genomic response.

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MATERIALS AND METHODS

Chemicals and reagents

2-aminoethoxydiphenylborate (2-APB), ethoxyresorufin and salicylamide were purchased from Sigma-Aldrich (St Louis, MO). TCDD was obtained from Cambridge Isotope Laboratories (Cambridge, MA) whereas 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA-AM**)**, the CaMK inhibitor KN-93 (2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine), and its inactive structural analogue KN-92 (2-[N-(4-Methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine), W7 (N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide), and ionomycin were obtained from Calbiochem (La Jolla, CA). Pluronic acid and Fura-2 acetoxymethylester (Fura-2-AM) were provided from Molecular Probes (Eugene, OR). Ficoll and TRIzol reagents were obtained from Life Technologies (Cergy Pontoise, France). GM-CSF (sp. act. 1.2 x 10^8 U/mg) was purchased from Schering-Plough (Lyon, France). Polyclonal goat anti-human CYP1A1/2 antibody (Ab) was obtained from Daiichi Pure Chemicals Co. Ltd. (Tokyo, Japan), polyclonal rabbit anti-AhR Ab from Biomol Research Labs (Plymouth, PA) and rabbit nonspecific IgG from Amersham Biosciences (Orsay, France). Monoclonal mouse anti-actin and polyclonal rabbit anti-p38 mitogen-activated protein kinase (MAPK), anti-GAPDH, anti-phospho-CaMKIα (Thr^{177}) and goat anti-lamin A/C Abs were purchased from Santa Cruz Biotechnology (La Perray en Yvelines, France). [γP^{32}] ATP was from Amersham Biosciences (Orsay, France). FITC-labelled anti-rabbit IgG Ab was purchased from Jackson Immunoresearch (Suffolk, UK). Chemicals were commonly used as stock solution in dimethyl sulfoxide (DMSO). Final concentration of solvent did not exceed 0.2% (v/v); control cultures received the same volume of solvent as for treated counterparts.

Cell culture

Human mammary MCF-7 cells were cultured in D-MEM medium with 4500 mg/L D-glucose, 110 mg/L sodium pyruvate and non-essential amino acids, supplemented with 100 U/ml penicillin, 100 U/ml streptomycin and 10% fetal calf serum. Primary human macrophages were obtained from GM-CSF-exposed blood monocytes and cultured as previously described (van Grevenynghe et al., 2003).

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Intracellular Ca^{2+} measurements

Variations in $[Ca^{2+}]i$ were analyzed by spectrofluorometry using the Ca^{2+}-sensitive probe Fura-2-AM, as previously reported (Le Ferrec et al., 2002). Briefly, MCF-7 cells were cultured in 24-well plates and incubated with 1.5 µM of the acetoxy cell-permeant form of Fura-2 (Fura-2 AM), for 30 minutes at 37 °C in Hepes-buffered medium (10 mM Hepes, 134.8 mM NaCl, 4.7 mM KCl, 1mM $MgCl_2$, 1.2 mM KH_2PO_4, 1 mM $CaCl_2$, 10 mM glucose, pH 7.4, at 37 °C), supplemented with 0.006% pluronic acid. After removing Fura-2-AM, cells were placed in the spectrofluorometer (SpectraMax Gemini SX, Molecular Devices, Toronto) and a well scan was performed at indicated times. Cells were irradiated alternately with 340 and 380 nm light, and fluorescence from the trapped dye was measured at 510 nm. The ratio of fluorescence intensities recorded after excitation at 340 nm and at 380 nm, defined as the F340/F380 ratio, was used to estimate $[Ca^{2+}]i$ changes, knowing that $[Ca^{2+}]i$ increase results in enhanced F340/F380 ratio (Le Ferrec et al., 2002; N'Diaye et al., 2006). Effects of chemical treatment on $[Ca^{2+}]i$ were expressed as Δ F340/F380 ratio, i.e. the F340/F380 ratio following the addition of chemicals minus the F340/F380 ratio measured prior to treatment.

Ethoxyresorufin O-deethylase (EROD) activity assay

EROD activity, corresponding to the O-deethylation of ethoxyresorufin, and mainly supfported by CYP1A1 enzyme in living MCF-7 cells, was measured as previously described (Sparfel et al., 2006). Briefly, MCF-7 cells were incubated in phosphate-buffered saline pH 7.4, containing 50 µM ethoxyresorufin and 1.5 mM salicylamide, and kinetic reading was performed at 37°C with a SpectraMax Gemini SX spectrofluorometer over a 30 minutes-period.

RNA isolation and analysis

Total RNAs, extracted using the TRIzol method (Invitrogen, Paisley, UK), were subjected to reverse transcription-real time quantitative polymerase chain reaction (RT-qPCR) analyses as previously described (Monteiro et al., 2007). Relative quantification of mRNA levels was performed after normalization of the total amount of cDNA tested to an 18 S RNA endogenous reference. The sequences of the primers used for RT-qPCR analysis were the following: CYP1A1 sense, 5'-GCACAGAGGTAGTCTCACTGCTTG-3', CYP1A1 antisense 5'-AAGGGCAGAGGAATGTGATGTT-3', CYP1B1 sense, 5'-TGATGGACGCCTTTATCCTC-3',

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CYP1B1 antisense 5'-CCACGACCTGATCCAATCT-3', interleukin-8 (IL-8) sense 5'-AAGAAACCACCGGAAGGAAC-3', IL-8 antisense 5'-AAATTTGGGGTGGAAAGGTT-3', CCL1 sense 5'-AGGCCTCTTTGCCTCTCTTC-3', CCL1 antisense 5'-ATGCAGATCATCACCACAGC-3', interleukin-1β (IL-1β) sense 5'-GGGCCTCAAGGAAAAGAATC-3', IL-1β antisense 5'-TTCTGCTTGAGAGGTGCTGA-3', β7 integrin (β7ITG) sense 5'-GAATCAACCAGACGGTGACTTTCT-3', β7ITG antisense 5'-GCCCGGAGCCTCAGGA-3', AhR repressor sense 5'-GTCAGTTACCTCCGGGTGAA-3', AhR repressor antisense 5'-TGGAAGCCCAGATAGTCCAC-3', 18S sense 5'-CGCCGCTAGAGGTGAAATTC-3', 18S antisense 5'-TTGGCAAATGCTTTCGCT-3'.

Reverse transfection of siRNA

For inhibiting expression of CaMKs, we first used "The human siArray Reverse Transfection Format siRNA library", targeting the various isoforms of the CaMK family and purchased from Dharmacon (Chicago, IL). This siRNA library corresponds to pools of four siRNA directed against different regions of targeted mRNA (Smart pool) for each CaMK isoform and spotted in 96 multiwell plates. Per well, 6.25 pmol dessicated siRNAs were rehydrated using 0.125µl of Dharmafect I reagent diluted in 25 µl of transfection medium (Opti-MEM, Invitrogen, Paisley, UK) for 40 minutes at room temperature. Next, 35000 MCF-7 cells, diluted in complete culture medium, were added per well. 24 hours later, transfection medium was replaced by fresh medium for an additional 48 hours. MCF-7 cells were next used for TCDD exposure and CYP1A1-related EROD activity assay. As control in siArray assays, the siCONTROL™ Non-Targeting siRNA (iNT1, 5'-UAGCGACUAAACACAUCAAtt-3') and 5 other siRNAs (provided with siRNA library, Dharmacon (Chicago, IL)) were used; values of TCDD-induced EROD activities obtained from cells-transfected with these six control siRNAs, were averaged, and arbitrary set at 100% as control values of EROD activity. For specific knock-down of CaMKIα isoform, we used the siRNA iCaMKIα (5'-GCGGUUACCCUCCCUUCUAtt-3') and the siRNA iNT1 as a non-targeting control, as reported above.

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Immunolocalization

Cells, fixed on coverslips with 4% paraformaldehyde for 30 minutes at 4°C, were incubated with the polyclonal IgG rabbit Ab anti-human AhR (2 µg/mL) or its recommended isotypic control at the same concentration overnight at 4°C. After washing, cells were incubated with FITC-labelled anti-rabbit IgG antibody (3 µg/mL) for 1 hour at room temperature. Coverslips were next mounted with PBS glycerol-Dabco. Pictures of fluorescent-labeled cells were finally captured with a DMRXA2 Leica microscope and a COOLSNAP HQ CCD camera, using Metavue software (Molecular Devices, Sunnyvale, CA).

Cellular protein extracts and immunoblotting analysis

Cellular protein extracts were prepared using a cell lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EGTA, 0.1% Tween 20, 10% glycerol, 100 µM phenylmethylsulfonyl fluoride and supplemented with an EDTA-free cocktail protease inhibitor (Roche Diagnostic, Meylan, France), whereas nuclear extracts were isolated from MCF-7 cells using the BD^{TM} Transfactor extraction kit (BD Biosciences, San Jose, CA). Protein samples (50 micrograms) were next subjected to electrophoresis in a 10% acrylamide gel and electrophoretically transferred to a nitrocellulose membrane (Bio-Rad, Marne la Coquette, France). After blocking with Tris-buffered saline containing 4% bovine serum albumin and 0.1% Tween 20 for 1 hour at room temperature, membranes were incubated overnight at 4°C with primary Ab. After incubation with appropriate horseradish peroxidase-conjugated secondary Ab for 1 hour, immuno-labelled proteins were visualized by autoradiography using chemiluminescence.

Reporter gene activity and transient transfection

The pCYP1A1-FL(-1566) construct containing a 1639-bp region (from -1566 to +73) of the human *CYP1A1* gene upstream of the *Firefly* luciferase reporter gene (a gift from Pr. Barouki, INSERM U747, Paris, France) and the pGL3-XRE3-FL construct containing only three XRE sequences from *CYP1A1* gene, have been previously described (Morel and Barouki, 1998). MCF-7 cells were cultured in 24-well plates and co-transfected with pRL-TK vector (Promega, Charbonnières, France) that codes for *Renilla* luciferase plus a plasmid carrying the *Firefly* luciferase or pCYP1A1-FL(-1566) or pGL3-XRE3-FL constructs. Transient transfection of MCF-7 cells was performed by the FuGENE[6]

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transfection reagent according to manufacturer's instructions (Roche Applied Science, Indianapolis, IN). Briefly, 250 µl of transfection medium (Opti-MEM) containing 225 ng of *Firefly* luciferase reporter plasmid was added per well along with 25 ng of the pRL-TK plasmid and 0.75 µl of FuGENE^{6} transfection reagent. After a 24-hours period, cells were exposed to TCDD for a 6-hours period. Dual luciferase assays (*Firefly* and *Renilla*) were then performed with a Promega kit according to the manufacturer's instructions. Data were expressed in arbitrary unit (a.u.), relatively to the value of luciferase activity levels found in TCDD-untreated cells, arbitrarily set at 1 unit.

CaMK assay

Cellular protein extracts were obtained using an extraction buffer containing 20 mM Tris-HCl, 2 mM EDTA, 2 mM EGTA, 20 µg/ml Soybean trypsin inhibitor (STI), 10 µg/ml aprotinin, 5 µg/ml leupeptin, 2 mM DTT, 25 mM benzamidin, and 1 mM phenylmethylsulfonyl fluoride. CaMK assay was then performed using the "SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase Assay System" kit (Promega, Charbonnières, France), according to the manufacturer's instructions. Briefly, a mix containing 2.5 µl of specific biotinylated peptide substrate of CaMK (0.5 mM), 5 µl of reaction buffer 5X, 5µl of activation buffer 5X or control buffer 5X, 5 µl ATP 0.5 mM and [γP^{32}] ATP (0.5 µCi specific activity), was added to cellular extracts. After a 5 minutes-period incubation at 30°C, the kinase reaction was stopped by adding 12.5 µl of termination buffer. The biotinylated peptide substrate was then captured by spotting 10 µl of the reaction on an individual square on the SAM® Biotin Capture Membrane. After various washing with 2 M NaCl and 2 M NaCl in 1% H_3PO_4, the membrane was dried and individual squares were placed in scintillation liquid for radioactivity counting.

Statistical analysis

Quantitative data are usually given as means ± s.d. of values from, at least, three independent experiments. Significant differences were routinely evaluated with the paired Student's *t*-test. The level of significance was $p<0.05$.

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RESULTS

$[Ca^{2+}]i$ changes are involved in TCDD-mediated up-regulation of CYP1A1 in MCF-7 cells

We first determined whether the cellular model mainly used in the present study, i.e. MCF-7 cells, was convenient for studying relationships between $[Ca^{2+}]i$ and TCDD-regulation of CYP1A1, used here as a prototypical AhR target. Using spectrofluorimetry and the Ca^{2+}-sensitive probe Fura-2-AM, exposure to 5 nM TCDD was firstly demonstrated to elicit a rapid increase of $[Ca^{2+}]i$ in MCF-7 cells (Fig. 1A). $[Ca^{2+}]i$ elevation was detectable as soon as 4 minutes after addition of TCDD and reached higher levels, closed to that obtained with the Ca^{2+} ionophore ionomycin (10 µM), after a 45-60 minutes-TCDD exposure. TCDD-elicited increase of $[Ca^{2+}]i$ in MCF-7 cells was fully abolished when cells were co-treated with the intracellular Ca^{2+} chelator, BAPTA-AM, or with the store-operated Ca^{2+} channel inhibitor 2-APB (Fig. 1A), which pointed to a major role of store-operated channels in TCDD-mediated Ca^{2+} movement in MCF-7 cells as already described in other cell models (N'Diaye et al., 2006; Tannheimer et al., 1997).

TCDD treatment was next shown to markedly increase both CYP1A1-mediated EROD activity and CYP1A1 mRNA expression in MCF-7 cells (Fig 1B,C). This up-regulation of CYP1A1 activity and expression was prevented by counteracting $[Ca^{2+}]i$ elevation using BAPTA-AM or 2-APB, thus linking CYP1A1 induction to $[Ca^{2+}]i$ changes in MCF-7 cells, in agreement with previous data in human macrophages (N'Diaye et al., 2006).

TCDD-mediated CYP1A1 up-regulation is impaired by chemical inhibition of the CaMK pathway

To test a putative role of Ca^{2+}/CaM/CaMK pathway in TCDD-mediated up-regulation of CYP1A1, we firstly analyzed the effect of TCDD on global CaMK activity in MCF-7 cells. As shown in Fig. 2A, TCDD triggered a marked and transient increase of CaMK activity, which reached levels similar to those observed in response to norepinephrine, a well-known activator of CaMK activity (Fatima et al., 2003). Chemical inhibition of the Ca^{2+}/CaM/CaMK signaling pathway using either the CaMK inhibitor KN-93 or the CaM antagonist W7 was next found to counteract TCDD-mediated induction of

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CYP1A1 activity (Fig. 2B) or expression at protein (Fig. 2C) or mRNA (Fig. 2D) levels in MCF-7 cells. By contrast, KN-92, an inactive chemical analogue of KN-93, failed to alter up-regulation of CYP1A1 activity (Fig. 2B) and expression (Fig. 2C) due to TCDD, thus highlighting the specificity of the effects of the CaMK inhibitor KN-93. This conclusion was further reinforced by the lack of effects of KN-93 towards $[Ca^{2+}]i$ changes occurring in MCF-7 cells exposed to TCDD (data not shown).

Knock-down of the CaMKIα isoform counteracts TCDD-mediated CYP1A1 induction

Owing to the fact that the CaMK family comprises several isoforms (Haribabu et al., 1995; Hook and Means, 2001), with at least some of them expressed in MCF-7 cells (Rodriguez-Mora et al., 2005), we were next interested in determining which isoform of the CaMK family was involved in the up-regulation of CYP1A1 upon TCDD exposure. To this purpose, we performed knock-down expression of 8 main CaMK isoforms (CaMKIα, δ, γ, CAMKIIα, β, δ, γ and CaMKIV) using the RNA interference methodology. We firstly used SMART pools of small interfering RNA from Dharmacon corresponding to pools of four siRNAs directed against each CaMK isoform. As indicated in Fig. 3A, only the SMART pool of siRNAs targeting the CaMKIα isoform (NM_003656) was found to significantly reduce TCDD-mediated up-regulation of CYP1A1 activity. We then separately analyzed the effects of the different siRNAs found in this SMART pool and selected the one that markedly down-regulated CaMKIα mRNA levels in MCF-7 cells without affecting those of other CaMK isoforms such as CaMKIIγ RNA (data not shown). Tranfection of MCF-7 cells by this siRNA, termed iCaMKIα, was further demonstrated to inhibit TCDD-triggered induction of CYP1A1 activity (Fig. 3B) and CYP1A1 expression at both protein (Fig. 3C) and mRNA (Fig. 3D) level. In addition, it concomitantly blocked TCDD-triggered increase of CaMK activity (Fig. 3E). Interestingly, exposure to TCDD resulted in increased CaMKIα (Thr^{177}) phosphorylation, and thereby, most likely in full CaMKIα activation (Haribabu et al., 1995) (Fig. 3F).

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TCDD-mediated activation of CYP1A1 promoter requires CaMKIα

To understand how CaMKIα regulates TCDD-induced CYP1A1 regulation, we performed transient transfection of MCF-7 cells with a plasmid construct corresponding to -1566 bp of the 5'-flanking region of the *CYP1A1* gene upstream to the luciferase reporter gene (pCYP1A1-FL construct), and containing five XRE consensus elements. TCDD treatment was found to induce activity of the reporter gene (Fig. 4A), in agreement with previous studies (Le Ferrec et al., 2002; Morel and Barouki, 1998). Co-treatment with the CaMK inhibitor KN-93 significantly abolished this induction (Fig. 4A). KN-93 also counteracted the TCDD-mediated increase of the luciferase activity when using a reporter gene construct driven by three XRE (pGL3-XRE3-FL construct) (Fig. 4B). This XRE-driven luciferase activity inducible by TCDD was also similarly inhibited by knock-down expression of CaMKIα (Fig. 4C).

CaMKIα inhibition and knock-down prevent TCDD-triggered nuclear import of AhR.

Down-regulation of TCDD-induced transcriptional activation of CYP1A1 by inhibition of CaMKIα expression and/or activity may be due to direct interference with the final nuclear step of the AhR signaling pathway, i.e. XRE-driven induction of CYP1A1 promoter activity, or alternatively, to an interaction with an upstream step of the AhR pathway such as AhR nuclear import in response to TCDD. To investigate this point, AhR cellular distribution in untreated and TCDD-treated MCF-7 cells was monitored by western-blotting and by immunofluorescence labelling (Fig. 5). Treatment by TCDD triggered an important increase of AhR nuclear content as demonstrated by western-blot analysis of nuclear fractions (Fig. 5A); similarly, immunolocalization studies indicated that AhR was found to be primarily localized in cytoplasm of untreated cells, whereas it was mainly detected in the nucleus of TCDD-exposed cells (Fig. 5B). This TCDD-induced AhR nuclear import was largely abrogated by co-treatment with KN-93 (Fig 5A,B). SiRNA-mediated knock-down of CaMKIα expression also markedly reduced TCDD-triggered nuclear translocation of AhR, as shown by western-blotting (Fig. 5C) and AhR immunolocalization studies (Fig. 5D).

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CaMK activity is involved in TCDD-mediated up-regulation of various AhR target genes in primary culture of human macrophages.

The last part of our study was designed to determine whether CaMK activity was required for TCDD-mediated regulation of various AhR target genes, in addition to CYP1A1. Using primary human macrophages, a convenient model for analyzing phenotypic effects of AhR ligands (Lecureur et al., 2005; Monteiro et al., 2007; N'Diaye et al., 2006; van Grevenynghe et al., 2004) and in which AhR ligands trigger $[Ca^{2+}]i$ increase (N'Diaye et al., 2006), we found that co-treatment with the CaMK inhibitor KN-93 was capable of counteracting the TCDD-mediated up-regulation of several AhR target genes such as the drug metabolizing CYP1B1, the cytokine IL-1β, the chemokines IL-8 and CCL1, the adhesion molecule β7 integrin and the AhR repressor (Fig. 6).

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DISCUSSION

Elevation of $[Ca^{2+}]i$ in response to AhR agonists has been reported in various cellular models, including lymphocytes (Archuleta et al., 1993; Burchiel et al., 1991; Pallardy et al., 1992), macrophages (N'Diaye et al., 2006; Pappas et al., 2003), intestinal (Le Ferrec et al., 2002) and mammary cells (Tannheimer et al., 1997), and can therefore be considered as an hallmark of AhR agonist exposure. Interestingly, this change of $[Ca^{2+}]i$ has been recently demonstrated to be required for regulation of the AhR target genes CYP1A1 and CCL1, in human Caco-2 intestinal cells (Le Ferrec et al., 2002) and human macrophages (N'Diaye et al., 2006), respectively. Likewise, chemical inhibition of TCDD-mediated $[Ca^{2+}]i$ increase in human mammary MCF-7 cells was also reported to counteract CYP1A1 induction in the present study. Taken together, these data strongly suggest that Ca^{2+}-related signaling pathway may take place, or interfere, in the AhR signaling pathway. The data reported in the present study, through identifying the requirement of the CaMK pathway, one of the major Ca^{2+}-related transduction pathway, for TCDD-mediated regulation of AhR target genes, fully support this hypothesis.

The possible implication of CaMK activity in the AhR signaling pathway was first pointed out by the fact that CaMK activity was increased in response to TCDD exposure in MCF-7 cells. Moreover, the specific CaMK inhibitor KN-93, unlike its inactive counterpart KN-92, prevented TCDD-mediated induction of both CYP1A1 activity and expression in MCF-7 cells. Besides CYP1A1 regulation, KN-93 also blocked TCDD-related induction of various AhR target genes in human macrophages, likely indicating that CaMK activity is essential for global AhR-mediated genomic response to TCDD exposure.

Involvement of the CaMK signaling pathway in AhR-genomic effects was moreover supported by the fact that W7, a CaM antagonist that prevents Ca^{2+}/CaM-triggered activation of CaMKs (Tanaka et al., 1983), nearly fully antagonised TCDD-mediated up-regulation of CYP1A1 activity and expression. Moreover, knock-down of CaMK expression by RNA interference inhibited CYP1A1 induction in response to TCDD. This use of siRNA is important to fully demonstrate the involvement of CaMK activity in TCDD-mediated CYP1A1 up-regulation since it allows to

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counteract any potential lack of specificity of the chemical inhibitors used in the study, including a putative AhR antagonist-like activity of KN-93, that should be formally considered. In addition, siRNA-related experiments allowed us to demonstrate that, among CaMK isoforms, CaMKIα is most likely the isoform whose expression/activity is required for AhR-dependent up-regulation of target genes in MCF-7 cells. Indeed, knock-down of the expression of this isoform markedly inhibited the up-regulation of CYP1A1 activity and expression occurring in TCDD-treated MCF-7 cells. In addition, it concomitantly abrogated the TCDD-related increase of CaMK activity, thus fully supporting a major role for CaMKIα isoform in TCDD-stimulated CaMK activity in MCF-7 cells. Moreover, TCDD treatment was found to notably result in CaMKIα activation, through the phosphorylation of the critical threonine residue at position 177 (Haribabu et al., 1995). Importantly, CaMKIα isoform has been demonstrated to be broadly distributed in various tissues (Haribabu et al., 1995), like AhR (Barouki et al., 2007), suggesting that it may participate to the AhR signaling pathway in a rather systemical manner.

Inhibition of CaMK activity and knock-down of CaMKIα expression markedly prevented TCDD-mediated induction of CYP1A1 promoter activity and of XRE-driven reporter activity, indicating that the CaMK way was required for transcriptional activity triggered by AhR in the nucleus. However, transcriptional activity of AhR depends, at least, on previous steps including AhR nuclear import and heterodimerization with ARNT. Since CaMKI has been implicated in cellular localization of several proteins (Kao et al., 2001), its effect towards AhR cellular distribution was checked. Regarding this point, we have found that both co-treatment by KN-93 and knock-down of CaMKIα expression inhibited TCDD-induced nuclear translocation of AhR, suggesting that AhR nuclear import may be an initial step of the AhR signaling pathway requiring CaMK activity. It should however be kept in mind that the initial cytosolic step of the AhR signaling cascade, that takes place before AhR nuclear import, can also constitute the primary target of the CaMK pathway; in this context, a putative interaction of CaMKI activity with cytosolic AhR partners, such as XAP2 (de Oliveira et al., 2007; Pollenz and Barbour, 2000) or HSP90 (Ogiso et al., 2004), may be interesting to

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consider. Further studies are therefore required to better understand the molecular mechanism by which the Ca^{2+}/CaM/CaMKIα pathway participates in the AhR-related genomic response.

In addition to CaMKIα, various protein kinases (Tan et al., 2004) have been shown to participate to transduction signaling elicited by AhR agonists. Some of these kinases such as the tyrosine kinases Fyn and Lck (Archuleta et al., 1993) are presumed to be involved in $[Ca^{2+}]$i increase in response to AhR agonists whereas others such as Src-kinase (Enan and Matsumura, 1996), protein kinase-C and MAPKs (Tan et al., 2002) may be more directly implicated in the control of AhR complex activity and in the regulation of AhR target genes. In addition, ERK has been shown to be involved in regulation of the AhR response (Lecureur et al., 2005; Tan et al., 2002) via the control of AhR degradation (Chen et al., 2005). Besides protein kinases, the proteolytic enzyme calpain, well-known to be activated by Ca^{2+}, has also been recently hypothesized to be involved in Ca^{2+}-related control of the AhR-signal transduction pathway (Dale and Eltom, 2006). These data are however controversial since calpain inhibitors did not affect TCDD-mediated up-regulation of CYP1A1 in several cell culture lines from different species (Pollenz, 2007); in agreement with these results, we have found that the calpain inhibitor III failed to alter TCDD-induced CYP1A1 expression in MCF-7 cells (data not shown), thus ruling out a major role for calpain in the AhR signaling pathways in such cells. Whether calpain may be implicated in the regulation of AhR target genes distinct from CYP1A1 remains to be determined. By contrast, we have found that CaMK activity, playing a role in the AhR signaling pathway in both normal and cancerous cell models, i.e. human primary macrophages and mammary tumoral MCF-7 cells, concerns regulation of various AhR target genes, including drug metabolizing enzymes and cytokines/chemokines.

In summary, the data reported in the present study indicate for the first time that the Ca^{2+}/CaM/CaMKIα pathway is involved in the AhR-mediated genomic response, notably in the AhR nuclear import step of the AhR signaling pathway.

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This article has not been copyedited and formatted. The final version may differ from this version.

REFERENCES

Archuleta MM, Schieven GL, Ledbetter JA, Deanin GG and Burchiel SW (1993) 7,12-Dimethylbenz[a]anthracene activates protein-tyrosine kinases Fyn and Lck in the HPB-ALL human T-cell line and increases tyrosine phosphorylation of phospholipase C-gamma 1, formation of inositol 1,4,5-trisphosphate, and mobilization of intracellular calcium. *Proc Natl Acad Sci U S A* **90**:6105-9.

Barouki R, Coumoul X and Fernandez-Salguero PM (2007) The aryl hydrocarbon receptor, more than a xenobiotic-interacting protein. *FEBS Lett*.

Braun AP and Schulman H (1995) The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. *Annu Rev Physiol* **57**:417-45.

Bunger MK, Moran SM, Glover E, Thomae TL, Lahvis GP, Lin BC and Bradfield CA (2003) Resistance to 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity and abnormal liver development in mice carrying a mutation in the nuclear localization sequence of the aryl hydrocarbon receptor. *J Biol Chem* **278**:17767-74.

Burchiel SW, Thompson TA and Davis DA (1991) Alterations in mitogen-induced calcium mobilization and intracellular free calcium produced by 7,12-dimethylbenz(a)anthracene in the Jurkat human T cell line. *Int J Immunopharmacol* **13**:109-15.

Chen S, Operana T, Bonzo J, Nguyen N and Tukey RH (2005) ERK kinase inhibition stabilizes the aryl hydrocarbon receptor: implications for transcriptional activation and protein degradation. *J Biol Chem* **280**:4350-9.

Dale YR and Eltom SE (2006) Calpain mediates the dioxin-induced activation and down-regulation of the aryl hydrocarbon receptor. *Mol Pharmacol* **70**:1481-7.

Davila DR, Davis DP, Campbell K, Cambier JC, Zigmond LA and Burchiel SW (1995) Role of alterations in Ca(2+)-associated signaling pathways in the immunotoxicity of polycyclic aromatic hydrocarbons. *J Toxicol Environ Health* **45**:101-26.

de Oliveira SK, Hoffmeister M, Gambaryan S, Muller-Esterl W, Guimaraes JA and Smolenski AP (2007) Phosphodiesterase 2A forms a complex with the co-chaperone XAP2 and regulates nuclear translocation of the aryl hydrocarbon receptor. *J Biol Chem* **282**:13656-63.

Enan E and Matsumura F (1996) Identification of c-Src as the integral component of the cytosolic Ah receptor complex, transducing the signal of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) through the protein phosphorylation pathway. *Biochem Pharmacol* **52**:1599-612.

Fatima S, Yaghini FA, Ahmed A, Khandekar Z and Malik KU (2003) CaM kinase IIalpha mediates norepinephrine-induced translocation of cytosolic phospholipase A2 to the nuclear envelope. *J Cell Sci* **116**:353-65.

Hankinson O (1995) The aryl hydrocarbon receptor complex. *Annu Rev Pharmacol Toxicol* **35**:307-40.

Haribabu B, Hook SS, Selbert MA, Goldstein EG, Tomhave ED, Edelman AM, Snyderman R and Means AR (1995) Human calcium-calmodulin dependent protein kinase I: cDNA cloning, domain structure and activation by phosphorylation at threonine-177 by calcium-calmodulin dependent protein kinase I kinase. *Embo J* **14**:3679-86.

Hook SS and Means AR (2001) Ca(2+)/CaM-dependent kinases: from activation to function. *Annu Rev Pharmacol Toxicol* **41**:471-505.

Ikuta T, Kobayashi Y and Kawajiri K (2004) Phosphorylation of nuclear localization signal inhibits the ligand-dependent nuclear import of aryl hydrocarbon receptor. *Biochem Biophys Res Commun* **317**:545-50.

Kao HY, Verdel A, Tsai CC, Simon C, Juguilon H and Khochbin S (2001) Mechanism for nucleocytoplasmic shuttling of histone deacetylase 7. *J Biol Chem* **276**:47496-507.

Le Ferrec E, Lagadic-Gossmann D, Rauch C, Bardiau C, Maheo K, Massiere F, Le Vee M, Guillouzo A and Morel F (2002) Transcriptional induction of CYP1A1 by oltipraz in human Caco-2 cells is aryl hydrocarbon receptor- and calcium-dependent. *J Biol Chem* **277**:24780-7.

Lecureur V, Ferrec EL, N'Diaye M, Vee ML, Gardyn C, Gilot D and Fardel O (2005) ERK-dependent induction of TNFalpha expression by the environmental contaminant benzo(a)pyrene in primary human macrophages. *FEBS Lett* **579**:1904-10.

Monteiro P, Gilot D, Le Ferrec E, Lecureur V, N'Diaye M, Le Vee M, Podechard N, Pouponnot C and Fardel O (2007) AhR- and c-maf-dependent induction of beta7-integrin expression in human macrophages in response to environmental polycyclic aromatic hydrocarbons. *Biochem Biophys Res Commun* **358**:442-8.

Morel Y and Barouki R (1998) Down-regulation of cytochrome P450 1A1 gene promoter by oxidative stress. Critical contribution of nuclear factor 1. *J Biol Chem* **273**:26969-76.

N'Diaye M, Le Ferrec E, Lagadic-Gossmann D, Corre S, Gilot D, Lecureur V, Monteiro P, Rauch C, Galibert MD and Fardel O (2006) Aryl hydrocarbon receptor- and calcium-dependent induction of the chemokine CCL1 by the environmental contaminant benzo[a]pyrene. *J Biol Chem* **281**:19906-15.

Ogiso H, Kagi N, Matsumoto E, Nishimoto M, Arai R, Shirouzu M, Mimura J, Fujii-Kuriyama Y and Yokoyama S (2004) Phosphorylation analysis of 90 kDa heat shock protein within the cytosolic arylhydrocarbon receptor complex. *Biochemistry* **43**:15510-9.

Pallardy M, Mishal Z, Lebrec H and Bohuon C (1992) Immune modification due to chemical interference with transmembrane signalling: application to polycyclic aromatic hydrocarbons. *Int J Immunopharmacol* **14**:377-82.

Pappas P, Sotiropoulou M, Karamanakos P, Kostoula A, Levidiotou S and Marselos M (2003) Acute-phase response to benzo[a]pyrene and induction of rat ALDH3A1. *Chem Biol Interact* **143-144**:55-62.

Pollenz RS (2007) Specific blockage of ligand-induced degradation of the Ah receptor by proteasome but not calpain inhibitors in cell culture lines from different species. *Biochem Pharmacol* **74**:131-43.

Pollenz RS and Barbour ER (2000) Analysis of the complex relationship between nuclear export and aryl hydrocarbon receptor-mediated gene regulation. *Mol Cell Biol* **20**:6095-104.

Rodriguez-Mora OG, LaHair MM, McCubrey JA and Franklin RA (2005) Calcium/calmodulin-dependent kinase I and calcium/calmodulin-dependent kinase kinase participate in the control of cell cycle progression in MCF-7 human breast cancer cells. *Cancer Res* **65**:5408-16.

Sparfel L, Van Grevenynghe J, Le Vee M, Aninat C and Fardel O (2006) Potent inhibition of carcinogen-bioactivating cytochrome P450 1B1 by the p53 inhibitor pifithrin alpha. *Carcinogenesis* **27**:656-63.

Swanson HI (2002) DNA binding and protein interactions of the AHR/ARNT heterodimer that facilitate gene activation. *Chem Biol Interact* **141**:63-76.

Tan Z, Chang X, Puga A and Xia Y (2002) Activation of mitogen-activated protein kinases (MAPKs) by aromatic hydrocarbons: role in the regulation of aryl hydrocarbon receptor (AHR) function. *Biochem Pharmacol* **64**:771-80.

Tan Z, Huang M, Puga A and Xia Y (2004) A Critical Role for MAP Kinases in the Control of Ah Receptor Complex Activity. *Toxicol Sci*.

Tanaka T, Ohmura T and Hidaka H (1983) Calmodulin antagonists' binding sites on calmodulin. *Pharmacology* **26**:249-57.

Tannheimer SL, Barton SL, Ethier SP and Burchiel SW (1997) Carcinogenic polycyclic aromatic hydrocarbons increase intracellular Ca2+ and cell proliferation in primary human mammary epithelial cells. *Carcinogenesis* **18**:1177-82.

van Grevenynghe J, Rion S, Le Ferrec E, Le Vee M, Amiot L, Fauchet R and Fardel O (2003) Polycyclic aromatic hydrocarbons inhibit differentiation of human monocytes into macrophages. *J Immunol* **170**:2374-81.

van Grevenynghe J, Sparfel L, Le Vee M, Gilot D, Drenou B, Fauchet R and Fardel O (2004) Cytochrome P450-dependent toxicity of environmental polycyclic aromatic hydrocarbons towards human macrophages. *Biochem Biophys Res Commun* **317**:708-16.

Zayzafoon M (2006) Calcium/calmodulin signaling controls osteoblast growth and differentiation. *J Cell Biochem* **97**:56-70.

Molecular Pharmacology Fast Forward. Published on December 18, 2007 as DOI: 10.1124/mol.107.043125
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FOOTNOTES

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LEGENDS TO FIGURES

Figure 1: Involvement of $[Ca^{2+}]i$ changes in TCDD-mediated up-regulation of CYP1A1 in MCF-7 cells. (A) MCF-7 cells, preloaded with Fura-2-AM, were exposed to 5 nM TCDD alone or in co-treatment with 10 µM BAPTA-AM or 100 µM 2-APB, or to 10 µM ionomycin at indicated times. $[Ca^{2+}]$i-sensitive F340/F380 ratio were then spectrofluorometrically measured. Data are expressed as Δ F340/F380 ratio, i.e. F340/F380 ratio in treated cells minus F340/F380 ratio measured in untreated counterparts; they are the means ± s.d. of three independent experiments. (B, C) MCF-7 cells were either untreated (UNT) or treated with 5 nM TCDD for 6 hours in the presence or absence of 10 µM BAPTA-AM or 100 µM 2-APB. CYP1A1-related EROD activity was then spectrofluorimetrically measured (B) whereas CYP1A1 mRNA levels were analyzed using RT-qPCR (C). Data are expressed as percent of the values of CYP1A1 activity or mRNA levels found in TCDD-treated cells, arbitrarily set to 100%. They correspond to the means ± S.D. of three independent experiments. *$p < 0.05$, when compared to untreated (A) or to TCDD-treated cells (B, C).

Figure 2: Impairment of TCDD-mediated CYP1A1 up-regulation by chemical inhibition of the Ca^{2+}/CaM/CaMK pathway. (A) CaMK activity was analyzed using cellular protein extracts from MCF-7 cells exposed to TCDD for various time (from 0 to 120 minutes); exposure to norepinephrine (NE, 10 µM) was used as positive control for CaMK activation. Data are expressed in arbitrary units (a.u.), relatively to the value of basal CaMK activity found in untreated cells, arbitrarily set to 1. (B, C, D) MCF-7 cells were either untreated or treated with 5 nM TCDD for 6 hours (B, C) or 2 hours (D) in the presence or absence of KN-92 or KN-93 (30, 40 or 50 µM), or W7 (40 µM). (B) CYP1A1-related EROD activity was then spectrofluorimetrically measured. Data are expressed as percent of the value of CYP1A1 activity found in TCDD-treated cells, arbitrarily set to 100%; they correspond to the means ± S.D. of three independent experiments. (C) CYP1A1 expression was analyzed by western blot analysis; detection of GAPDH expression was used as loading control. The data shown are representative of three independent experiments. (D) CYP1A1 mRNA levels were analyzed using RT-qPCR. Data are expressed as percent of the value of CYP1A1 mRNA levels found in TCDD-treated

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cells, arbitrarily set to 100%; they correspond to the means ± s.d. of three independent experiments. *p < 0.05, when compared to untreated (A) or to TCDD-treated cells (B, D).

Figure 3: Effects of CaMKIα knock-down on TCDD-induced CYP1A1 up-regulation in MCF-7 cells. MCF-7 cells were transfected with siRNAs directed against various CaMK isoforms (A-E), or against CaMKIα (B-E) or with control siRNAs (CT siRNA corresponding to six different CaMK-unrelevant siRNA sequences for A and iNT1 corresponding to a non-targeting siRNA for B-E). Consequences of expression knock-down were assessed by evaluating CYP1A1-related EROD activity (A, B), CYP1A1 protein (C) and mRNA (D) expression or CaMK activity (E), in cells either untreated or exposed to 5 nM TCDD for 6 hours (A-C), 2 hours (D) or 30 minutes (E and F). (A, B, D) EROD activity or CYP1A1 mRNA level data are expressed as percent of the values of CYP1A1-related EROD activity or CYP1A1 mRNA levels found in CT siRNA or iNT1-transfected cells exposed to TCDD, arbitrarily set to 100%; they correspond to the mean ± s.d. of three independent experiments. *, $p<0.05$, when compared to CT siRNA or iNT1-transfected cells exposed to TCDD. (C) The data shown are representative of three independent western-blot analyses; detection of p38 MAPK expression was used as loading control. (E) CaMK activity data are expressed in arbitrary units (a.u.), relatively to the value of CaMK activity levels found in TCDD-untreated iNT1-transfected cells, arbitrarily set to the value of 1 unit; they correspond to the means ± s.d. of three independent experiments. *p < 0.05, when compared to untreated cells. (F) MCF-7 cells were untreated or exposed to 5 nM TCDD for 30 minutes. Phospho-CaMKIα on threonine 177 (P-CaMKIα) was then analyzed by western-blotting; detection of actin expression was used as loading control. The data shown are representative of three independent experiments.

Figure 4: CaMKIα implication in TCDD-mediated activation of CYP1A1 promoter. KN-93-treated or -untreated MCF-7 cells (A, B) and iNT1- or iCaMK1α-transfected MCF7 cells (C) were transfected with pCYP1A1-FL (-1566) (A) or pGL3-XRE3 luciferase constructs (B, C). Cells were then either untreated or treated with 5 nM TCDD for 6 hours. Construct-related luciferase activity was next determined as described in Experimental Procedures. Data are expressed in arbitrary units (a.u.),

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relatively to the value of luciferase activity found in KN-93-untreated (A, B) or control iNT1-transfected (C) cells not exposed to TCDD, arbitrarily set to the value of 1 unit. The data correspond to the means ± s.d. of three independent experiments. *, $p < 0.05$, when compared to TCDD-untreated cells.

Figure 5: Implication of CaMKIα in nuclear import of AhR in response to TCDD. KN-93-treated or -untreated MCF-7 cells (A, B) and iNT1- or iCAMKIα-transfected MCF-7 cells (C, D) were either untreated or treated with 5 nM TCDD for 30 minutes. (A, C) Nuclear contents of AhR and lamin A/C (used here as loading control) were analyzed by western blotting in MCF-7 nuclear fractions. (B, D) For AhR immunolocalization experiments, MCF-7 cells were fixed and stained with the polyclonal IgG rabbit anti-human AhR Ab or its recommended isotypic control, followed by incubation with FITC-labeled anti-rabbit IgG secondary Ab. Cells were photographed with a digital camera using identical exposure. The scale bar represents 10 µm. (A-D). Data shown are representative of three independent experiments.

Figure 6: Inhibition of TCDD-mediated up-regulation of various AhR target genes by the CaMK inhibitor KN-93 in primary human macrophages. Primary human macrophages were either untreated or treated with 5 nM TCDD for 6 hours in the presence or absence of 50 µM KN-93. Levels of AhR target gene mRNAs were then analyzed using RT-qPCR. Data are expressed in arbitrary units (a.u.), relatively to the value of mRNA levels found in untreated cells (UNT), arbitrarily set to the value of 1 unit. They correspond to the means ± s.d. of three independent experiments. *$p < 0.05$, when compared to untreated counterparts. β7ITG, β7 integrin; AhRR, AhR repressor.

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Figure 1

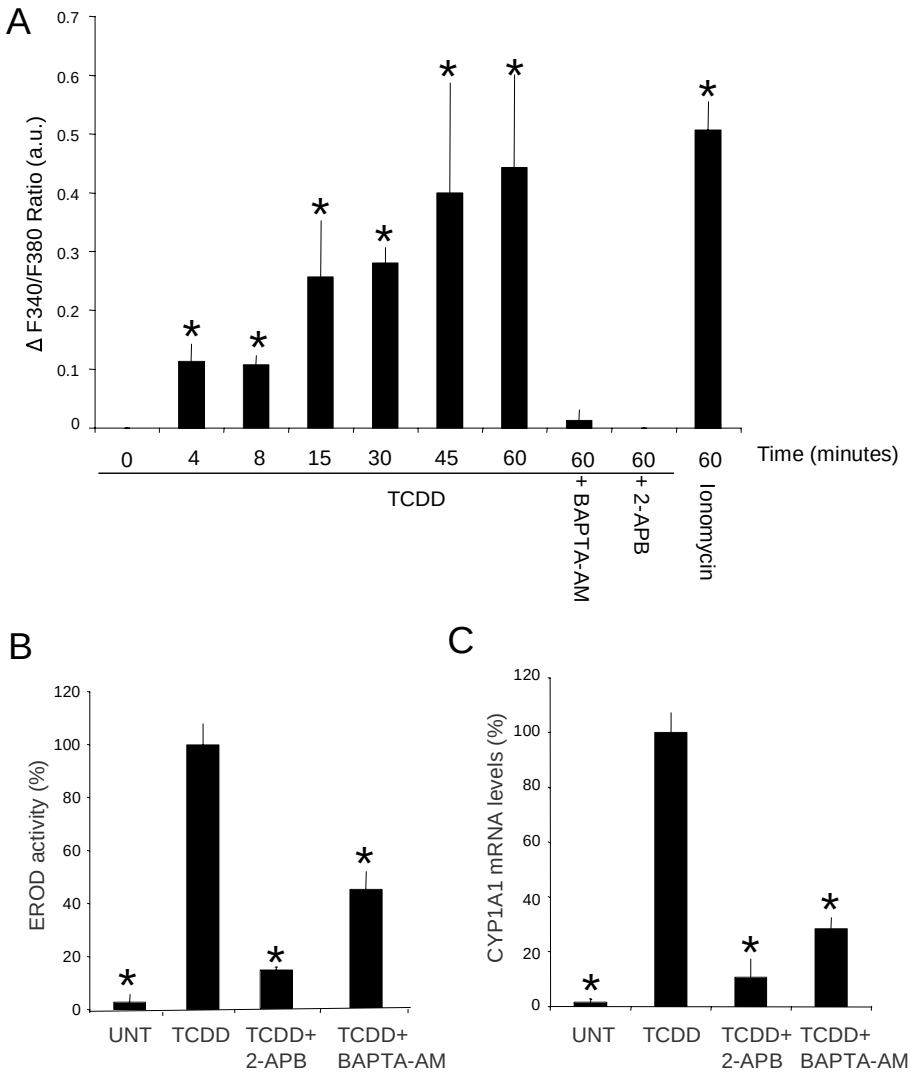

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Figure 2

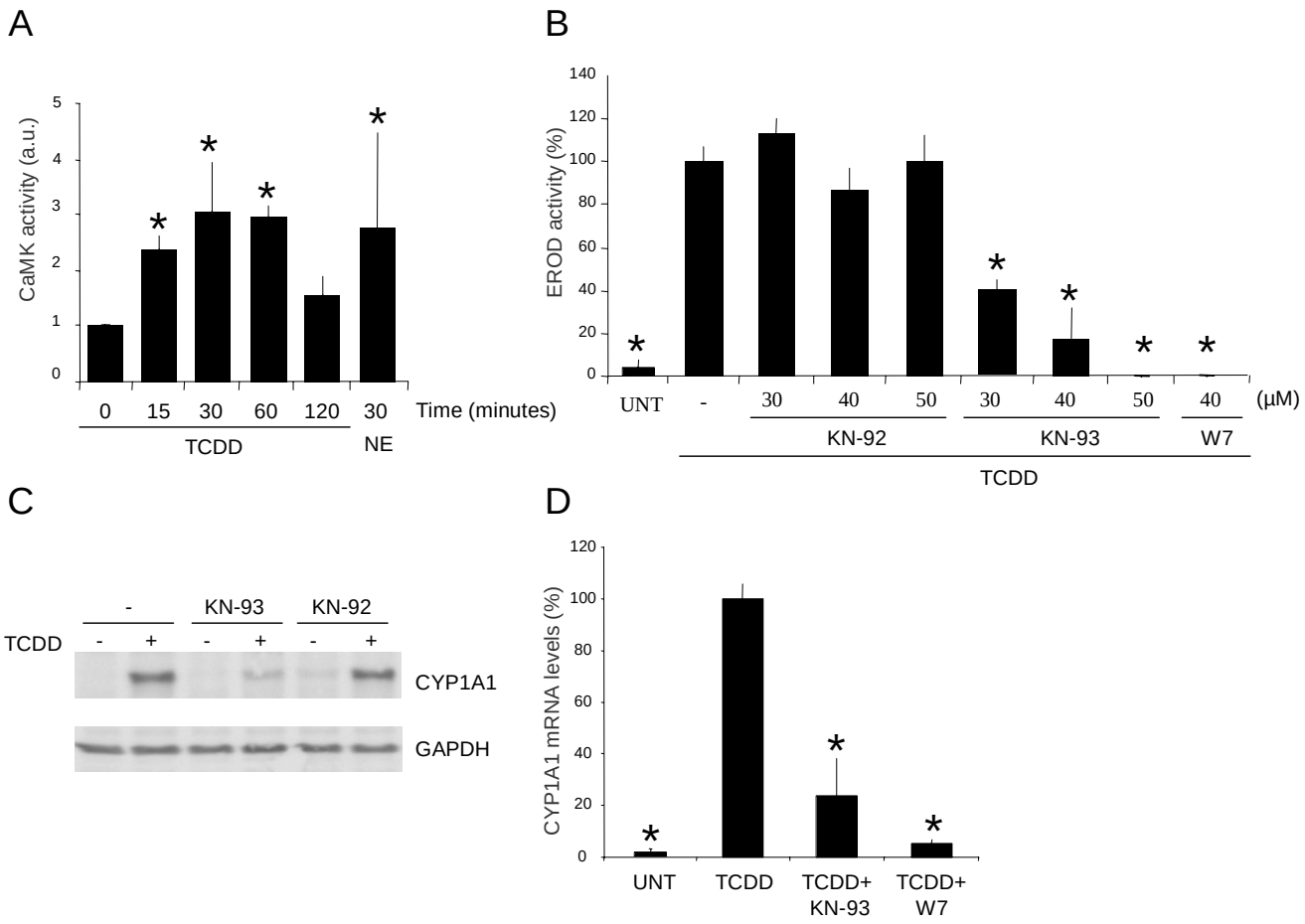

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Figure 3

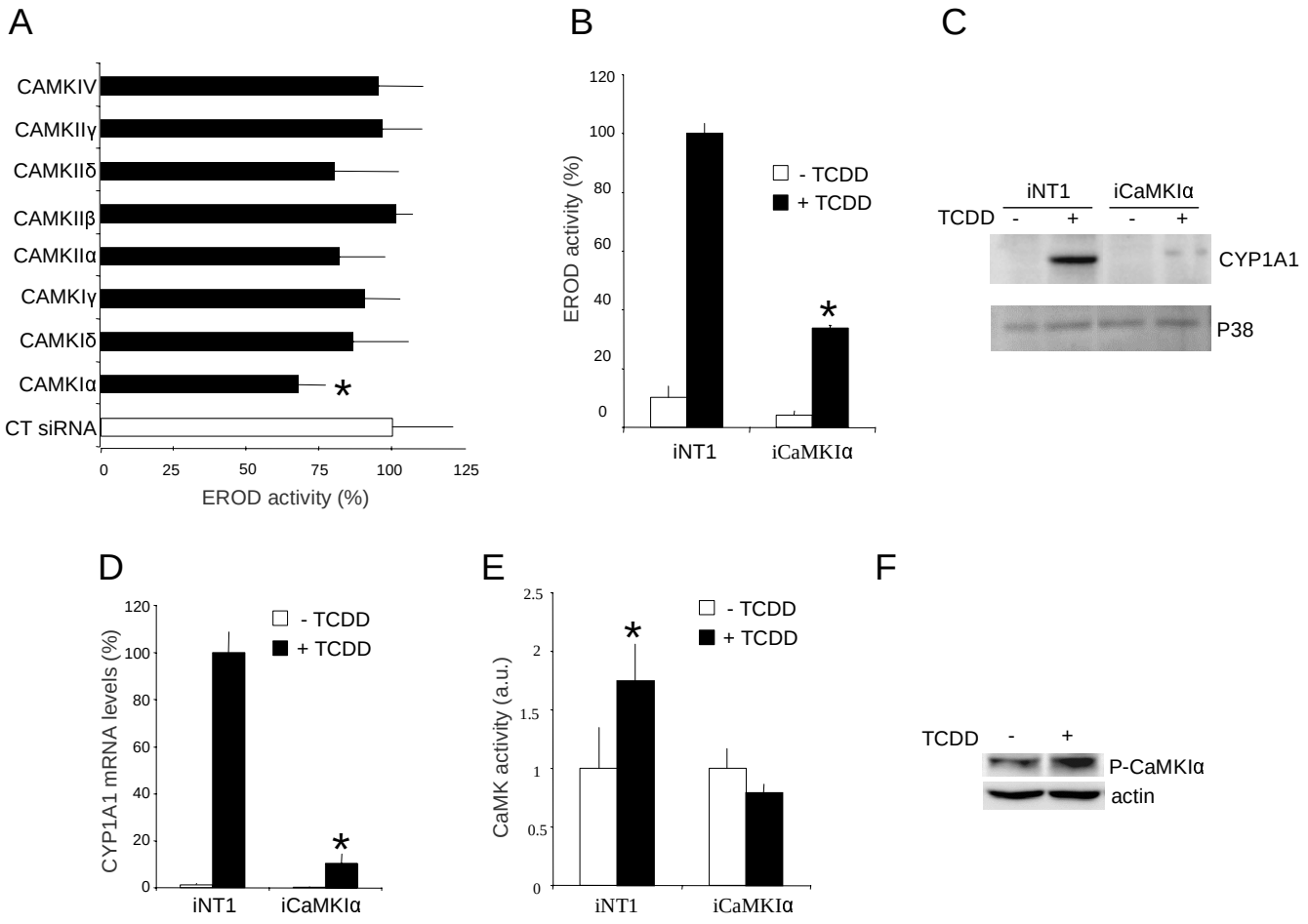

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Figure 4

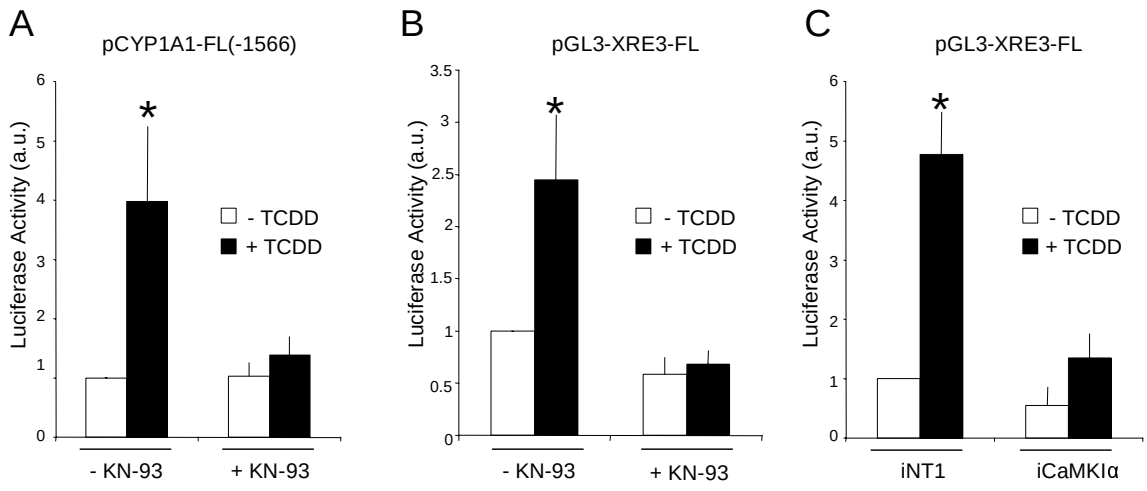

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Figure 5

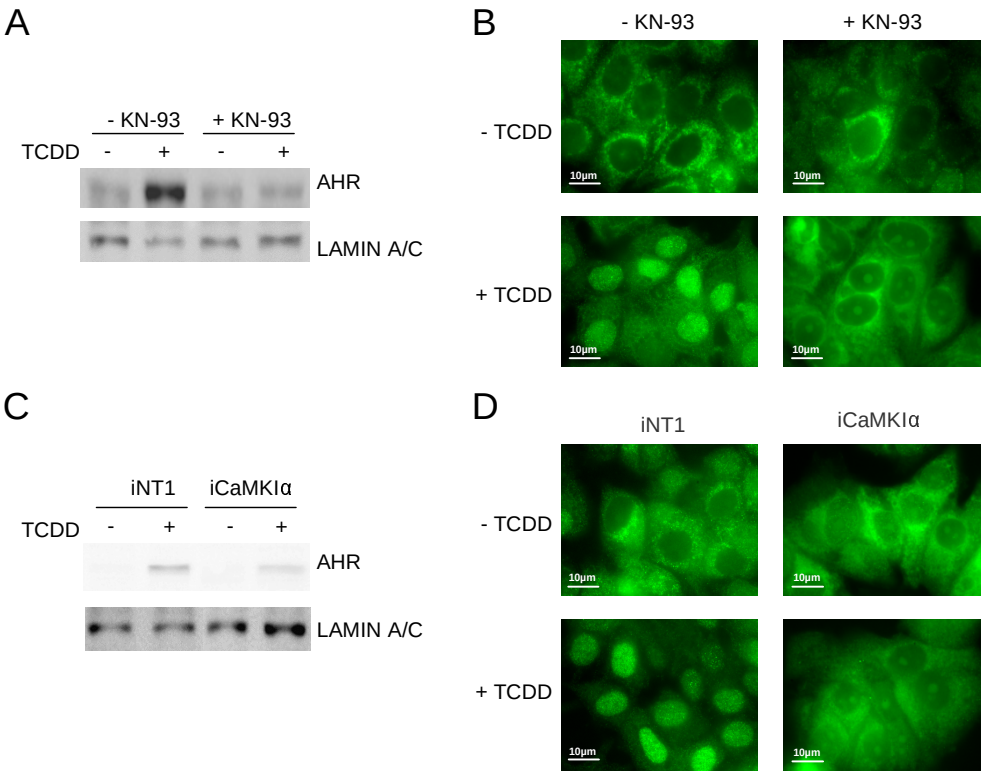

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Figure 6