0026-895X/08/7303-769–777$20.00
MOLECULAR PHARMACOLOGY
Vol. 73, No. 3
43125/3311269
Mol Pharmacol 73:769–777, 2008
Printed in U.S.A.

Dioxin-Mediated Up-Regulation of Aryl Hydrocarbon Receptor Target Genes Is Dependent on the Calcium/Calmodulin/CaMKIα Pathway

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Received November 5, 2007; accepted December 17, 2007

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 the 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 *CYP1A1*. Small interfering RNA (siRNA)-mediated knockdown 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.

The aryl hydrocarbon receptor (AhR) is a 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 90-kDa heat shock protein, p23, and X-associated protein 2 (XAP2), is primarily located in the cytosol (Hankinson, 1995). After ligand binding, AhR moves to the nucleus, dissociates from the chaperone complex, and forms a heterodimer with the AhR nuclear translocator. 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). In this way, TCDD and other AhR agonists [such as the carcinogenic environmental polycyclic aromatic hydrocarbons (PAHs)] markedly induce expression of the drug-metabolizing enzyme CYP1A1, known to detoxify but also to bioactivate carcinogens, including PAHs, and commonly considered a paradigm of AhR gene targets (Barouki et al., 2007).

In addition to activation of AhR, TCDD and PAHs have been shown to increase intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) (Burchiel et al., 1991; Archuleta et al., 1993; Davila et al., 1995; Tannheimer et al., 1997; Le Ferrec et al., 2002). It is noteworthy that 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-talk between Ca^{2+} variations

This work was supported by grants from the Ligue Nationale contre le Cancer, the University of Rennes I, and the Agence Francaise de Sécurité Sanitaire de l'Environnement et du Travail (AFSSET).

P.M. received a fellowship from the Ligue Nationale contre le Cancer

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.107.043125.

ABBREVIATIONS: AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XAP2, X-associated protein 2; XRE, enobiotic responsive elements; PAH, polycyclic aromatic hydrocarbon; CaM, calmodulin; CaMK, Ca^{2+}/CaM-dependent protein kinase; 2-APB, 2-Aminoethoxydiphenylborate; BAPTA-AM, 1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl) ester; KN-93, 2-[*N*-(2-hydroxyethyl)]-*N*-(4-methoxybenzenesulfonyl)amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine; KN-92, 2-[*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; AM, acetoxymethyl ester; Ab, antibody; EROD, ethoxyresorufin *O*-deethylase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; siRNA, small interfering RNA.

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 that play important roles in proliferation (Rodriguez-Mora et al., 2005) and differentiation (Zayzafoon, 2006). It is noteworthy that 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 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.

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 analog 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-AM were provided from Invitrogen (Carlsbad, CA). Ficoll and TRIzol reagents were obtained from Invitrogen. Granulocyte macrophage–colony-stimulating factor (specific activity, 1.2×10^8 U/mg) was purchased from Schering-Plough (Lyon, France). Polyclonal goat antihuman CYP1A1/2 antibody (Ab) was obtained from Daiichi Pure Chemicals Co. Ltd. (Tokyo, Japan), polyclonal rabbit anti-AhR Ab from BIOMOL Research Laboratories (Plymouth Meeting, PA) and rabbit nonspecific IgG from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). Monoclonal mouse anti-actin and polyclonal rabbit anti-p38 mitogen-activated protein kinase, anti-glyceraldehyde-3-phosphate dehydrogenase, anti-phospho-CaMKIα (Thr^{177}), and goat anti-lamin A/C Abs were purchased from Santa Cruz Biotechnology (La Perray en Yvelines, France). [γ-^{32}P] ATP was from Amersham Biosciences. FITC-labeled anti-rabbit IgG Ab was purchased from Jackson Immunoresearch (Suffolk, UK). Chemicals were commonly used as stock solution in dimethyl sulfoxide. 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 Dulbecco's modified Eagle's medium with 4500 mg/l D-glucose, 110 mg/l sodium pyruvate, and nonessential amino acids, supplemented with 100 U/ml penicillin, 100 U/ml streptomycin, and 10% fetal calf serum. Primary human macrophages were obtained from granulocyte macrophage-colony-stimulating factor–exposed blood monocytes and cultured as described previously (van Grevenynghe et al., 2003).

Intracellular Ca^{2+} Measurements. Variations in $[Ca^{2+}]_i$ were analyzed by spectrofluorometry using the Ca^{2+}-sensitive probe Fura-2-AM, as reported previously (Le Ferrec et al., 2002). In brief, MCF-7 cells were cultured in 24-well plates and incubated with the acetoxy cell-permeant form of Fura-2 (Fura-2 AM; 1.5 μM) for 30 min at 37°C in HEPES-buffered medium (10 mM HEPES, 134.8 mM NaCl, 4.7 mM KCl, 1 mM $MgCl_2$, 1.2 mM KH_2PO_4, 1 mM $CaCl_2$, and 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 XS; Molecular Devices, Toronto, ON, Canada) and a well scan was performed at indicated times. Cells were irradiated alternately with light at wavelengths of 340 and 380 nm, and fluorescence from the trapped dye was measured at 510 nm. The ratio of fluorescence intensities recorded after excitation at 340 nm and 380 nm, defined as the F340/F380 ratio, was used to estimate $[Ca^{2+}]_i$ changes, knowing that $[Ca^{2+}]_i$ increase results in an 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 ΔF340/F380 ratio (i.e., the F340/F380 ratio after the addition of chemicals minus the F340/F380 ratio measured before treatment).

Ethoxyresorufin *O*-deethylase Activity Assay. EROD activity, corresponding to the *O*-deethylation of ethoxyresorufin, and mainly supported by CYP1A1 enzyme in living MCF-7 cells, was measured as described previously (Sparfel et al., 2006). In brief, 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 min-period.

RNA Isolation and Analysis. Total RNAs, extracted using the TRIzol method (Invitrogen), were subjected to reverse transcription-real-time quantitative polymerase chain reaction (RT-qPCR) analyses as described previously (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 are given in Table 1.

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 (Dharmacon RNA Technologies, Lafayette, CO). 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. In each well, 6.25 pmol of desiccated siRNAs were rehydrated using 0.125 μl of Dharmafect I reagent diluted in 25 μl of transfection medium (Opti-MEM; Invitrogen) for 40 min at room temperature. Next, 35,000 MCF-7 cells diluted in complete culture medium were added to each well. Twenty-four hours later, transfection medium was replaced by fresh medium for an

TABLE 1
Sequences of the primers used for RT-qPCR analysis

CYP1A1	
Sense	5′-GCACAGAGGTAGTCTCACTGCTTG-3′
Antisense	5′-AAGGGCAGAGGAATGTGATGTT-3′
CYP1B1	
Sense	5′-TGATGGACGCCTTTATCCTC-3′
Antisense	5′-CCACGACCTGATCCAATCT-3′
Interleukin-8	
Sense	5′-AAGAAACCACCGGAAGGAAC-3′
Antisense	5′-AAATTTGGGGTGGAAAGGTT-3′
CCL1	
Sense	5′-AGGCCTCTTTGCCTCTCTTC-3′
Antisense	5′-ATGCAGATCATCACCACAGC-3′
Interleukin-1β	
Sense	5′-GGGCCTCAAGGAAAAGAATC-3′
Antisense	5′-TTCTGCTTGAGAGGTGCTGA-3′
β7 integrin	
Sense	5′-GAATCAACCAGACGGTGACTTTCT-3′
Antisense	5′-GCCCGGAGCCTCAGGA-3′
AhR repressor	
Sense	5′-GTCAGTTACCTCCGGGTGAA-3′
Antisense	5′-TGGAAGCCCAGATAGTCCAC-3′
18S	
Sense	5′-CGCCGCTAGAGGTGAAATTC-3′
Antisense	5′-TTGGCAAATGCTTTCGCT-3′

additional 48 h. MCF-7 cells were next used for TCDD exposure and CYP1A1-related EROD activity assay. As control in siArray assays, the siCONTROL nontargeting siRNA (iNT1, 5′-UAGCGACUAAACACAUCAAtt-3′) and five other siRNAs (provided with siRNA library from Dharmacon) were used; values of TCDD-induced EROD activities obtained from cells-transfected with these six control siRNAs were averaged and arbitrarily set at 100% as control values for EROD activity. For specific knockdown of CaMKIα isoform, we used the siRNA iCaMKIα (5′-GCGGUUACCCUCCCUUCUAtt-3′) and the siRNA iNT1 as a nontargeting control, as reported above.

Immunolocalization. Cells, fixed on coverslips with 4% paraformaldehyde for 30 min 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 fluorescein isothiocyanate-labeled anti-rabbit IgG antibody (3 μg/ml) for 1 h at room temperature. Coverslips were next mounted with PBS glycerol/1,4-diazabicyclo[2.2.2]-octane. Finally, pictures of fluorescence-labeled cells were captured with a DMRXA2 Leica microscope and a COOLSNAP HQ charge-coupled device 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, and 100 μM phenylmethylsulfonyl fluoride, supplemented with an EDTA-free cocktail protease inhibitor (Roche Diagnostic, Meylan, France), whereas nuclear extracts were isolated from MCF-7 cells using the BD Transfactor extraction kit (BD Biosciences, San Jose, CA). Protein samples (50 μg) were next subjected to electrophoresis in a 10% acrylamide gel and electrophoretically transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Marne la Coquette, France). After blocking with Tris-buffered saline containing 4% bovine serum albumin and 0.1% Tween 20 for 1 h at room temperature, membranes were incubated overnight at 4°C with primary Ab. After incubation with appropriate horseradish peroxidase-conjugated secondary Ab for 1 h, immunolabeled proteins were visualized by autoradiography using chemiluminescence.

Reporter Gene Activity and Transient Transfection. The pCYP1A1-FL(−1566) construct containing a 1639-base-pair 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 described previously (Morel and Barouki, 1998). MCF-7 cells were cultured in 24-well plates and cotransfected with pRL-TK vector (Promega, Charbonnières, France) that codes for *Renilla reniformis* 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 transfection reagent according to manufacturer's instructions (Roche Applied Science, Indianapolis, IN). In brief, 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-h period, cells were exposed to TCDD for a 6-h period. Dual luciferase assays (firefly and *R. reniformis*) were then performed with a kit according to the manufacturer's instruc-

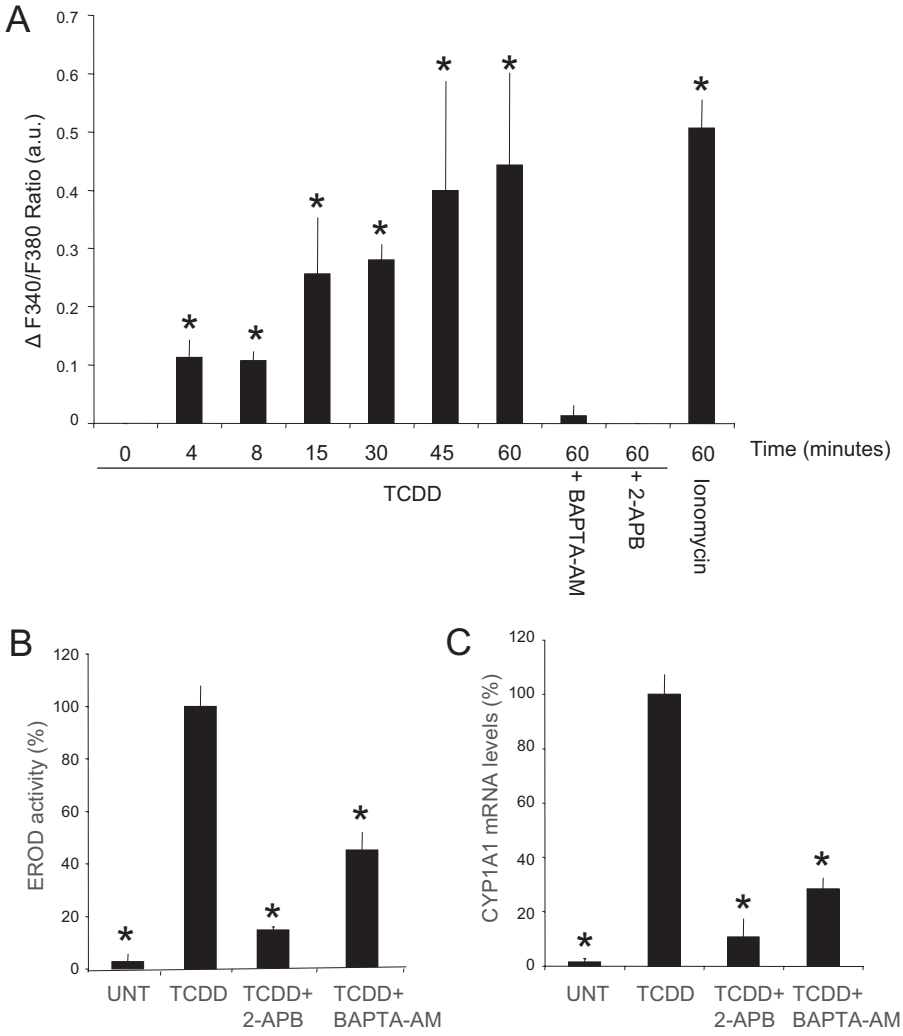

Fig. 1. Involvement of $[Ca^{2+}]_i$ changes in TCDD-mediated up-regulation of CYP1A1 in MCF-7 cells. A, MCF-7 cells, preloaded with Fura-2-AM, were exposed to 5 nM TCDD alone or in cotreatment with 10 μM BAPTA-AM or 100 μM 2-APB or to 10 μM ionomycin at indicated times. $[Ca^{2+}]_i$-sensitive F340/F380 ratios were then spectrofluorometrically measured. Data are expressed as ΔF340/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 and C, MCF-7 cells were either untreated (UNT) or treated with 5 nM TCDD for 6 h in the presence or absence of 10 μM BAPTA-AM or 100 μM 2-APB. CYP1A1-related EROD activity was then spectrofluorometrically measured (B), whereas CYP1A1 mRNA levels were analyzed using RT-qPCR (C). Data are expressed as percentage 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. *, $p < 0.05$, compared with untreated (A) or TCDD-treated cells (B and C).

tions (Promega). Data were expressed in arbitrary unit (a.u.), relative 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, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 2 mM dithiothreitol, 25 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride. CaMK assay was then performed using the "SignaTECT Calcium/Calmodulin-Dependent Protein Kinase Assay System" kit (Promega), according to the manufacturer's instructions. In brief, a mix containing 2.5 μl of specific biotinylated peptide substrate of CaMK (0.5 mM), 5 μl of reaction buffer 5×, 5 μl of activation buffer 5× or control buffer 5×, 5 μl of 0.5 mM ATP, and [γ-^{32}P]ATP (specific activity, 0.5 μCi), was added to cellular extracts. After a 5-min 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 $p < 0.05$.

Results

$[Ca^{2+}]_i$ Changes Were 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 spectrofluorometry and the Ca^{2+}-sensitive probe Fura-2-AM, exposure to 5 nM TCDD was first demonstrated to elicit a rapid increase of $[Ca^{2+}]_i$ in MCF-7 cells (Fig. 1A). $[Ca^{2+}]_i$ elevation was detectable as early as 4 min after addition of TCDD and reached higher levels, close to that obtained with the Ca^{2+} ionophore ionomycin (10 μM), after a 45- to 60-min TCDD exposure. TCDD-elicited increase of $[Ca^{2+}]_i$ in MCF-7 cells was fully abolished when cells were cotreated 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

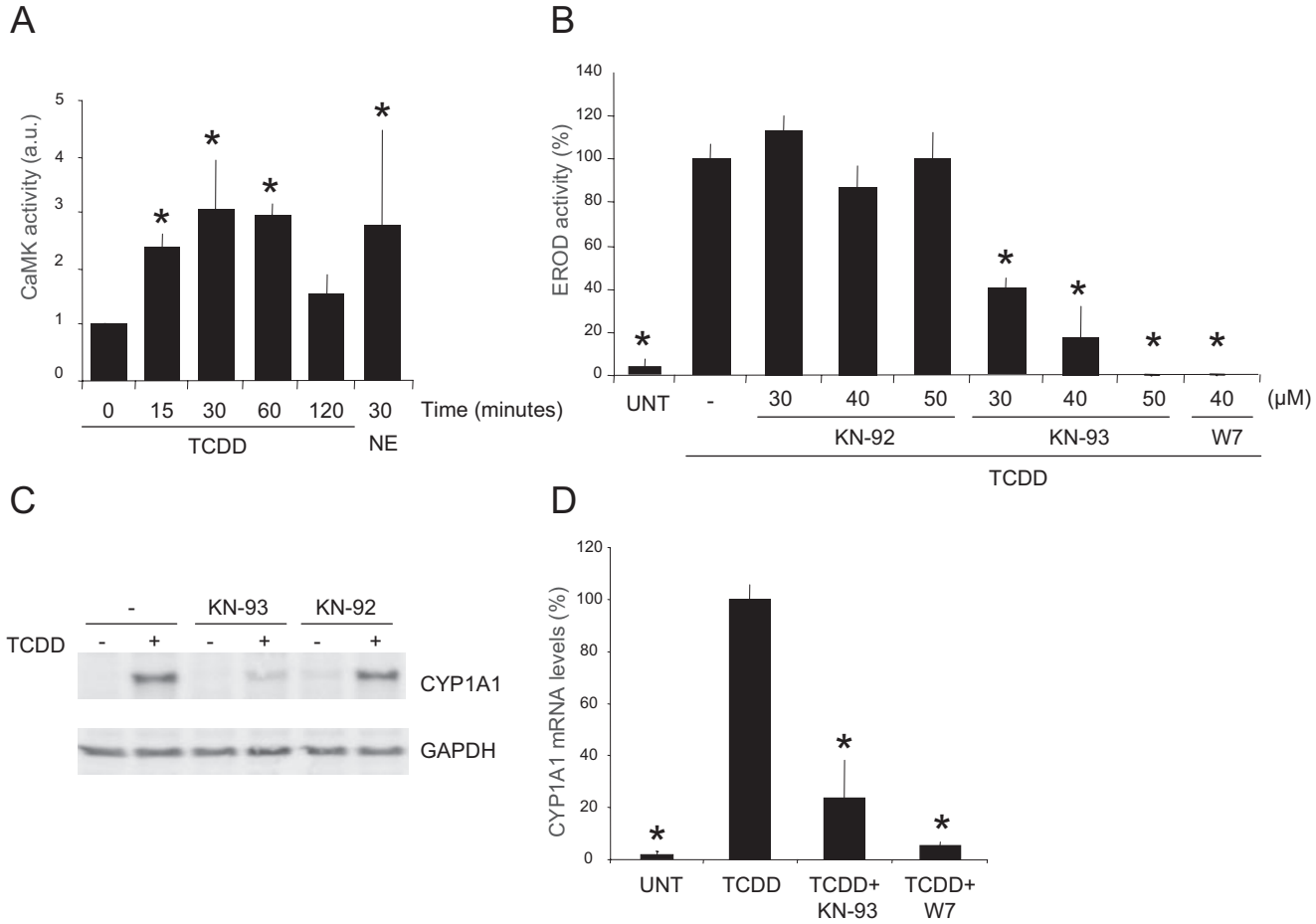

Fig. 2. Impairment of TCDD-mediated CYP1A1 up-regulation by chemical inhibition of the Ca^{2+}/CaM/CaMK pathway. A, CaMK activity was analyzed using cellular protein extracts from MCF-7 cells exposed to TCDD for various times (from 0 to 120 min); exposure to norepinephrine (NE, 10 μM) was used as positive control for CaMK activation. Data are expressed in arbitrary units (a.u.), relative to the value of basal CaMK activity found in untreated cells, arbitrarily set to 1. B–D, MCF-7 cells were either untreated or treated with 5 nM TCDD for 6 h (B and C) or 2 h (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 spectrofluorometrically measured. Data are expressed as percentage 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; detection of glyceraldehyde-3-phosphate dehydrogenase 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 percentage of the value of CYP1A1 mRNA levels found in TCDD-treated cells, arbitrarily set to 100%; they correspond to the means ± S.D. of three independent experiments. *$p < 0.05$, compared with untreated cells (A) or TCDD-treated cells (B and D).

of store-operated channels in TCDD-mediated Ca^{2+} movement in MCF-7 cells as described previously in other cell models (Tannheimer et al., 1997; N′Diaye et al., 2006).

TCDD treatment was next shown to markedly increase both CYP1A1-mediated EROD activity and CYP1A1 mRNA expression in MCF-7 cells (Fig. 1, B and 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 Was 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 first 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 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 analog of KN-93, failed to alter the up-regulation of CYP1A1 activity (Fig. 2B) and the 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 toward $[Ca^{2+}]_i$ changes occurring in MCF-7 cells exposed to TCDD (data not shown).

Knockdown of the CaMKIα Isoform Counteracted TCDD-Mediated CYP1A1 Induction. Because 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

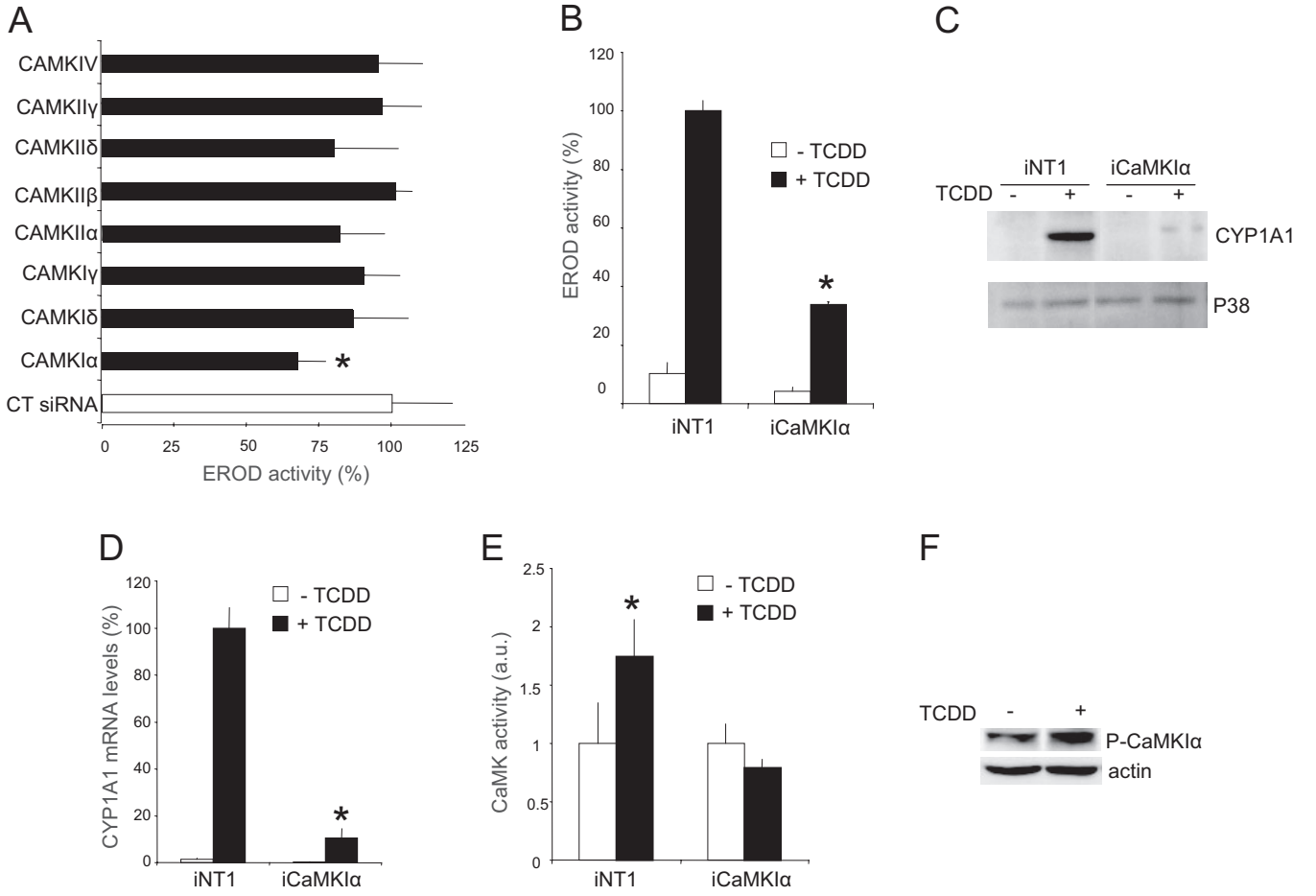

Fig. 3. Effects of CaMKIα knockdown on TCDD-induced CYP1A1 up-regulation in MCF-7 cells. MCF-7 cells were transfected with siRNAs directed against various CaMK isoforms (A), against CaMKIα (B–E), or with control siRNAs (CT siRNA corresponding to six different CaMK-unrelevant siRNA sequences for A and iNT1 corresponding to a nontargeting siRNA for B–E). Consequences of expression knockdown were assessed by evaluating CYP1A1-related EROD activity (A and B), CYP1A1 protein (C) and mRNA (D) expression or CaMK activity (E) in cells either untreated or exposed to 5 nM TCDD for 6 h (A–C), 2 h (D), or 30 min (E). A, B, and D, EROD activity or CYP1A1 mRNA level data are expressed as percentage 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. *, $p < 0.05$ compared with 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.), relative 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. *, $p < 0.05$, compared with untreated cells. F, MCF-7 cells were untreated or exposed to 5 nM TCDD for 30 min. 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.

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 knockdown expression of eight main CaMK isoforms (CaMKIα, -δ, and -γ; CAMKIIα, -β, -δ, and -γ; and CaMKIV) using RNA interference. We first 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 (GenBank accession number 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). Transfection 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). It is noteworthy that exposure to TCDD

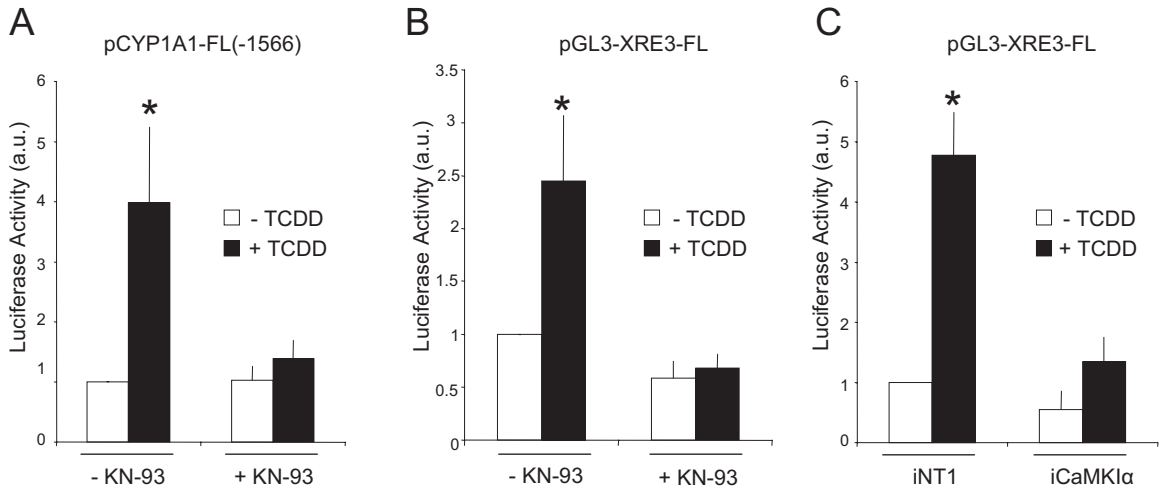

Fig. 4. CaMKIα implication in TCDD-mediated activation of CYP1A1 promoter. KN-93-treated or -untreated MCF-7 cells (A and B) and iNT1- or iCaMK1α-transfected MCF7 cells (C) were transfected with pCYP1A1-FL (−1566) (A) or pGL3-XRE3 luciferase constructs (B and C). Cells were then either untreated or treated with 5 nM TCDD for 6 h. Construct-related luciferase activity was next determined as described under *Experimental Procedures*. Data are expressed in arbitrary units (a.u.) relative to the value of luciferase activity found in KN-93-untreated (A and 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. *, $p < 0.05$, compared with TCDD-untreated cells.

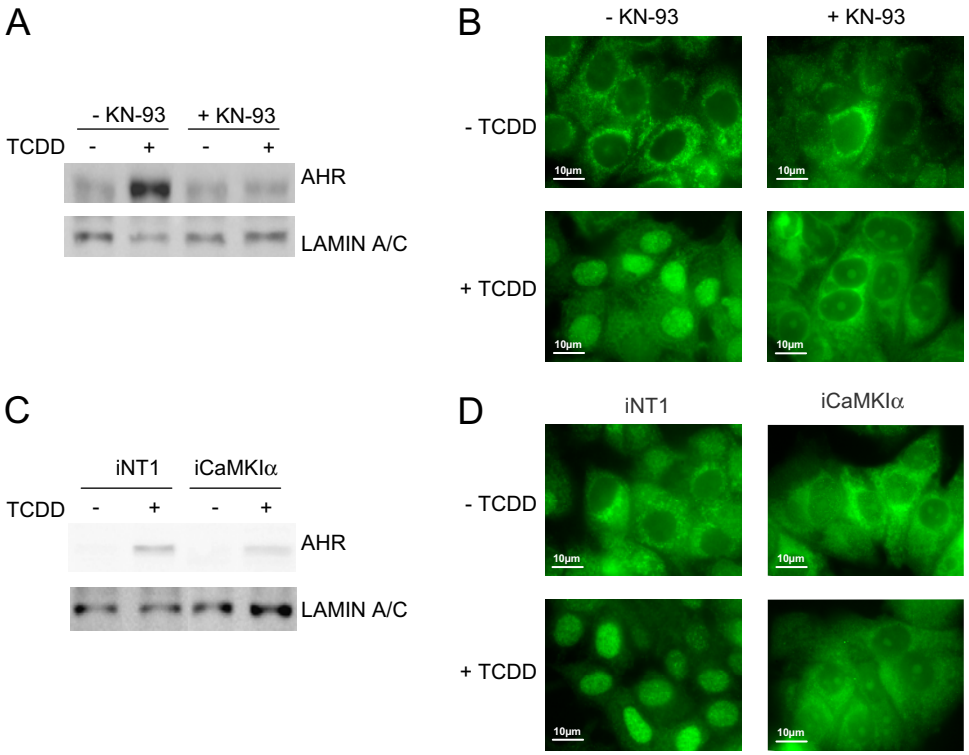

Fig. 5. Implication of CaMKIα in nuclear import of AhR in response to TCDD. KN-93-treated or -untreated MCF-7 cells (A and B) and iNT1- or iCAMKIα-transfected MCF-7 cells (C and D) were either untreated or treated with 5 nM TCDD for 30 min. A and C, nuclear contents of AhR and lamin A/C (used here as loading control) were analyzed by Western blotting in MCF-7 nuclear fractions. B and 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 fluorescein isothiocyanate-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.

resulted in increased CaMKIα (Thr^{177}) phosphorylation and probably, thereby, in full CaMKIα activation (Haribabu et al., 1995) (Fig. 3F).

TCDD-Mediated Activation of CYP1A1 Promoter Required 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 base pairs 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 (Morel and Barouki, 1998; Le Ferrec et al., 2002). Cotreatment 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 knockdown expression of CaMKIα (Fig. 4C).

CaMKIα Inhibition and Knockdown Prevented 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 labeling (Fig. 5). Treatment by TCDD triggered an important increase of AhR nuclear content as demonstrated by Western blot analysis of nuclear fractions (Fig. 5A); likewise, 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 cotreatment with KN-93 (Fig. 5, A and B). SiRNA-mediated knockdown 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).

CaMK Activity Was 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 (van Grevenynghe et al., 2004; Lecureur et al., 2005; N′Diaye et al., 2006; Monteiro et al., 2007) and in which AhR ligands trigger $[Ca^{2+}]_i$ increase (N′Diaye et al., 2006), we found that cotreatment 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 interleukin-1β, the chemokines interleukin-8 and CCL1, the adhesion molecule β7 integrin, and the AhR repressor (Fig. 6).

Discussion

Elevation of $[Ca^{2+}]_i$ in response to AhR agonists has been reported in various cellular models, including lymphocytes (Burchiel et al., 1991; Pallardy et al., 1992; Archuleta et al., 1993), 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 a hallmark of AhR agonist exposure. It is noteworthy that 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

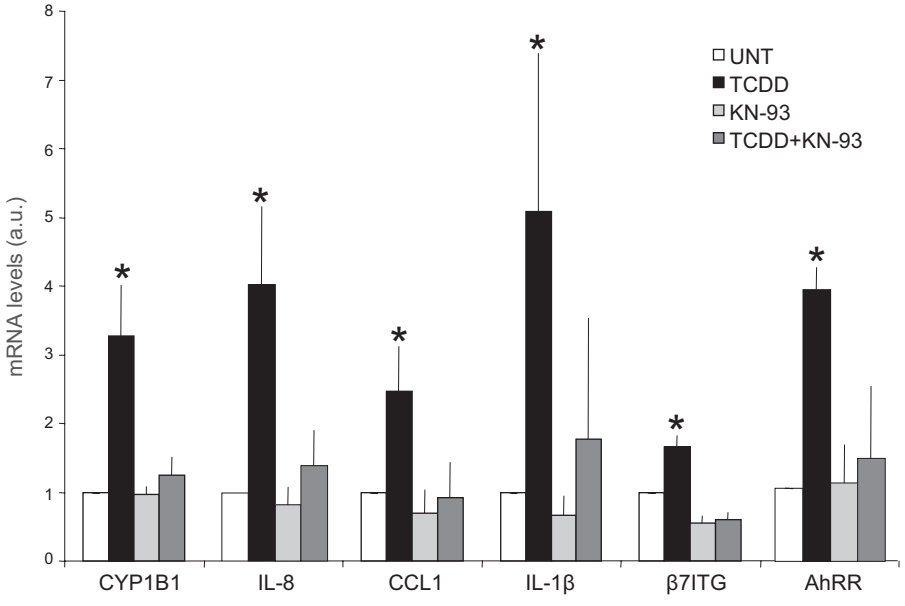

Fig. 6. Inhibition of TCDD-mediated up-regulation of various AhR target genes by the CaMK inhibitor KN-93 in primary human macrophages. Primary human macrophages were either untreated or treated with 5 nM TCDD for 6 h in the presence or absence of 50 μM KN-93. Levels of AhR target gene mRNAs were then analyzed using RT-qPCR. Data are expressed in arbitrary units (a.u.), relative 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. *, $p < 0.05$, compared with untreated counterparts. β7ITG, β7 integrin; AhRR, AhR repressor.

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 antagonized TCDD-mediated up-regulation of CYP1A1 activity and expression. Moreover, knockdown 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 because it allows neutralization of any potential lack of specificity of the chemical inhibitors used in the study, including a putative AhR antagonist-like activity of KN-93, which should be formally considered. In addition, siRNA-related experiments allowed us to demonstrate that, among CaMK isoforms, expression/activity of the CaMKIα isoform is most likely to be required for AhR-dependent up-regulation of target genes in MCF-7 cells. Indeed, knockdown 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). It is noteworthy that the 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 systemic manner.

Inhibition of CaMK activity and knockdown of CaMKIα expression markedly prevented TCDD-mediated induction of CYP1A1 promoter activity and of XRE-driven reporter activity, indicating that the CaMK pathway was required for transcriptional activity triggered by AhR in the nucleus. However, transcriptional activity of AhR, at least, depends on previous steps, including AhR nuclear import and heterodimerization with AhR nuclear translocator. Because CaMKI has been implicated in cellular localization of several proteins (Kao et al., 2001), its effect toward AhR cellular distribution was checked. Regarding this point, we have found that both cotreatment by KN-93 and knockdown 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 be kept in mind, however, that the initial cytosolic step of the AhR signaling cascade, which 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 the 90-kDa heat shock protein (Ogiso et al., 2004), may be interesting to 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 mitogen-activated protein kinases (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, extracellular signal-regulated kinase has been shown to be involved in regulation of the AhR response (Tan et al., 2002; Lecureur et al., 2005) 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 controversial, however, because 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.

Acknowledgments

We thank Stephanie Dutertre (IFR 140) for fluorescence microscopy experiments.

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