BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **229,** 231–237 (1996)
ARTICLE NO. 1785

Induction of CYP1A1 by GABA Receptor Ligands

Marianne D. Sadar,*,†,[1] Anna Westlind,‡ Fredrik Blomstrand,§
and Tommy B. Andersson*,‡

**Department of Zoophysiology and §Institute of Anatomy and Cell Biology, University of Göteborg, S 40031 Göteborg, Sweden; †Department of Biomedical Sciences, University of Bradford, Bradford, West Yorkshire BD7 1DP, United Kingdom; and ‡Department of Pharmacokinetics and Drug Metabolism, Astra Hässle AB, S 43183 Mölndal, Sweden*

Received October 31, 1996

The induction of CYP1A1 is mediated via the aromatic hydrocarbon (Ah) receptor. Studies from our laboratory show CYP1A1 induction by picrotoxin and phenobarbital which prompted us to examine if other ligands of the γ-aminobutyric acid (GABA) receptor could also induce CYP1A1. Here we report the nuclear translocation of the Ah receptor and its DNA binding activity to radiolabeled double-stranded synthetic xenobiotic response elements (XREs) in nuclear extracts, increased accumulation of CYP1A1 mRNA, and alterations in intracellular calcium concentrations in cells exposed to GABA receptor ligands.

The basic helix-loop-helix (bHLH) Ah receptor is a ligand activated transcription factor which mediates the induction of *CYP1A1* gene expression and has been correlated to carcinogenesis by various compounds including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (1,2). Phenobarbital (PB) is a classic CYP2B inducer in mammals, while in fish investigators have been unsuccessful in detecting CYP2B induction despite the presence of a *CYP2B*-like gene (3). Curiously, for twenty years PB has been shown to induce *CYP1A1* gene expression in mammals, both *in vivo* (4,5) and *in vitro* (5-8), and recently *in vitro* in fish (9). Teleost hepatic CYP1A1 is similar to its mammalian counterpart in its ability to be induced by numerous polycyclic aromatic hydrocarbons (10,11). The Ah receptor (12), its nuclear translocation (9,13) and the presence of xenobiotic response elements (XREs) on the *CYP1A1* gene (14) have been detected in fish.

PB and picrotoxin have been shown to induce CYP1A1 in primary cultures of rainbow trout hepatocytes (9,15). These compounds do not comply to the theory that CYP1A1 inducers must be planar and rigid molecules that bind to the Ah receptor, and therefore their effects on CYP1A1 may primarily be via signal transduction pathways. Both PB and picrotoxin are ligands of the γ-aminobutyric acid (GABA) receptor (16), which has been detected in peripheral tissues outside of the central nervous system (CNS) both in mammals (17) and fish (18,19). The $GABA_A$ receptor and a GABA transport system have been detected on isolated rat hepatocytes (20). The purpose of this study was to examine if other GABA receptor ligands, in addition to PB and picrotoxin, could alter *CYP1A1* gene expression in a primary culture of rainbow trout hepatocytes.

MATERIALS AND METHODS

Cell culture and treatments. All chemicals were purchased from Sigma, unless stated otherwise. Hepatocytes were isolated and cultivated from juvenile, cultured rainbow trout (*Oncorhynchus mykiss*) as described previously (9). Cells

[1] Corresponding author. Department of Cancer Endocrinology, B.C. Cancer Agency, 600 West 10th Avenue, Vancouver, B.C., Canada V5Z 4E6. Fax: (604) 877-6011.

0006-291X/96 $18.00

were counted in a Bürker chamber, assessed for cell viability by the Trypan Blue exclusion test, and seeded at 10^7 cells per plate (10 cm) twenty-four hours before inducers were added. PB, GABA, δ-aminovaleric acid (DAVA) were added to cultures in Medium 199 (Gibco), while TCDD (Cambridge Isotope Laboratories) and actinomycin D were dissolved in dimethyl sulfoxide (DMSO; final concentration of 0.1%). Control cultures for actinomycin D and TCDD received DMSO only (0.1%). All experiments were performed at least three times using different cell culture preparations, and the data presented represents a single experiment using replicate culture plates.

RNA isolation and analysis. Total RNA was extracted from cultured hepatocytes using the RNAzol (Cinna/Biotecx Lab. Inc) solution method, which employs acid guanidium thiocyanate-phenol-chloroform, as described (21,22). Total RNA (10 μg) was fractionated by agarose gel electrophoresis. Equal loading and the integrity of each sample was assessed by staining the gel with ethidium bromide and quantification of 18S rRNA. Membranes were hybridized with a cDNA probe that was specific for rainbow trout CYP1A1 mRNA (pSG-15 plasmid with 2553 bp of the cDNA for rainbow trout CYP1A1). This probe amplified by polymerase chain reaction (PCR) with the upper primer recognizing position 661 bp of the gene (5′-GGCTTGGTGAACATGAGT-3′) and the lower primer recognizing the sequence at 1433 bp of the gene (5′-GCCAAGAGGAAGACC-3′) and was labeled with digoxygenin for detection of CYP1A1 mRNA with the DIG Luminescent Detection Kit (Boehringer Mannheim). The chemiluminescent signal was recorded on x-ray film and the CYP1A1 mRNA bands were quantitated by scanning with a densitometer (LKB 2222-020 Ultroscan XL Laser Densitometer).

Immunoblots. Nuclear extracts were prepared as previously described (23), with the modification that the nuclear lysis buffer contained 0.4 M NaCl instead of KCl, and 10% glycerol was present during cell lysis. Western blots were performed using protein from nuclear extracts, as described previously (24). Nitrocellulose blots were blocked for 30 minutes with 5% dry milk (w/v) in 20 mM Tris-HCl, pH 7.4 containing 500 mM NaCl (TBS). The nitrocellulose blots were incubated overnight with anti-Ah receptor protein (M. Ingelman-Sundberg, Karolinska, Sweden) (1:300), then washed and incubated an additional 2 hours with the secondary antibody (1:1000). Ah receptor proteins were detected using the ECL luminescence kit (Amersham). The protein content of cell extracts was determined as described previously (25).

Electrophoretic mobility shift assay (EMSA). Cells were treated with 100 pM TCDD, 10 mM GABA, 10 mM DAVA, or DMSO (0.1%) for various time periods before harvesting. Nuclei were prepared and protein extracted as described above. DNA-binding reactions were carried out in 18 μl, with 8 μg of nuclear protein at a final concentration of 25 mM HEPES (pH 7.5), 0.2 mM EDTA, 75 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerol, 0.8 μg poly(dI-dC) (Pharmacia). Thirty-six femtomoles of a double-stranded, ^{32}P-labeled oligonucleotide (5′-tacacctttgCA-CGCtatcgaaatct-3′) corresponding to -1364 to -1338 of the rainbow trout *CYP1A1* gene were used. For competition experiments indicated, 100-fold excess unlabeled oligonucleotide was used. DNA-protein complexes were separated under non-denaturing conditions in a 6% polyacrylamide gel (30:1) containing 2.5% glycerol, run in Tris-glycine-EDTA (50 mM Tris, 380 mM glycine, and 2.0 mM EDTA) at room temperature.

Intracellular calcium analyses. Cultured hepatocytes grown on glass coverslips were loaded with 5 μM Fura-2/AM, at a temperature of 12°C for 1 hour. The cells were washed with salmon buffer (26) and fixed into a chamber mounted on the stage of a inverted epifluorescence microscope (Nikon Diaphot), equipped with a fluorescence 40× objective, interfaced with a SPEX CM-X microspectrofluorimeter. $[Ca^{2+}]_i$ was determined from the ratio of two excitation wavelengths, 340 and 380 nm, with the emission wavelength centered at 505 nm. $[Ca^{2+}]_i$ fluxes were detected by a shift occurring in the excitation spectrum of Fura-2/AM from 380 nm in its unbound state to 340 nm when it is bound to calcium. $[Ca^{2+}]_i$ (nM) was estimated by comparing the experimental 340/380 ratio with a calibration curve for free Ca^{2+}, obtained by using Ca^{2+} calibration buffers with known concentrations of Ca^{2+} and Fura-2/AM.

RESULTS

According to the mechanism for the central nervous system $GABA_A$ receptor-ionophore complex, PB modulates GABA stimulation (16). To determine if GABA could modulate PB or TCDD induction of *CYP1A1* expression we examined the accumulation of CYP1A1 mRNA in cells exposed to various combinations of these compounds. Heptocytes exposed to 2 mM PB for 24 hours showed an increase in accumulation of CYP1A1 mRNA that was 2.2-fold higher than control levels (Fig. 1A). After 24 hours, the addition GABA (0.1 mM to 50 mM) enhanced the PB induced accumulation of CYP1A1 mRNA. A concentration of 0.1 mM GABA increased PB induction of CYP1A1 mRNA to 3.8-fold over control levels, and 1 mM GABA increased PB induction of CYP1A1 mRNA levels to 5.2-fold over the control levels. GABA administered alone showed no effect on the levels of CYP1A1 mRNA, as compared to control levels. Treatment of hepatocytes with a saturating concentration of TCDD for 24 hours caused a 5.4-fold induction of CYP1A1 mRNA levels over controls. The simultaneous addition of TCDD with GABA did not affect the induction of CYP1A1 mRNA levels as compared to the

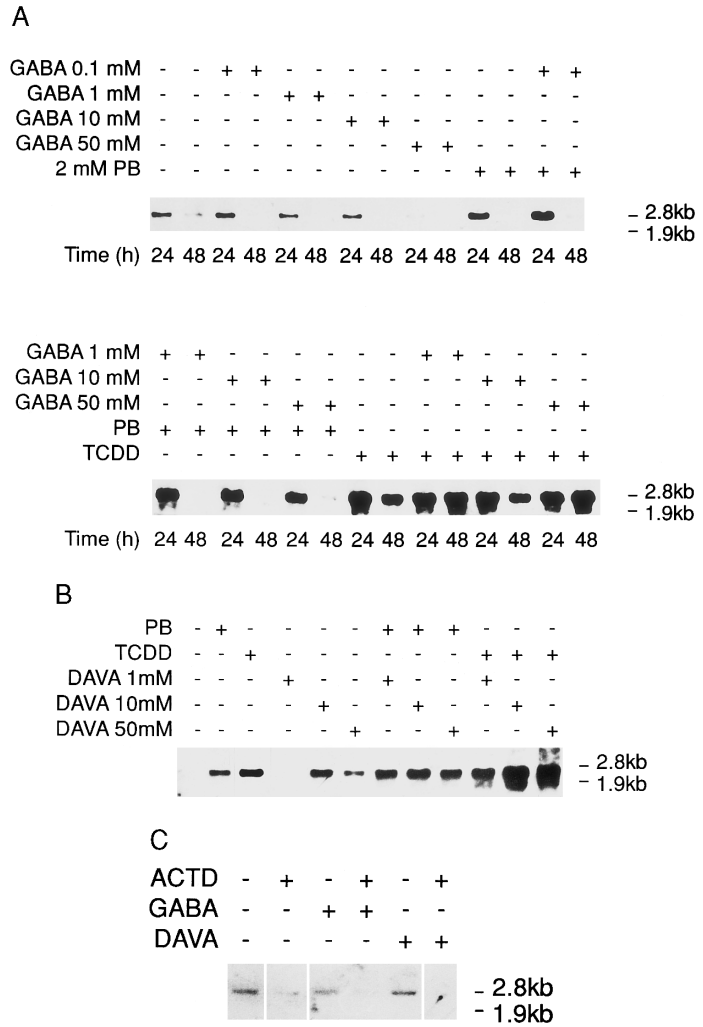

FIG. 1. Regulation and effects of GABA receptor ligands on CYP1A1 mRNA in rainbow trout hepatocytes maintained in primary culture. (A) Primary cultures of rainbow trout hepatocytes were exposed to PB (2 mM), TCDD (100 pM), varying concentrations of GABA (0.1 - 50 mM), or combinations of GABA with either PB, or TCDD, for 24 or 48 hours before the cells were harvested and Northern blots prepared. (B) Primary cultures of rainbow trout hepatocytes were exposed to PB (2 mM), TCDD (100 pM), varying concentrations of DAVA (1, 10, and 50 mM), or combinations of DAVA with either PB, or TCDD, for 24 hours before the cells were harvested and Northern blots prepared. (C) CYP1A1 mRNA levels in cells exposed to an inhibitor of transcription (actinomycin D (ACTD), 2.5 μg/ml) in the co-presence of either GABA (50 mM), or DAVA (50 mM). Variation between two samples was not more than 10% in CYP1A1 mRNA quantifying.

TCDD response. After 48 hours of exposure of hepatocytes to PB and GABA, CYP1A1 mRNA dropped to levels comparable with the control. TCDD induced levels of CYP1A1 mRNA remained elevated after 48 hours of treatment and these levels were unaffected by the simultaneous addition of GABA.

It should be noted that due to the quick metabolism of GABA in the liver (20) and the rate of metabolism of the compound playing a key role in determining its potency (27), relatively high concentrations of GABA and DAVA, as compared to TCDD, were required to observe an induction of CYP1A1.

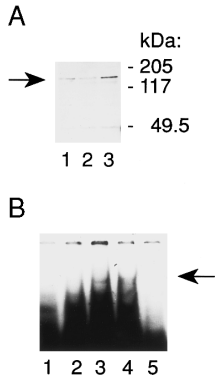

FIG. 2. DAVA induces both nuclear translocation of the Ah receptor (A) and DNA-binding activity of the Ah receptor to synthetic CYP1A1 XREs (B) in nuclear extracts from rainbow trout hepatocytes. (A) Immunoblot analysis of the Ah receptor in nuclear extracts from primary cultures of rainbow trout hepatocytes exposed to DAVA for 12 hours. Total liver nuclear protein (40 μg) was loaded to each lane, before denaturing SDS-PAGE, Western blotting, and immunodetection using a peptide corresponding to amino acids 12 to 31 of the murine dioxin receptor (coupled to ovalbumin and antisera produced in rabbits). (B) Specific DNA-binding activity of the Ah receptor in nuclear extracts from rainbow trout hepatocytes exposed to GABA (10 mM) and DAVA (10 mM) 12 hours. Lanes: 1, Control; 2, GABA (10 mM); 3, DAVA (10 mM); 4, TCDD (100 pM); 5, TCDD (100 pM) with 100X excess unlabeled XRE. Arrow indicates Ah receptor/DNA complex.

Exposure of cells for 24 hours with DAVA (10 mM) caused a pronounced increase in the accumulation of CYP1A1 mRNA when applied to cells solely (7.5-fold increase as compared to control levels), or in combinations with PB (4.9-fold increase over PB levels) or TCDD (5.3-fold over TCDD levels) (Fig. 1B).

Transcriptional regulation of CYP1A1 induction by DAVA was investigated by application of actinomycin D, an inhibitor of transcription, to cells for twelve hours. Actinomycin D prevented accumulation of CYP1A1 mRNA by DAVA (Fig. 1C), thereby indirectly suggesting that DAVA does not stabilize CYP1A1 mRNA but rather induces CYP1A1 at a transcriptional level. Consistent with results in Figure 2A, GABA did not induce accumulation of CYP1A1 mRNA.

Both PB and TCDD have been shown to cause the nuclear translocation of the Ah receptor in primary cultures of rainbow trout hepatocytes (9). To determine if DAVA and/or GABA were acting through the Ah receptor we examined if these compounds could increase the nuclear levels of Ah receptor protein. Results presented in Fig. 2A show DAVA (lane 3) to induce the nuclear levels of Ah receptor protein after 12 hours of exposure. The levels of Ah receptor in nuclear extracts from cells exposed to GABA (lane 2) for 12 hours were not elevated over the constitutive levels observed in the control (lane 1). The Ah receptor was calculated to have a molecular weight of 145 kDa which is in agreement with other reports (28,29). Specificity of the band detected was confirmed by competition experiments using 1 mg/ml of antigen pre-incubated with the primary antibody (data not shown).

In support of nuclear translocation of the Ah receptor, we show the activation of the DNA-binding activity of the Ah receptor by employing the electrophoretic mobility shift assay (EMSA) with nuclear extracts of cells exposed to solely DAVA. Results presented in Fig. 2B show specific DNA-binding activity in cells exposed to DAVA for 12 hours (lane 3) as also seen with TCDD-treated cells (lane 4).

To assess a possible connection via a second messenger between a putative plasma membrane receptor and the Ah receptor, we examined the effects of PB, GABA, and DAVA upon the $[Ca^{2+}]_i$ in rainbow trout hepatocytes employing the calcium probe, Fura-2/AM. The addition

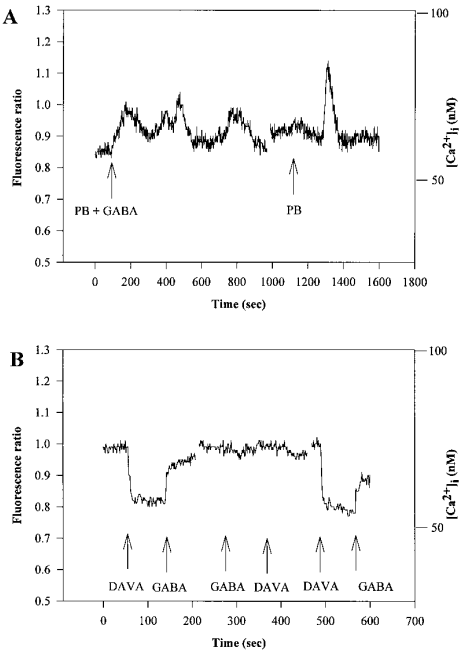

FIG. 3. Effects of GABA, PB, and DAVA on $[Ca^{2+}]_i$ in primary cultures of rainbow trout hepatocytes. (A) PB (2 mM); GABA (0.1 mM). (B) DAVA (50 mM); GABA (50 mM). $[Ca^{2+}]_i$ was measured in a single hepatocyte in salmon buffer containing 1.5 mM $CaCl_2$. Compounds were added where the arrow depicts and the cells were washed and allowed to rest where the trace is discontinued. The fluorescence ratio employed was 340 nm/380 nm.

of GABA (0.1 mM to 50 mM) did not have any effect upon $[Ca^{2+}]_i$. PB was shown to cause a transient increase in $[Ca^{2+}]_i$ after a lag period of two to three minutes (Fig. 3A). This increase was characteristically a single spike, which after approximately 1.5 minutes in width, effectively returned to the normal $[Ca^{2+}]_i$ resting levels (74 nM). When a mixture of 0.1 mM GABA and 2 mM PB was added to hepatocytes, the $[Ca^{2+}]_i$ increased in sinusoidal oscillations. The first two oscillations were approximately 4.5 minutes apart and the subsequent oscillations were progressively further apart. The first oscillation was approximately 3.5 minutes in width and was an immediate response to the addition of GABA and PB.

The addition of DAVA (50 mM) to cells induced a decrease in $[Ca^{2+}]_i$ (Fig. 3B). However, if GABA was subsequently added to cells initially exposed to DAVA (DAVA still present), the $[Ca^{2+}]_i$ was observed to immediately return to resting levels. When we reversed the order of addition of DAVA and GABA, (i.e., DAVA followed the addition of GABA) no change in $[Ca^{2+}]_i$ was observed in response to the inclusion of either compound.

DISCUSSION

The present study shows that GABA and the GABA receptor antagonist DAVA modulate CYP1A1 expression. Previously we showed induction of CYP1A1 in rainbow trout hepatocytes by the GABA receptor ligands, picrotoxin (15) and PB (9). Interaction between DAVA and GABA with either PB or TCDD showed dramatic differences in regards to CYP1A1 induction. Exposure of cells to GABA for 12 hours did not result in an increase in nuclear Ah receptor levels, nor could DNA-binding activity be observed by incubating nuclear extracts with a synthetic XRE at this time point. Consistent with these data, GABA did not induce CYP1A1 mRNA by itself, nor did it affect TCDD induction, but GABA did potentiate PB induction of

CYP1A1 mRNA to levels comparable to those obtained with TCDD. The effect of GABA on PB induction is probably on the transcriptional rate and not by a general stabilization of mRNA since GABA did not have any effect on TCDD induced mRNA. In addition these results argue against GABA producing a generalized effect upon metabolism which positively influences CYP1A1 mRNA transcription. Similar to GABA effects on TCDD induction, PB has also been shown not to enhance TCDD induction of CYP1A1 mRNA (9).

The mechanisms by which DAVA affects CYP1A1 induction may include other additional components than the mechanism for GABA since DAVA was shown to potentiate TCDD induction of CYP1A1 mRNA. Such an alternative mechanism does not appear to involve stabilization of mRNA as we demonstrate in Figure 1C through application of actinomycin D which completely blocked DAVA induction of CYP1A1. Picrotoxin, another GABA antagonist was previously shown to synergistically increase TCDD induction of CYP1A1 mRNA (15). Collectively, these data show GABA antagonists, DAVA and picrotoxin, to enhance TCDD induction of CYP1A1 mRNA and to induce CYP1A1 mRNA by themselves.

Transient increases in $[Ca^{2+}]_i$ have been suggested to be a possible early event in TCDD induction of CYP1A1 (30). We similarly demonstrated PB to induce transient increases in $[Ca^{2+}]_i$ which was characteristically observed as a single spike, while GABA appeared to have no effect on $[Ca^{2+}]_i$. Remarkably, a cocktail of PB and 100 μM GABA resulted in sinusoidal oscillations in $[Ca^{2+}]_i$. However, in apparent contradiction to the generalized observation of CYP1A1 inducers increasing $[Ca^{2+}]_i$, DAVA caused a decrease in $[Ca^{2+}]_i$. Whether, these changes in $[Ca^{2+}]_i$ do in fact play an important role in the regulation of CYP1A1 remains to be established. The apparent opposite effects of DAVA and PB on $[Ca^{2+}]_i$ may involve the participation of different receptor ionophore-complexes since PB has been shown to bind $GABA_A$ receptors and DAVA is known to bind $GABA_A$ and $GABA_B$ receptors in the CNS (16). In addition it should be stressed that what is presently understood for neuronal GABA receptors may not extend to peripheral receptors. This point was illustrated in a study with mammalian adrenal medulla reporting that picrotoxin and barbiturates elicited similar patterns of nicotinic response through their action on the GABA receptor-ionophore complex (31).

The present studies show CYP1A1 induction to be modulated by DAVA, PB, and GABA, plus picrotoxin was previously shown to induce CYP1A1 (15). All of these compounds are known ligands of the $GABA_A$ receptor and do not have molecular structures which would predict them being Ah receptor ligands. PB has been shown to have poor affinity for the Ah receptor (32). Similarly other compounds, such as omeprazole (a compound which inhibits ion transport via a plasma membrane receptor), also shows poor affinity for the Ah receptor but induces CYP1A1 (33-36), as does alterations in cell-shape and adhesion (37), hyperoxia (38), and hydrodynamic shear (39). The effects of the GABA ligands upon CYP1A1 investigated in this study may reflect subtle changes in cell morphology as this has been shown to induce CYP1A1 via the Ah receptor (37) or involve the mitogen activated protein kinase (MAPK) cascade which is known to be stimulated by cell-shape, shear stress, and inhibition of protein synthesis (40-43). The participation of MAPK and CYP1A1 induction is presently being investigated in our laboratories.

ACKNOWLEDGMENTS

The authors express special thanks to E. Nilsson and J. Sundqvist for their excellent technical assistance. M. Sadar is grateful to the European Science Foundation for supporting this research through a fellowship providing financial assistance. T. Andersson is grateful to the Swedish Natural Science Research Council for financial support.

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