Biochemical Pharmacology, Vol. 40, No. 8, pp. 1815–1819, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00
© 1990. Pergamon Press plc

EFFECT OF PHENOBARBITAL ON THE GLUCOCORTICOID RECEPTOR IN RAT HEPATOMA CELLS

SYLVETTE CHASSEROT-GOLAZ,*† GISÈLE BECK,* ANIKO VENETIANER‡ and LAURENT CORCOS§

* Institut de Biologie Moléculaire et Cellulaire, 15 Rue Descartes, 67000 Strasbourg, France; ‡ Institute of Genetics, Biological Research Center, 6701 Szeged, P.O.B. 521, Hungary; and § Unité de Génétique de la Différenciation, Département de Biologie Moléculaire, Institut Pasteur, 25 Rue du Dr Roux, 75015 Paris, France

(*Received* 8 *March* 1990; *accepted* 9 *June* 1990)

Abstract—Phenobarbital is a potent inducer of several liver-specific genes such as those encoding detoxication enzymes, including cytochromes P450. However, the mechanisms of action of the barbiturate are poorly understood. Since both, phenobarbital and glucocorticoids, are capable of inducing the same cytochrome P450 species, we asked whether the glucocorticoid receptor could participate to the phenobarbital induced responses. The results presented here show that phenobarbital was able to induce a two-fold increase in the affinity of the glucocorticoid receptor for the binding of dexamethasone, as well as a 30% increase of the receptor number in Reuber rat hepatoma cells of the Fao line. These effects may have a biological significance since they were paralleled by an enhancement of the dexamethasone-induced tyrosine aminotransferase activity, a glucocorticoid inducible function in rat hepatoma cells and in rat liver. To our knowledge, phenobarbital is the first compound shown to be able to induce, in intact cells, an increase in the affinity of the glucocorticoid receptor for the binding of its ligand.

Phenobarbital (PB‖) has been shown to induce an increase in the expression of a large number of liver-specific genes such as those encoding detoxication enzymes involved in xenobiotic metabolism [1]. PB is able to act at both the transcriptional and post-transcriptional levels [2]. In addition, the possibility that PB acts directly via a specific receptor is rather unlikely since millimolar doses of the compound are necessary to induce the observed effects. The fact that some barbiturate analogs that evoke the same biological responses as PB are able to bind a cytosolic component in mouse liver makes it possible that such compounds act directly through a specific receptor molecule [3]. However, such binding has not been observed in rat liver [4]. Up to now, none of the mechanisms of PB-induced modulation of cellular functions has been elucidated, due to an acute lack of suitable *in vitro* mammalian cell systems responsive to the barbiturate.

A way of approaching the mechanism(s) of action of PB comes from the observation that PB and steroid compounds can induce the expression of the same genes in rat hepatoma cells and in rat primary hepatocytes. Indeed, it has recently been shown that in Reuber rat hepatoma cells of the Fao line, accumulation of the mRNA encoding cytochrome P450-PB 1 can be enhanced about 10–15-fold by dexamethasone (DXM) and PB, respectively [5]. Furthermore, PB, DXM, pregnenolone 16-α-carbonitrile and corticosterone are able to induce the expression of the rat P450p gene in primary rat hepatocytes [6]. In this case, it was shown that PB was able both to increase the synthesis and to decrease the degradation of the cytochrome P450p protein [7]. Thus, there are similarities in the responses induced by PB and synthetic or natural steroids, with respect to the expression of P450 genes.

The regulation of expression of DXM-responsive genes mostly involves interaction of glucocorticoid receptor (GR)–ligand complexes with DNA sequences named glucocorticoid response elements (GREs) generally located in the 5′ non-coding regions of these genes [8]. In steroid treated tissues or cultured cells, GREs are bound by GR–ligand complexes. The final effect is either positive or negative on the transcriptional activity of these genes [9]. With the notable exception of P450 genes, DXM and PB usually affect the expression of distinct genes. In addition, half-maximal induction of P450s by glucocorticoids requires higher doses than induction of other steroid-responsive genes such as that encoding the tyrosine aminotransferase (TATase) activity [10]. Although it seems unlikely that induction of the P450 and TATase gene involve the same mechanism, the possibility that the glucocorticoid receptor participates to the induction process of P450 genes by PB remains an open question.

The sensitivity of cells to glucocorticoids has been shown to be dependent upon the number of receptors [11]. This number can be increased by cAMP in HTC rat hepatoma cells [12]. Moreover, the quantity of available receptors appears to be limiting since its increase is paralleled by an increase in expression of DXM-inducible genes in these cells.

† Author to whom correspondence should be addressed.

‖ Abbreviations: Ah, aromatic hydrocarbon; dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; DXM, dexamethasone; GR, glucocorticoid receptor; GRE, glucocorticoid response element; PB, phenobarbital; PBS, phosphate buffered saline; P450, cytochrome P450; TATase, tyrosine aminotransferase (EC 2.6.1.5); TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

BP 40:8-J

The number of a different steroid-like receptor species, the dioxin receptor (Ah receptor), can be increased in rat liver cytosol, upon treatment of animals with PB [13, 14]. Since both the glucocorticoid- and the Ah-receptors exhibit many structural and functional similarities [15], we asked whether PB would also be able to modulate the number of glucocorticoid receptors in liver derived cells. For this purpose, we took advantage of the availability of PB-responsive cells from the Reuber rat hepatoma.

MATERIALS AND METHODS

Chemicals. Dexamethasone phosphate (DXM) was kindly provided by Roussel-Uclaf, Romainville. Labeled [^{3}H]DXM (88 Ci/mmol) was purchased from Amersham. Phenobarbital (PB) was from Specia.

Cell culture. The Fao cells, derived from H4IIEC3 Reuber rat hepatoma cells, express several liver-specific functions [16]. The cells were grown in NCTC 135/Ham's F12 (1 : 1, v/v) medium supplemented by 5% foetal calf serum, in 5% CO_2 atmosphere, as described by Wiebel *et al.* [17]. They were plated in 75 cm^2 plastic flasks; the medium was changed every day and the cells were used after 5 days of culture.

Cell line H56-26 was isolated from H56 cell clone which is a spontaneous dedifferentiated derivative of the H4IIEC3 cell line [16]. The growth of H56 cells is inhibited by DXM [18]. H56-26 is a DXM-resistant subclone of H56 cells which contains a drastically reduced level of GR—less than 10% of the parental clone [19]. The cells were grown in Ham's F12 medium supplemented by 5% foetal calf serum, in 5% CO_2 atmosphere.

Analysis of [^{3}H]*DXM uptake.* The specific binding of [^{3}H]DXM to Fao cells was measured by a whole cell binding assay [20]. Cells were grown in 24-well plates. The medium was removed and [^{3}H]DXM was added at final concentrations ranging from 1 to 30 nM in Minimum Essential Medium supplemented with 0.1% bovine serum albumin. Experiments were carried out on duplicate plates, in the presence of a 100-fold molar excess of unlabeled DXM to assess non-specific binding. After a 1 hr incubation period at 37°, the medium was removed and replaced by 1 mL phosphate buffered saline (PBS) for 30 min at room temperature. The cells were subsequently washed three times with 1 mL of ice-cold PBS. Finally, 0.5 mL ethanol was added to extract the labeled steroid. Following a further 30 min incubation at room temperature, the radioactivity was counted in 3 mL Beckman "ready gel" scintillation fluid, with a 35% efficiency.

Assay of tyrosine aminotransferase activity. Cells were washed with PBS, and collected after trypsinization into PBS containing 0.2 mM pyridoxal phosphate, 1 mM EDTA, 5 mM α-ketoglutarate. Cells were lysed by three cycles of freezing–thawing. Following centrifugation, the cytosolic fraction was assessed for TATase activity according to the method of Diamondstone [21]. Protein concentration was determined by using the Biorad protein assay kit.

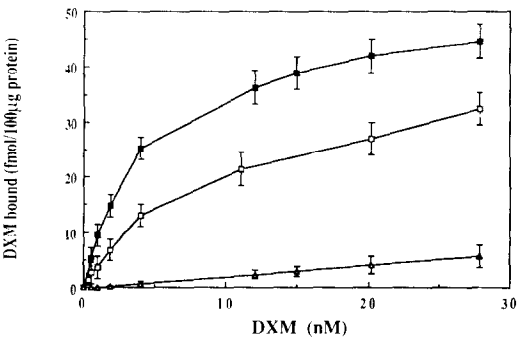

Fig. 1. Uptake of [^{3}H]DXM in Fao cells treated with 2 mM PB (■) or untreated (□). Monolayer cultures were incubated for 1 hr at 37° with various concentrations of [^{3}H]DXM plus or minus a 100-fold molar excess of unlabelled DXM to assess non-specific binding. The specific binding was determined by subtracting from the total binding (■, □) the non-specific binding which is the same in PB-treated as in control cells (△). The results presented correspond to the mean values of 10 independent experiments.

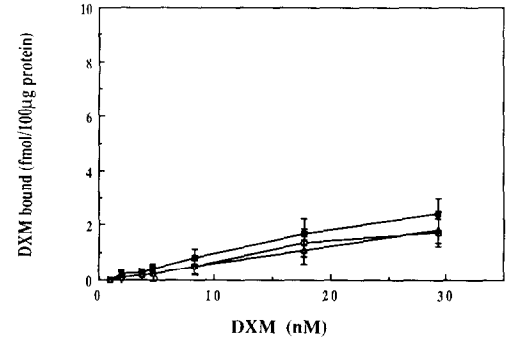

Fig. 2. Uptake of [^{3}H]DXM in the hepatoma cell line H56-26. The cells have been treated (■) or not (□) by 2 mM PB. Monolayer cultures were incubated for 1 hr at 37° with various concentrations of [^{3}H]DXM plus or minus a 100-fold molar excess of unlabelled DXM to assess non-specific binding. The specific binding was determined by subtracting from the total binding (■, □) the non-specific binding which is the same in PB-treated as in control cells (△). The results presented correspond to the mean values of six independent experiments.

RESULTS

PB increases the uptake of [^{3}H]*DXM in Fao hepatoma cells*

Glucocorticoid binding sites were determined by whole cell binding assays. Cells were treated with 2 mM PB for 3 days, before [^{3}H]DXM was added at increasing concentrations. The saturation binding curves obtained (Fig. 1) showed that PB treatment led to an increase in glucocorticoid binding capacity. This effect could reflect an increase either in the number of receptors or in their ligand binding capacity. In rat hepatoma cells that contain almost no GR, no specific binding of [^{3}H]DXM could be detected, regardless of PB treatment (Fig. 2).

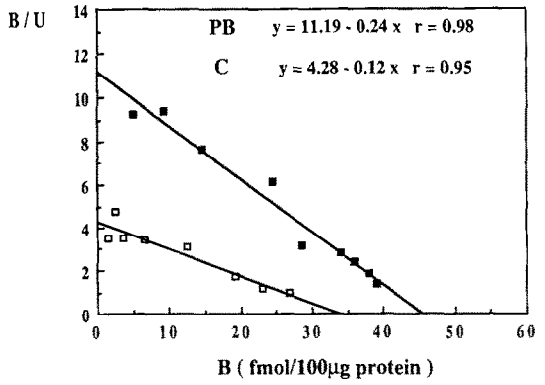

Fig. 3. Representative Scatchard plots comparing binding of [^{3}H]DXM in whole Fao cells treated by 2 mM PB (■) or untreated (□). B designates the quantity of [^{3}H]DXM specifically bound expressed as fmol/100 μg cellular protein, U the free steroid concentration (nM).

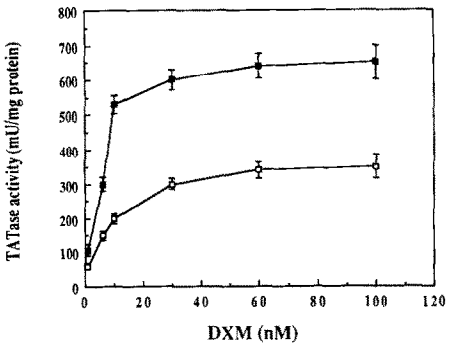

Fig. 4. Dose–response curves of TATase induction by DXM in Fao cells treated with PB (■) or untreated (□). TATase activity was determined in Fao cells incubated for 3 days in presence (■) or absence (□) of PB (2 mM) and by adding increasing concentrations of DXM during the last 18 hr of incubation. Each value corresponds to the mean of eight different experiments.

PB increases the binding capacity of DXM in Fao hepatoma cells

Scatchard representation [22] of [^{3}H]DXM binding in Fao cells showed linear plots suggesting that only a single major class of ligand binding sites existed (Fig. 3). In untreated cells, the K_d value was 8 nM and the number of [^{3}H]DXM binding sites was approximately 34 fmol/100 μg total cell protein (Table 1). These values were in close agreement with those previously reported by others [23]. In PB-treated cells, a decrease of the K_d value to 4 nM and a slight increase (30%) in the number of binding sites to 45 fmol/100 μg total cell protein were observed. No significant variations in the GR-mRNA level were detected in PB treated cells (data not shown).

The PB-induced increase in the binding affinity of [^{3}H]DXM precedes the enhancement of [^{3}H]DXM binding sites

In order to analyse the kinetics of the PB response, Fao cells were incubated with PB (2 mM) for various periods of time before the binding capacity of [^{3}H]DXM was determined. The effect of PB became detectable only after an incubation of 18 hr, time at which a decrease of the K_d value to 5.6 nM was already apparent, while the number of [^{3}H]DXM binding sites remained unchanged (Table 1).

PB potentiates the inducibility of the TATase gene by DXM

In order to determine whether the effects of PB over the GR would be paralleled by an increase in the expression of a liver function positively regulated by DXM, we have analysed DXM-induced TATase activity in cells pre-treated or not by PB (2 mM) for 3 days (Fig. 4). PB alone was able to induce a two-fold increase in TATase activity (from 50 to 100 mUnits/mg total cell protein). Such an enhancement was not observed for an incubation time of 18 hr (data not shown). This finding suggests that the effects of PB on TATase activity were a consequence of the action of PB on the GR. It can be hypothesized that the increase in TATase activity following PB treatment could reflect the binding of an endogenous steroid compound to the modified GR. The increase in TAT activity was fully prevented by the addition of RU486, a potent antagonist of DXM [24] which bind the GR with a high affinity in both PB-treated and untreated cells (data not shown).

An 18 hr-treatment of the cells with DXM alone, induced a six-fold increase in TATase activity. When cells were treated by a combination of PB (for 3 days) and DXM (for the last 18 hr), the TATase activity underwent another two-fold increase, giving a specific activity of 600 mUnits/mg total cell protein. In addition, the half-maximal response in TATase activity following DXM treatment was obtained for lower concentrations of DXM when cells had been pre-treated by PB, as compared to untreated cells (5 nM DXM versus 10 nM DXM).

Table 1. Time–course of the effects of PB treatment (2 mM) on [^{3}H]DXM binding in Fao cells

PB treatment (hr)	0	3	6	18	72
K_d (nM)	8 ± 0.3	8 ± 0.2	8 ± 0.2	5.6 ± 0.2	4 ± 0.2
DXM bound (fmol/100 μg protein)	34 ± 3	34 ± 3	34 ± 5	37 ± 2	45 ± 4

The specific [^{3}H]DXM binding was measured in whole cell binding assays as described in the legend to Fig. 1. Scatchard analyses were performed and the binding parameters were calculated. Values are the mean ± SD of three separate experiments.

DISCUSSION

The results presented here show that the affinity of a steroid receptor for its ligand can be increased in liver-derived Reuber rat hepatoma cells following treatment with PB. The increase in affinity was already apparent after 18 hr and was maximal after 3 days of exposure to the barbiturate. In addition, PB was able to induce a 30% increase in the overall receptor number upon 3 day exposures. These effects may have a biological significance since PB was able to potentiate the induction by DXM of a positively regulated liver function, the TATase activity. Indeed, half-maximum induction of the activity was obtained for a two-fold lower DXM concentration in PB-treated cells as compared to untreated cells. In addition, the maximum DXM-induced TATase activity was higher following PB treatment. The 30% increase of the receptor number is not likely to account for the two-fold increase in TATase activity, since a similar effect over TATase expression had been shown to require a two-fold increase in the receptor number in rat hepatoma cells [11]. Therefore, the observed increase in DXM-induced TATase activity following PB treatment may essentially reflect the increase in affinity of the GR for DXM. If this assumption is correct, it should be concluded that the glucocorticoid–receptor complexes would have a higher potency toward transcriptional activation in PB-treated versus untreated cells.

The possibility that the GR participates to the induction of P450 genes by PB remains to be investigated. We are currently testing this hypothesis by using glucocorticoid agonists and antagonists.

The kind of modification that led to the observed increase in affinity of the GR for its ligand is unknown; it probably required several steps, since no effect could be detected before 16 to 18 hr. The increase in the receptor number, apparent at 72 hr, could reflect a stabilization of the protein possibly related to the nature of the modification that led to the increase in affinity.

PB is a tumor promoting agent such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) which is able to activate protein kinase C [25]. Moreover, TPA has been shown to potentiate the induction of the TATase activity by DXM in rat liver and in rat hepatocytes [26]. This effect was restricted to TPA since other phorbol esters which are not tumor promoters and are unable to induce protein kinase C activity were inefficient. Therefore, it was suggested that TPA could affect the degree of phosphorylation of the GR via a specific kinase activity. Indeed GR are phosphoproteins, and their conversion from the non-hormone-binding to the hormone-binding form requires ATP or ADP [27]. We do not know if PB is able to induce a kinase activity in rat hepatoma cells; nevertheless it is tempting to speculate that the barbiturate could induce additional and/or new phosphorylations of the GR which in turn could be responsible of the observed PB-induced increase in affinity of the receptor for DXM.

In the absence of ligand, the GR and Ah-receptors are part of distinct cytosolic protein complexes that mostly contain a core protein of 90 kD which is a heat shock protein. Fixation of the ligand is accompanied by a dissociation of the 90 kD heat shock protein from the complex [28]. Since PB is able to modify the number and/or the affinity of both the GR and the Ah receptors, the molecular events implicated could conceivably involve modifications in the complexes formed between these receptors and the 90 kD species. Further experiments are needed to test this possibility.

To our knowledge, PB is the first agent shown to be able to modify the affinity of the GR for its ligand. Our results are thus in favour of the hypothesis that GR, or closely related members of the same family of receptors, participate in a "non-classical" way to the response of liver cells to PB. Presently, we do not know whether the modification evidenced here reflects a general effect that PB would be able to exert over several members of the superfamily of steroid receptors. We do not know if this effect depends on tissue-specific constraints. Transfection of expression vectors coding for these receptors, in either liver or non-liver derived cells, may allow to directly address these questions.

Acknowledgements—This work has been supported by a grant (No. 6496) and a fellowship to L. Corcos from the Association pour la Recherche Contre le Cancer. We are grateful to A. Poland for critical reading of the manuscript.

REFERENCES

1. Hardwick JP, Gonzalez FJ and Kasper CB, Transcriptional regulation of rat liver epoxide hydratase, NADPH-cytochrome P-450 oxidoreductase, and cytochrome P-450b genes by phenobarbital. *J Biol Chem* **258**: 8081–8085, 1983.
2. Simmons DL, McQuiddy P and Kasper CB, Induction of the hepatic mixed-function oxidase system by synthetic glucocorticoids. *J Biol Chem* **262**: 326–332, 1987.
3. Poland A, Mak I, Glover E, Boatman RJ, Ebetino FH and Kende AS, 1,4-Bis [2-(3,5-dichloropyridyloxy)]-benzene, a potent phenobarbital-like inducer of microsomal monooxygenase activity. *Mol Pharmacol* **18**: 371–580, 1980.
4. Poland A, Mak I and Glover E, Species differences in responsiveness to 1,4-bis [2-(3,5-dichloropyridyloxy)]-benzene, a potent phenobarbital-like inducer of microsomal monooxygenase activity. *Mol Pharmacol* **20**: 442–450, 1981.
5. Corcos L and Weiss MC, Phenobarbital, dexamethasone and benzanthracene induce several cytochrome P450 and mRNAs in rat hepatoma cells. *FEBS Lett* **233**: 37–40, 1988.
6. Schuetz EG and Guzelian PS, Induction of cytochrome P450 by glucocorticoids in rat liver. II. Evidence that glucocorticoids regulate induction of cytochrome P450 by a nonclassical receptor mechanism. *J Biol Chem* **259**: 2007–2012, 1984.
7. Schuetz EG, Wrighton SA, Safe SH and Guzelian PS, Regulation of cytochrome P-450 by phenobarbital and phenobarbital-like inducers in adult rat hepatocytes in primary monolayer culture and "*in vivo*". *Biochemistry* **25**: 1124–1133, 1986.
8. Strähle U, Schmid W and Schütz G, Synergistic action of the glucocorticoid receptor with transcription factors. *EMBO J* **7**: 3389–3395, 1988.
9. Dong Y, Poellinger L, Gustafsson JA and Okret S, Regulation of glucocorticoid receptor expression: evidence for transcriptional and post-transcriptional mechanisms. *Mol Endocrinol* **2**: 1256–1264, 1988.

10. Thompson EB, Tomkins GM and Curran JF, Induction of the tyrosine amino-transferase by steroid hormones in a newly established tissue culture cell line. *Proc Natl Acad Sci USA* **56**: 296–303, 1966.
11. Vanderbilt JN, Miesfeld R, Male B and Yamamoto K, Intracellular receptor concentration limits glucocorticoid-dependent enhancer activity. *Mol Endocrinol* **1**: 68–74, 1987.
12. Dong Y, Aronsson M, Gustafsson JA and Okret S, The mechanism of cAMP-induced glucocorticoid receptor expression. *J Biol Chem* **256**: 13679–13683, 1989.
13. Okey AB and Vella LM, Elevated binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 3-methylcholanthrene to the Ah-receptor in hepatic cytosols from phenobarbital-treated rats and mice. *Biochem Pharmacol* **33**: 531–538, 1984.
14. Denomme MA, Leece B, Li A, Towner R and Safe S, Elevation of 2,3,7,8-tetra chlorodibenzo-*p*-dioxin (TCDD) polychlorinated biphenyls. Structure–activity relationships. *Biochem Pharmacol* **35**: 277–282, 1986.
15. Prokipcak RD, Faber LE and Okey AB, Characterization of the Ah-receptor for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: use of chemical crosslinking antibody directed against a 59-k-Da protein associated with steroid receptors. *Arch Biochem Biophys* **274**: 648–658, 1989.
16. Deschatrette J and Weiss MC, Characterization of differentiated and dedifferentiated clones from a rat hepatoma. *Biochimie* **56**: 1603–1611, 1974.
17. Wiebel FJ, Kiefer F and Murdia VS, Phenobarbital induces cytochrome P450 dependent monooxygenases in rat hepatoma cells. *Chem Biol Interact* **52**: 151–162, 1984.
18. Venetianer A, Pinter Z and Gal A, Examination of glucocorticoid sensitivity and receptor content of hepatoma cell lines. *Cytogenet Cell Genet* **28**: 280–283, 1980.
19. Venetianer A and David D, Regulation of liver expression in dexamethasone resistant hepatoma cells. In: *Activation of Hormone and Growth Factor Receptors. Molecular Mechanisms and Consequences* (Eds. Alexis MN and Sekeris CE), Nato ASI Series 295, pp. 173–193. Kuwer Academic Publisher, 1990.
20. Taylor CM, Blanchard B and Zava DT, A simple method to determine whole cell uptake of radiolabelled oestrogen and progesterone and their subcellular localization in breast cancer cell lines in monolayer culture. *J Steroid Biochem* **20**: 1083–1088, 1984.
21. Diamondstone TI, Assay of TAT activity by conversion of *p*-hydroxyphenylpyruvate to *p*-hydroxybenzaldehyde. *Anal Biochem* **16**: 395–401, 1966.
22. Scatchard G, The attraction of proteins for small molecules and ions. *Ann NY Acad Sci* **51**: 660–672, 1949.
23. Gagne D, Labhilili M and Pons M, Description and analysis of differential sensitivity to glucocorticoids in Fao cells. *J Steroid Biochem* **31**: 917–925, 1988.
24. Moguilewsky M and Philibert D, RU38486: a potent antiglucocorticoid activity correlated with strong binding to the cytosolic glucocorticoid receptor followed by an impaired activation. *J Steroid Biochem* **20**: 271–276, 1984.
25. Nishizuka Y, Studies and perspectives of protein kinase C. *Science* **233**: 305–312, 1986.
26. Kido H, Fukusen N and Katunuma N, Tumor-promoting phorbol ester amplifies the induction of tyrosine aminotransferase and ornithine decarboxylase by glucocorticoid. *Biochemistry* **26**: 2353–2354, 1986.
27. Barnett CA, Palmour RM, Litwack G and Seegmiller JE, *In vitro* stabilization of the unoccupied glucocorticoid receptor by adenosine 5′-diphosphate. *Endocrinology* **112**: 2059–2068, 1983.
28. Denis M, Wilhelmsson A, Cuthill S, Poellinger L and Gustafsson JA, Structural differences between the glucocorticoid, dioxin and oxysterol receptors from rat liver cytosol. *Biochem Biophys Res Commun* **163**: 444–451, 1989.