Protein serine/threonine phosphatase inhibitors suppress phenobarbital-induced *Cyp2b10* gene transcription in mouse primary hepatocytes

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Using a primary hepatocyte culture in which the mouse *Cyp2b10* gene transcription is activated by phenobarbital (PB)-type inducers, we examined the cellular signalling mechanisms associated with PB induction. Low nanomolar concentrations of protein serine/threonine phosphatase inhibitors okadaic acid (OA) and calyculin A blocked the induction of CYP2B10 mRNA. Nuclear run-on assays indicated that OA suppressed *Cyp2b10* gene transcription. Pretreatment of the cells with an inhibitor of Ca^{2+}/calmodulin-dependent protein kinases {1-[*N*,*O*-bis-(5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62)} or with a flavonoid, naringin, were completely or partly protective respectively against the OA-mediated suppression of CYP2B10 mRNA. Several other established modulators of protein kinase activities did not greatly affect the induction of CYP2B10 mRNA, nor could they prevent the suppressive effect of OA. Our results indicate that specific protein phosphorylation–dephosphorylation is required for the induction of *Cyp2b10* gene expression, which is modulated through multiple endogenous and exogenous signals.

INTRODUCTION

Phenobarbital (PB), a classic inducer of drug- and carcinogen-metabolizing enzymes, represents a group of structurally unrelated chemicals that share the property of inducing several genes within cytochrome *P*-450 (CYP) subfamilies 2B, 2C and 3A. The hepatic levels of these CYP mRNAs increase quickly *in vivo* after PB administration owing to the activation of *CYP* gene transcription, leading to the enhanced metabolism of both exogenous and endogenous compounds such as steroids, drugs and carcinogens. The components of the cellular signalling pathway through which PB and other PB-like inducers act are largely unknown because there are no reliable PB-responsive *in vitro* models and difficulties in studying this pathway *in vivo* (reviewed in [1–3]).

Recently, the induction by PB of CYP2B mRNA species and enzyme activities has been reproduced in several different primary hepatocyte cultures [4–7]. In these systems the induction of rat CYP2B mRNA species, and *CYP* gene expression in general, seems to be inhibited by growth factors and cytokines. For instance, epidermal growth factor (EGF) and interleukin 1 effectively decreased CYP2B, CYP1A and CYP2C mRNA species in rat hepatocytes [6,8,9], and growth hormone (GH) inhibited the expression of rat CYP2B mRNA species in hepatocyte culture [4] or in liver tissue [10]. These decreases in CYP mRNA species are presumably due to the growth factor-elicited activation of intracellular protein kinase signal transduction pathways. However, there is a lack of information on the involvement of any specific signalling pathway in PB induction; only recently, elevated cAMP levels were shown to inhibit the accumulation of rat CYP2B mRNA species by PB [11]. Additionally, it has seldom been demonstrated that the observed inhibition had occurred at the level of gene transcription. It is therefore possible that some treatments might have affected the stability and/or processing of CYP mRNA species during the often long culture period. Another complicating factor is that the different culture conditions might dictate the phenotype of the cells, thus determining which signalling pathway dominates over the others.

We recently described a mouse primary hepatocyte culture system in which the endogenous *Cyp2b10* gene was activated within 8 h, mimicking the rapid kinetics *in vivo* [12]. We show here that the transcription of the *Cyp2b10* gene in hepatocytes can be suppressed by low nanomolar concentrations of okadaic acid (OA) and calyculin A, inhibitors of protein serine/threonine phosphatases related to isoforms PP2A and PP1 [13], and that this suppression could be prevented by some but not all protein kinase inhibitors. Our results suggest that relatively specific protein phosphorylation mechanisms are involved in the induction of *CYP2B* genes by PB.

EXPERIMENTAL

Reagents

The sources of radioisotopes and molecular biology reagents were as described [12]. Cell culture media and media supplements were from Gibco BRL (Gaithersburg, MD, U.S.A.); collagenase type I was from Sigma Chemical Co. (St. Louis, MO, U.S.A.); tissue culture dishes, EGF and TGFβ1 were obtained from Becton Dickinson Labware (Bedford, MA, U.S.A.). 1-[*N*,*O*-Bis-(5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62), calphostin C and H-89 were from Calbiochem (San Diego, CA, U.S.A.); calyculin A was from Boehringer Mannheim (Indianapolis, IN, U.S.A.); nor-okadaone and OA were from LC Services (Woburn, MA, U.S.A.). 1,4-Bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) was synthesized, puri-

Abbreviations used: CYP, cytochrome *P*-450; EGF, epidermal growth factor; GH, growth hormone; H7, 1-(5-quinolinesulphonyl)-2-methylpiperazine-HCl; H89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline sulphonamide; KN-62, 1-[*N*,*O*-bis-(5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine; OA, okadaic acid; PB, phenobarbital; TCPOBOP, 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene.

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fied and verified as described previously [12]. All other chemicals were from Sigma.

Hepatocyte culture

Primary hepatocytes were isolated from 8–12-week-old male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME, U.S.A.) by a two-step collagenase perfusion (approx. 0.3 mg/ml in Hanks balanced salt solution supplemented with 10 mM Hepes, pH 7.4, and 0.5 μM pig insulin) and repeated low-speed centrifugations to a viability of at least 85 %. Immediately after harvesting, the cells were washed and suspended (10^6 per ml) in prewarmed William's E medium supplemented with 7 % (v/v) foetal bovine serum, ITS supplement (Sigma), 30 mM pyruvate and penicillin G/streptomycin (100 i.u./ml) (modified from [12]). Aliquots (3 ml) were dispensed on 60-mm culture dishes. After 30 min at 37 °C under air/CO_2 (19 : 1), the unattached cells were removed and the dishes were washed with PBS. The cells then received fresh medium without serum but containing 5 nM dexamethasone.

Test compounds or their vehicles (DMSO, ethanol or saline) were then added 30 or 60 min before inducers or solvents for inducers (saline for PB, DMSO for TCPOBOP). The cells were incubated in the presence of inducers for 8 h. The interval between the removal of serum-containing medium and the addition of PB or TCPOBOP varied from 0 to 90 min, depending on the pretreatments in a particular experiment. However, the basal level and the inducibility of CYP2B10 mRNA were not affected by this difference. Organic vehicles (at most 0.3 % final concentration) had no effect on CYP2B10 mRNA in pilot studies. With several test compounds, both PB and TCPOBOP were used as inducers with similar results. Because both compounds seem to activate the mouse *CYP* genes via the same mechanism (see [12] and the Discussion section), and because TCPOBOP was considered superior to PB owing to its stability, pH-independent solubility and high potency, TCPOBOP was used in later studies for clarity of the results.

Analysis of cellular mRNA species

The cells were lysed after 8 h of inducer treatment or at specified times in time-course experiments with Trireagent (Molecular Research Center, Cincinnati, OH, U.S.A.). The CYP2B10 and albumin mRNA species were analysed by Northern blotting with 360 bp CYP2B10 and 180 bp albumin cDNA probes as described [12].

Analysis of gene transcription

Approx. 8×10^6 hepatocytes were seeded on 100-mm dishes (four to six dishes per treatment) and cultured as above; 4 h after inducer addition, cells were washed, scraped into ice-cold PBS and pelleted for 1 min at 300 ***g***. Nuclei were purified by centrifugation through 0.9 M sucrose cushions [14] modified only by decreasing Nonidet P40 to 0.4 % (v/v) in the homogenization buffer. Purified nuclei were used in nuclear run-on assays essentially as described previously [12], with 3 μg of linearized cDNA probes immobilized on nylon membranes [12].

Other assays

Protein determinations [15] were performed in accordance with the method cited. Intracellular cAMP levels were measured with a Biotrak radioreceptor assay kit (Amersham, Arlington Heights, IL, U.S.A.) in accordance with the manufacturer's instructions.

RESULTS

Regulation of CYP2B10 mRNA in mouse hepatocytes

Northern hybridization showed that the 2.2 kb CYP2B10 mRNA was barely detectable in unexposed cells but it appeared at 2 h, with maximum levels seen at 8 h after PB (Figure 1A) or TCPOBOP treatment (results not shown). A dose–response study conducted at 8 h indicated that the maximal induction in this cell system was reached at approx. 20 nM TCPOBOP (Figure 1A) and at approx. 0.5 mM PB (results not shown). The albumin mRNA (Figures 1A and 1B) and the 2.7 kb CYP2B-related mRNA (Figure 1B) were not affected, which indicated the specificity of PB and TCPOBOP as inducers. Transcriptional inhibitors actinomycin D and α-amanitin blocked the appearance of both 2.2 and 2.7 kb bands but cycloheximide was without effect (Figure 1B), confirming our earlier finding that CYP2B10 mRNA induction is not dependent on continuous protein

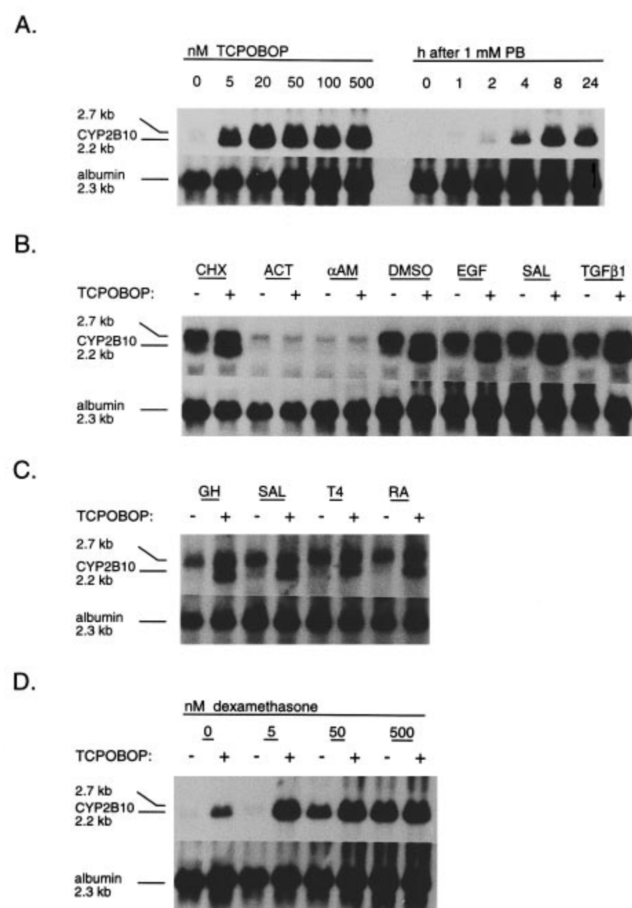


Figure 1 Regulation of CYP2B10 mRNA in primary hepatocytes

Total RNA (10 μg per lane) was analysed by Northern blotting for the presence of 2.2 kb CYP2B10 and 2.7 kb CYP2B-related mRNA species (upper panels) and 2.3 kb albumin mRNA (lower panels) as described in the Experimental section. (**A**) The cells were treated with the indicated concentrations of TCPOBOP for 8 h (left panel), or with 1 mM PB for the indicated times (right panel). (**B**) The cells were preincubated for 30 min with 35 μM cycloheximide (CHX), 0.5 μM actinomycin D (ACT), 1 μM α-amanitin (αAM), DMSO, 50 ng/ml EGF, saline (SAL) or 3 ng/ml TGFβ1 before the addition of vehicle (−) or 50 nM TCPOBOP (+) for 8 h. (**C**) The cells were preincubated for 30 min with 500 ng/ml GH, saline, 1 μM thyroid hormone (T4) or 1 μM all-*trans*-retinoic acid (RA) before the addition of vehicle (−) or 50 nM TCPOBOP (+) for 8 h. (**D**) The cells were incubated in culture medium containing the indicated concentrations of dexamethasone in the absence (−) or presence (+) of 50 nM TCPOBOP.

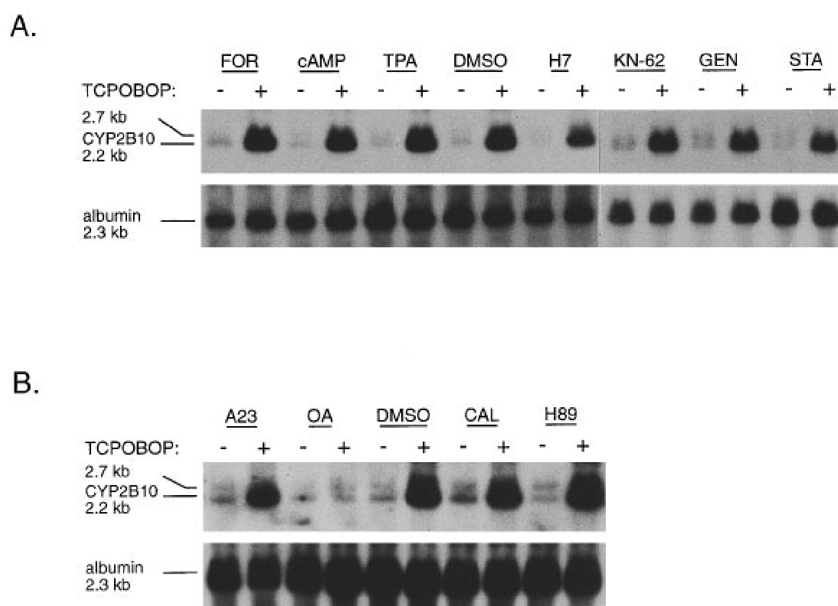


Figure 2 Effects of modulators of protein phosphorylation on CYP2B10 mRNA accumulation

(**A**) The cells were preincubated for 30 min (60 min for H7 and staurosporine) with 75 μM forskolin (FOR), 0.2 mM dibutyryl cAMP (cAMP), 0.3 μM PMA, DMSO, 20 μM H7, 10 μM KN-62, 2 μM genistein (GEN) or 0.2 μM staurosporine (STA) before the addition of vehicle ($-$) or 50 nM TCPOBOP ($+$) for 8 h. (**B**) The cells were preincubated for 30 min with 2 μM A23187 (A23), 4 nM OA, DMSO, 0.5 μM calphostin C (CAL) or 10 μM H89 before the addition of vehicle ($-$) or 50 nM TCPOBOP ($+$) for 8 h.

synthesis [12]. In this experiment the film was overexposed to see the lowest levels of CYP2B expression. When compared with our previous results, the inclusion of pyruvate seemed to improve the inducibility of CYP2B10 mRNA. The ratio between the 2.7 kb CYP2B-related and CYP2B10 mRNA species varied between cell batches, perhaps owing to slight differences in the age of the mice and variations in the cell preparation, which was optimized for CYP2B10 expression only. However, the response of CYP2B10 mRNA to inducers or test compounds was not influenced by these differences (results not shown).

Effects of peptide growth factors and hormones

Several peptide factors and hormones (at concentrations covering the effective range established in other studies with primary hepatocytes) were tested for their effects on CYP2B10 mRNA inducibility. Even high doses of EGF (50 ng/ml; usually effective below 20 ng/ml) [6], TGFβ1 (3 ng/ml; usually effective at 1 ng/ml) [16] or GH (500 ng/ml; effective at 50–500 ng/ml) [4,17] did not appreciably affect the basal or induced levels of CYP2B10 mRNA (Figures 1B and 1C). The addition of thyroid hormone or all-*trans*-retinoic acid at 1 μM, which level is sufficient to activate the respective nuclear receptors [18], did not affect the CYP2B10 mRNA levels (Figure 1C). Increasing the pretreatment time with these test compounds to 60 min or using PB as an inducer with EGF and GH gave similar results (results not shown). Low nanomolar concentrations of dexamethasone were necessary for optimal induction of CYP2B10 mRNA, whereas at higher concentrations dexamethasone was an inducer by itself (Figure 1D).

Effects of modifiers of protein phosphorylation

We next screened several known activators or inhibitors of protein kinases for their possible effects on CYP2B10 mRNA expression. The specificities of these kinase modulators have been reviewed [19]. We selected the doses on the basis of reportedly effective concentrations in experiments performed predominantly with primary hepatocytes from a large number of studies (results not shown). Even though these modulators have been used in numerous studies with well-established effects [19], the possibility that some of these compounds might have non-specific effects should be borne in mind.

Table 1 Levels of cAMP in primary mouse hepatocytes after a 30 min exposure to selected chemicals

The hepatocytes were incubated for 30 min with the indicated chemicals, washed extensively with PBS, then lysed by boiling in buffer containing 10 mM EDTA to inhibit phosphoesterases. The cAMP levels were then determined with a radioreceptor assay kit (Amersham). The results are means for two independent experiments with less than 20% difference.

Chemical	Concentration (M)	cAMP level (pmol/mg of protein)	Fold increase
None (DMSO)	–	< 0.2	–
TCPOBOP	5×10^{-8}	< 0.2	1.0
Forskolin	7.5×10^{-5}	4.4	> 22
Dibutyryl cAMP	2×10^{-4}	39.0	> 185

Figure 2(A) shows that forskolin and dibutyryl cAMP, which activate protein kinase A by increasing intracellular cAMP levels, had no effect on CYP2B10 inducibility. Both compounds have been reported to decrease rat CYP2B mRNA [11] and increase mouse CYP2A mRNA [20] at 10 μM; in our experiments, intracellular cAMP levels were increased markedly by both compounds (see Table 1). Neither the protein kinase C activator PMA [21–23], the Ca^{2+}/calmodulin-dependent kinase inhibitor KN-62 [22,24] nor the tyrosine kinase inhibitor genistein [23,24] had any effect on CYP2B10 mRNA at doses that have

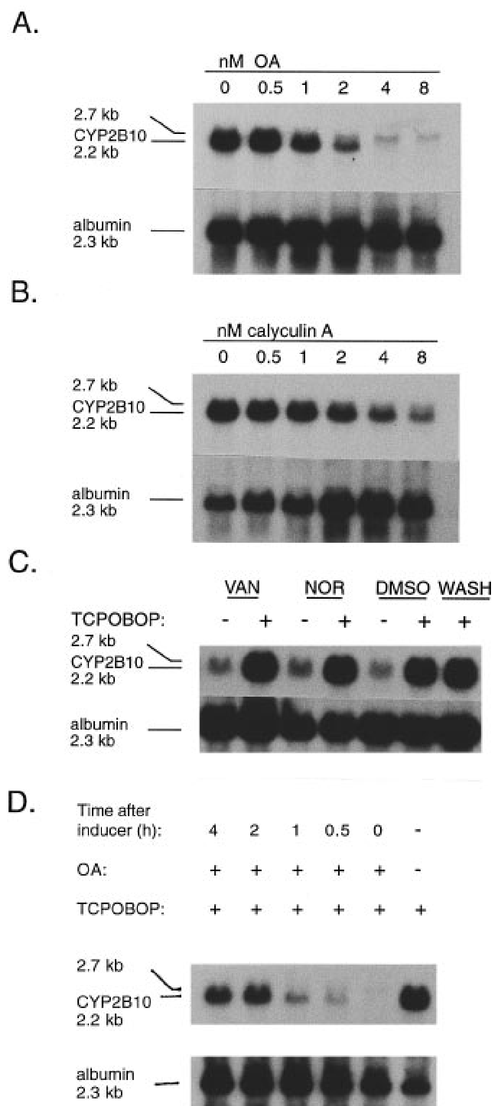


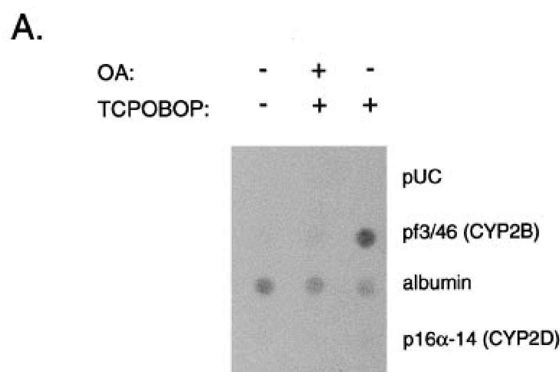
Figure 3 Effects of inhibitors of protein phosphatases on CYP2B10 mRNA accumulation

(**A**, **B**) The cells were preincubated for 30 min with the indicated concentrations of OA (**A**) or calyculin A (**B**) before the addition of vehicle (−) or 50 nM TCPOBOP (+) for 8 h. (**C**) The cells were preincubated for 30 min with 0.2 mM vanadate (VAN), 16 nM nor-okadaone (NOR) or DMSO before the addition of vehicle (−) or 50 nM TCPOBOP (+) for 8 h, or the cells were preincubated with 4 nM OA for 2 h and then washed four times with medium over a 1 h period before the addition of 50 nM TCPOBOP for 8 h (WASH). (**D**) The cells were incubated with (+) or without (−) 50 nM TCPOBOP; 4 nM OA was then added at the indicated times relative to inducer addition.

been found effective in hepatic cells (Figure 2A). PMA and cAMP were also ineffective when PB was the inducing agent (results not shown). A partial inhibition of CYP2B10 mRNA expression was seen when longer (60 min) preincubation times and higher concentrations of 1-(5-quinolinesulphonyl)-2-methylpiperazine-HCl (H7) (20 μM) [22] or staurosporine (0.2 μM) [20,23] were used (Figure 2A). H7 preferentially inhibits protein kinases A, G and C, whereas staurosporine is an even more general inhibitor [19]. Treatments with the Ca^{2+} ionophore A23187, which increases cytosolic Ca^{2+} levels [21,25], or with more specific inhibitors for protein kinase C (calphostin C) or protein kinase A {*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoline sulphonamide (H89)} [19,24] did not affect CYP2B10 mRNA induction, whereas calphostin C increased the basal CYP2B10 mRNA level approx. 2-fold. The protein phosphatase inhibitor OA (4 nM), which inhibits the PP1 isoform at high concentrations and is selective for PP2A at the low nanomolar concentrations used here [13,23,25], completely abolished the inducer-dependent accumulation of CYP2B10 mRNA, whereas basal levels were not significantly affected (Figure 2B). Again, the PB-induced CYP2B10 mRNA was suppressed by OA, indicating that the effects of modifiers of protein phosphorylation are not dependent on the specific inducer (results not shown).

Dose–response studies indicated that OA and calyculin A (an equally effective inhibitor of both PP1 and PP2A [13,26]) blocked CYP2B10 mRNA without much effect on 2.7 kb CYP2B-related or albumin mRNA species (Figures 3A and 3B). Because this inhibition occurred at low nanomolar concentrations of OA, which do not significantly inhibit the PP1 isoform [13], these results indicate that PP2A and/or a related phosphatase is involved in PB induction. In these experiments, the 2.7 kb band is overlapped in some cases by the large amounts of 2.2 kb CYP2B10 mRNA owing to the strong induction of the latter by TCPOBOP. However, our previous results [12] and results in Figures 1 and 2 indicate that neither TCPOBOP, PBn or OA influence the expression of the 2.7 kb mRNA but affect only the CYP2B10 mRNA. As control for the specificity of OA action, CYP2B10 mRNA was not suppressed by 0.2 mM vanadate, a tyrosine phosphatase inhibitor [27] nor by nor-okadaone, an OA derivative devoid of any capacity for phosphatase inhibition (Figure 3C). The inhibitory effect of OA was reversible because CYP2B10 mRNA was induced in cells that were first incubated with 4 nM OA for 2 h and then washed extensively with culture medium (Figure 3C, WASH). Because of this reversibility and because (1) OA did not change the morphology or the attachment of the cells within the culture period and (2) the viability of the cells was not changed (results not shown), the suppressive effect on CYP2B10 mRNA is unlikely to be due to any toxicity of OA. This was in line with reports that even 20–30 nM OA did not interfere with protein synthesis, glucose utilization or ATP levels in primary hepatocytes [22,25]. The addition of OA after the inducer was still capable of suppressing the induction of CYP2B10 mRNA (Figure 3D). Taken together, these results suggest that continuing protein serine/threonine dephosphorylation is required for efficient CYP2B10 mRNA induction. In addition, PP2A-like phosphatases are probably involved in this process, although the participation of other OA-sensitive phosphatases cannot yet be excluded.

Run-on assays showed that *Cyp2b10* gene transcription was activated more than 10-fold by TCPOBOP in 4 h; this activation was completely abolished by OA. Transcription of *albumin* or *Cyp2d9/10* genes was not affected by TCPOBOP or by OA (Figure 4A). This result was confirmed by studying the stability of CYP2B10 mRNA allowed to accumulate for 8 h. The subsequent gene transcription was stopped by the addition of actinomycin D and α-amanitin (1 μM each). Because the CYP2B10 mRNA was very stable, its half-life could not be accurately determined because of the rounding and partial detachment of OA-treated cells in prolonged (more than 24 h) incubations. Nevertheless the decay of CYP2B10 was not altered in cells exposed to 4 nM OA compared with controls during 8 h of culture (Figure 4B) when the effects of OA on mRNA levels should be readily observed (see Figures 2 and 3). These experiments show that OA specifically inhibited the induction of CYP2B10 mRNA at the transcriptional level. It should be noted here that the run-on assays were performed on nuclei from male



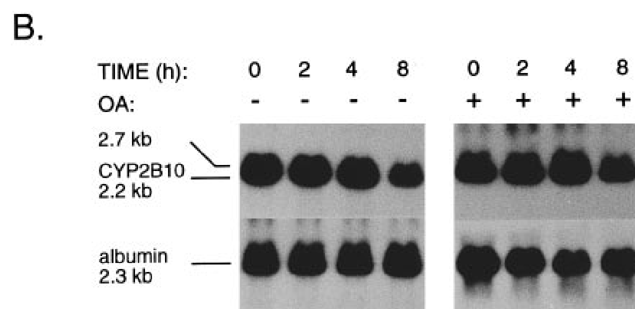


Figure 4 Effect of OA on transcription and stability of CYP2B10 mRNA

(**A**) The cells were incubated in the absence (−) or presence (+) of 4 nM OA and 50 nM TCPOBOP for 4 h. Isolations of nuclei and run-on assays were performed as described in the Experimental section, and the nascent ^{32}P-labelled transcripts were hybridized with filter-bound linear plasmids containing the following inserts [12]: 2.7 kb pUC13, 2.0 kb CYP2B10 (pf3/46), 0.18 kb mouse albumin and 1.8 kb CYP2D9 (p16α-14) cDNA species. (**B**) The cells were incubated for 8 h with 50 nM TCPOBOP to induce CYP2B10 mRNA fully. Actinomycin D and α-amanitin (1 μM each) were then added to block further transcription in the absence (−) or presence (+) of 4 nM OA. Cellular mRNA species were analysed at the indicated times after OA addition.

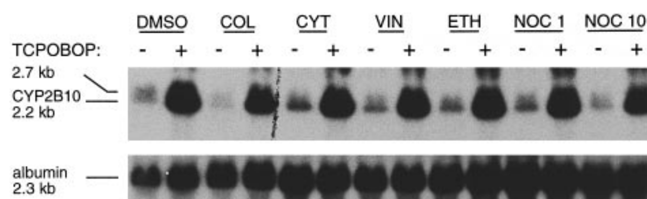


Figure 6 Effects of cytoskeleton-disrupting agents on CYP2B10 mRNA accumulation

The cells were preincubated for 30 min with DMSO, 1 μM colchicine (COL), 10 μM cytochalasin D (CYT), 10 μM vinblastine (VIN), ethanol (ETH), 1 μM or 10 μM nocodazole (NOC) before the addition of vehicle (−) or 50 nM TCPOBOP (+) for 8 h.

mice from which the expression of the 80% similar, female-specific *Cyp2b9* gene is absent, and in which the CYP2B9 mRNA has not been detected in hepatocytes [12], thus minimizing any significant cross-hybridization artifacts.

On the basis of the above results, it was reasonable to assume that the presence of OA caused an accumulation of a phosphorylated protein factor that interfered with PB-type induction. This accumulation is the net effect between dephosphorylation and phosphorylation reactions; theoretically it could be prevented by inhibition of the phosphorylation reaction catalysed by a protein kinase. Therefore we tested whether any of the kinase inhibitors could prevent the OA-mediated suppression of CYP2B10 mRNA induction. Hepatocytes were preincubated for 60 min with staurosporine, H89, genistein, calphostin C, KN-62 and naringin before OA addition, and the inducer was added 30 min after OA. Northern blotting of RNA samples collected after 8 h of inducer treatment (Figure 5) indicated that only 10 μM KN-62 and 100 μM naringin provided complete and partial protection respectively against the OA-dependent suppression of CYP2B10 mRNA. These compounds had no major stimulatory or inhibitory effect of their own with the exception of staurosporine and H7 (see Figure 2). It is notable that the latter two non-specific kinase inhibitors alone attenuated CYP2B10 mRNA expression by approx. 60% but were unable to provide any protection against the action of OA. Together with the results in Figure 2, these results indicate that relatively specific kinase–phosphatase systems seem to participate in CYP2B10 mRNA regulation but that protein kinases A and C do not seem to have a major role in this culture system.

Because OA can cause the disorganization of cytoskeleton structures by hyperphosphorylation of associated proteins [26], we added known cytoskeleton-disruptive agents at established concentrations [26,28] to hepatocytes before the inducer. Despite the rapid rounding of the cultured cells by these agents, only 1 μM colchicine and 10 μM nocodazole were slightly inhibitory to CYP2B10 mRNA induction, whereas cytochalasin D and vinblastine at 10 μM [28] were without effect (Figure 6). This result suggests that cytoskeletal structures themselves are not

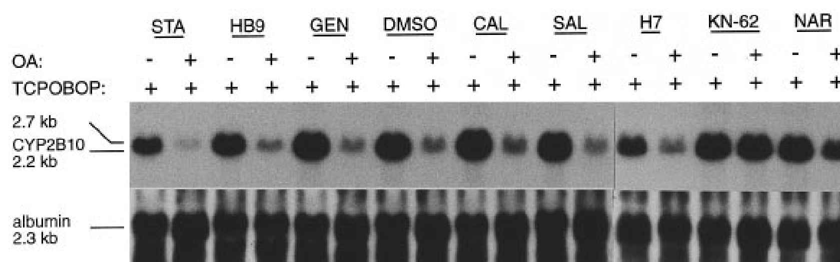


Figure 5 Effects of inhibitors of protein kinases on OA-mediated CYP2B10 mRNA suppression

The cells were preincubated for 60 min with 0.2 μM staurosporine (STA), 10 μM H89, 2 μM genistein (GEN), DMSO, 0.5 μM calphostin C (CAL), saline (SAL), 20 μM H7, 10 μM KN-62 or 100 μM naringin (NAR) before the addition of vehicle (−) or 4 nM OA (+); 30 min after OA addition, all cells were exposed to 50 nM TCPOBOP for 8 h.

important for induction. In this respect, the cell shape was unaffected by the inducer alone or in combination with other test compounds (results not shown).

DISCUSSION

Hepatocyte culture

The signalling mechanisms involved in PB-induced transcription of *CYP* genes, and in particular *CYP2B* genes, have not yet been discovered. Continuous hepatoma-derived cell lines in general do not express *CYP2B* genes constitutively or in response to PB-type inducers. This probably reflects the highly differentiated nature of *CYP* gene expression in normal quiescent hepatocytes. Earlier studies indicated that even primary hepatocytes can rapidly lose their liver-specific functions; the development of extracellular Matrigel matrix as a cell substratum proved beneficial for the expression of rat CYP2B mRNAs [4]. Because considerable difficulties have been documented in developing culture conditions for *CYP2B* gene expression and induction, it would be tempting to speculate that the cell shape and cell interactions as governed by cytoskeleton and/or extracellular matrix are crucial for induction. Later studies, however, have indicated that induction can be reproduced to some extent in a variety of culture systems such as special media with or without collagen coating, denatured collagen or Matrigel substrata, or aggregated hepatocyte (spheroid) cultures [4,5,7,29], or on untreated plastic with minimal exposure to serum [12]. Additionally, in the present study, cytoskeleton-disrupting agents did not inhibit CYP2B10 mRNA accumulation even though cell shape was altered markedly and the phosphatase inhibitors did not change the cell shape during the 8 h culture, whereas CYP2B10 mRNA was greatly suppressed. These differences in the cell environment and cell shape suggest that the induction of *Cyp2b10* gene is not strongly dependent on cell–cell or cell–matrix interactions, unlike some other genes [30,31].

Growth factors, hormones and *Cyp2b10* gene expression

Several growth factors and cytokines are known repressors of CYP2B mRNA, although their mode of action has not been established (see, for example, [6,9]). With respect to GH and contrary to rat hepatocytes [4], we did not detect any repression by GH of mouse *Cyp2b10* gene expression. One might argue that during our short culturing period membrane-associated receptors might have not recovered and thus the cells would not respond to GH. This is unlikely because a similar isolation and culture protocol yielded GH-responsive cells [17] and because GH was also ineffective in mouse spheroid culture allowed to recover for several days before inducers and GH were added [7]. More importantly, the expression of *CYP2B* genes *in vivo* is quite independent of GH in the mouse [12,32], contrary to reports on the rat liver [4,10], suggesting that the transduction pathway activated by the GH receptor is not a major component of PB induction in mice. Nevertheless it is possible that factors involved in PB signalling and PB-induced gene transcription might be substrates for (and inhibited by) MAP kinase. MAP kinase participates in several signalling cascades and it is known to be activated by GH and also by OA [33,34]. Therefore, depending on the species, the sensitivity to GH of factors involved in PB induction could differ. This question warrants additional studies. Low concentrations of dexamethasone have been reported to be essential for a good induction response both in rat and mouse hepatocytes [5,7,35], which was confirmed by the present studies. Furthermore we could reproduce the induction of CYP2B mRNA by dexamethasone *in vivo* [32] in our culture system.

Taken together, our mouse primary hepatocyte system mimics the *in vivo* PB-type induction in its rapidity and responses to GH and dexamethasone. The advantages of the present mouse hepatocyte system are the rapid transcriptional activation of the *Cyp2b10* gene, which compares favourably with culture systems requiring 48 h or more for optimal mRNA induction [4,6,7], the simple requirements for culture medium and supplements, and its compatibility with DNA transfection either before or after cell plating [12]. This culture system should therefore prove valuable in other studies on *CYP2B* gene regulation.

Protein phosphorylation and *Cyp2b10* gene expression

PB induction of rat CYP2B mRNAs was reportedly blocked by cAMP [11]. cAMP is also known to maintain hepatocyte gap junctions and to inhibit hepatocyte DNA replication. These two processes are regulated in the opposite way by PB [16,36,37]. Thus it might seem that the counteracting effects of PB and cAMP on hepatocyte growth and *CYP2B* gene expression are somehow associated with same signalling mechanisms. In contrast, the induction of other PB-responsive genes such as mouse *Cyp2a5* [20] and rat haem biosynthetic enzymes [38] was enhanced by PB. With respect to the *Cyp2b10* gene, activators of protein kinases A and C did not repress CYP2B10 mRNA even at high concentrations, and the specific protein kinase A and C inhibitors H89 and calphostin C had no major effect on the CYP2B10 mRNA, nor could they protect against the suppression by OA. These results indicate that protein kinases A and C do not have a major role in *Cyp2b10* gene regulation, but their participation as gene-specific factors in PB induction cannot be completely ruled out. The observed species differences indicate that the cAMP-responsive components of the PB induction pathway are probably more dominant in the rat than in the mouse, or that they become more dominant under the different culture conditions.

We did not find any major effect on CYP2B10 mRNA induction by EGF or GH, which can activate receptor-tyrosine kinases [34], or by genistein, which inhibits some of these kinases [19]. Neither could vanadate, a general inhibitor of protein tyrosine phosphatases [34], inhibit CYP2B10 mRNA accumulation. Even though our findings imply that tyrosine phosphorylation is not involved in *Cyp2b10* gene regulation, some protein serine/threonine phosphatases are activated by tyrosine phosphorylation [34], suggesting that the latter pathway cannot yet be completely eliminated.

Similar results were obtained regardless of the inducer, indicating that the ineffectiveness of protein kinases A and C and the EGF- or GH-activated signal transduction pathways (or the effectiveness of OA) is not inducer-dependent but consistent with the view that PB-type inducers act through the same general mechanism [12,39].

OA and *Cyp2b10* gene expression

Low nanomolar concentrations of OA completely inhibited the inducible *Cyp2b10* gene transcription. One facet of OA action is its ability to increase the phosphorylation of CREB, C/EBPβ and other transcription factors [27,40]. The hyperphosphorylation of regulatory proteins is generally associated with enhanced gene transcription [27,34,40]. However, OA has been reported to down-regulate the glucose-induced transcription of the *S14* gene [25], indicating that OA has multiple gene-specific effects in transcriptional regulation. For technical reasons, including partial cell detachment and extremely strong activation by OA of heterologous thymidine kinase and SV40 promoters that interfered with the normalization of reporter gene assays, we were

not able to test convincingly whether OA acted on PB-responsive DNA elements of the *Cyp2b10* gene [39] or whether other elements governing e.g. the basal promoter activity [12] are involved. Administration of OA was reported to increase the protein binding to a positive DNA element at −98/−68 bp of the rat *CYP2B2* gene and increase the cell-free transcription governed by this element [41]. In striking contrast, and even though no reference genes were included in the run-on assay, administration of OA to rats *in vivo* did not enhance endogenous *CYP2B1/2* gene transcription in the same study [41].

Because low concentrations of OA inhibit PP2A primarily, but potentially also other members of the PP gene family [13,34], it is not yet possible to determine exactly the OA-sensitive components of *Cyp2b10* gene regulation, especially because inhibitors more specific for each PP isoform are not available. Nevertheless our results show clearly that factors involved in induction by PB are targets for specific protein phosphorylation because, among many kinase inhibitors, only KN-62 could prevent the OA-mediated suppression of CYP2B10 mRNA. It is noteworthy that both KN-62-sensitive kinases (CaMK II and IV) and OA-sensitive phosphatases (PP2A, 4 and 5) are present in cell nuclei [34,42]. It is possible that the actual mechanism involves multiple kinases and/or phosphatases and such cascade models are well known [34]. Nevertheless our results emphasize the importance of PP2A-like phosphatases and Ca^{2+}/calmodulin-dependent kinases relative to other enzymes and indicate one priority for future work on *CYP2B* gene regulation.

We thank Rick Moore and Kim Washburn for their help in liver perfusion and RNA gels; Dr. Cary Weinberger and Dr. Gordon Ibeanu for their comments; and M.Sc. Maarit Korhonen for her critical reading of the manuscript.

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Received 22 September 1997/29 October 1997; accepted 20 November 1997