Diphenylhydantoin: An Alternative Ligand of a Glucocorticoid Receptor Affecting Prostaglandin Generation in A/J Mice

Abstract. *Evidence for the binding of 5,5-diphenylhydantoin and glucocorticoids to a common receptor is presented for pulmonary and hepatic cytosols and thymocytes of A/J female mice. The 5,5-diphenylhydantoin–protein complex is adsorbed by DNA cellulose, and is incorporated into nuclei. 5,5-Diphenylhydantoin, like glucocorticoids, inhibits the production of prostaglandins in thymocytes. Thus a common receptor is probably responsible for the inhibitory and teratogenic effects of these drugs.*

5,5-Diphenylhydantoin (DPH), an anticonvulsant, causes malformation in rodents (*1*) and in humans (*2*). However, the mechanism of malformation is not completely understood. Several studies indicate that the production of cleft palate by DPH has a common pathway with that produced by glucocorticoids. When given with cortisone, DPH does not increase the frequency of cleft palate (*3*), and a probit analysis of cleft palate produced by DPH and cortisone indicates an identical mechanism (*4*). Susceptibility to cleft palate induced by both drugs is influenced by the H-2 histocompatibility locus (*5*). Both drugs reduce fetal movements during palatal differentiation, and delay shelf elevation from a vertical to horizontal position (*6*). Both cortisone (*7*) and DPH (*8*) cause an inhibition of RNA and protein synthesis in mouse fetal palates, and susceptibility to DPH-induced clefting correlates with the inhibition (*8*). Since glucocorticoids probably produce cleft palate by a glucocorticoid-receptor mechanism (*9, 10*), we have hypothesized that DPH and glucocorticoids may bind to a common receptor (*11*). In the study reported here, we conducted competitive binding analyses to determine whether DPH and dexamethasone bind to the same receptor in the lungs and livers of A/J female mice, a strain highly susceptible to the induction of cleft palate by DPH (*1, 5*).

The glucocorticoid receptor mechanism appears to involve inhibition of the synthesis of prostaglandins or thromboxanes (*12*). Therefore, we also studied the effect of both DPH and dexamethasone on the synthesis of 6-ketoprostaglandin $F_{1\alpha}$ and thromboxane B_2 in A/J thymocytes.

In the experiment illustrated in Fig. 1, the drug was dissolved in a solution containing 25 m*M* Na_2CO_3 and 120 m*M* NaCl (*p*H 10.5), and mixed with nine volumes of buffered cytosol preparation (final *p*H, 7.8 to 7.9). When the DPH was dissolved in ethanol, propylene glycol, or dimethyl sulfoxide, saturable binding of the drug did not occur. Solubilizing the DPH with Na_2CO_3 did not change the mobility of the drug when it was tested by thin-layer chromatography with three kinds of solvent systems. In pulmonary cytosol, the number of DPH binding sites (mean ± standard deviation, 72 ± 21 fmole per milligram of protein, $N = 5$) was significantly less than the number of dexamethasone binding sites (144 ± 23 fmole per milligram of protein, $N = 4$, $P = .002$, based on a *t*-test). The number of DPH binding sites was also significantly less in hepatic cytosol: 44 ± 11 fmole per milligram of protein, $N = 5$, for DPH, and 224 ± 82 fmole per milligram of protein, $N = 4$ ($P = .002$) for dexamethasone. The DPH binding was significantly greater in lungs than in livers ($P = .029$).

The binding of DPH was also effectively blocked by other glucocorticoids and by androgen to some degree, but not by estrogen or progesterone. Percentage inhibitions were determined with 40 n*M* [^{3}H]DPH and 1 μ*M* concentrations of competitors, and with hepatic cytosol. The order of the inhibitory effect (average of four experiments) on DPH binding was as follows: dexamethasone = triamcinolone acetonide = cortexolone = corticosterone > cortisol = cortisone > 5α-dihydrotestosterone > testosterone > 17β-estradiol > diethylstilbestrol = progesterone (0 percent).

When DPH competed with [^{3}H]dexamethasone binding to cytosols of lung and liver, specific binding was blocked by only 37 and 59 percent, respectively. Molecular seiving with Sephadex G-200 of [^{3}H]dexamethasone binding to either cytosol demonstrated two peaks that could be adsorbed with DNA-cellulose. The Stokes' radius of the peaks was 61 Å and 53 Å. The [^{3}H]DPH bound only to the 61-Å peak in each cytosol. Two [^{3}H]dexamethasone peaks with a similar Stokes' radius have been demonstrated with Sephadex G-200 in extracts of nuclei of rabbit fetal lungs treated with [^{3}H]dexamethasone (*13*).

The adsorption of the [^{3}H]DPH-carrying protein of pulmonary cytosol to

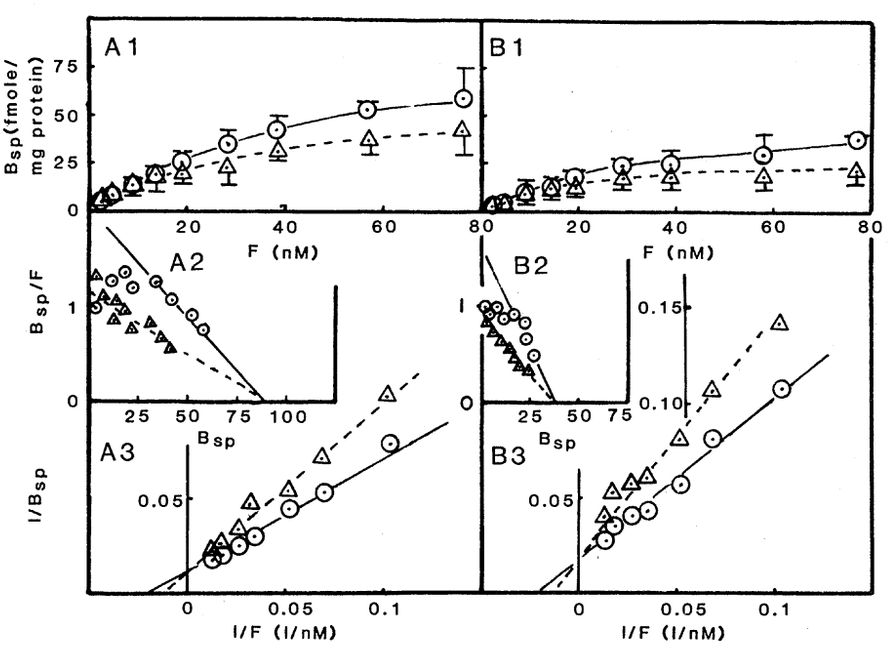

Fig. 1. Competition between DPH and dexamethasone for the same binding site. Pulmonary and hepatic cytosols were prepared as described (*15*). Portions of [^{3}H]DPH in ethanol were mixed with 0.2 pmole of nonradioactive DPH, 0.1 pmole of dexamethasone, or no competitor; and were evaporated under N_2. The residue was dissolved in 20 μl of a solution of 25 m*M* Na_2CO_3 and 0.12*M* NaCl, and was incubated at 5°C for 2 hours. The ligand binding was started by adding 180 μl of cytosolic preparation. The cytosols were incubated at 5°C for 2 hours, and then the protein-bound ligand was separated from free (*F*) ligand by treatment with dextran-coated charcoal (*15*). The specifically bound [^{3}H]DPH (B_{sp}) was calculated by subtracting the value measured with nonradioactive DPH from the value without it. (A1, A2, and A3) Binding of DPH to pulmonary cytosol. (A1) Saturation curve, (A2) Scatchard plot, and (A3) double reciprocal plot. (B1, B2, and B3) The corresponding results with hepatic cytosol. Symbols: ○, [^{3}H]DPH binding without competitor; △, [^{3}H]DPH binding in the presence of 0.5 μ*M* dexamethasone.

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DNA-cellulose was studied according to Kalimi *et al.* (*14*). The adsorption by DNA-cellulose was significantly higher than that by plain cellulose: 3.06 ± 0.69 fmole/mg protein compared to 1.16 ± 0.69 fmole per milligram of protein ($N = 4$, $P = .015$, t-test). Furthermore, the DNA-cellulose adsorption was characteristic of the cytosolic binding proteins but not of bovine serum albumin (data not shown).

Nuclear incorporation of DPH was also observed in thymocytes. The thymocytes were incubated in RPMI-1640 medium containing 10 m*M* Hepes and 30 n*M* [^{3}H]DPH with or without 1 μ*M* nonradioactive DPH at 37°C for 30 minutes. The nuclei were then separated as previously described (*15*). The incorporation of [^{3}H]DPH into both whole cells and nuclei in the absence of nonradioactive DPH was significantly higher than that observed in the presence of nonradioactive DPH (whole cells, 3.33 ± 1.45 pmole per milligram of DNA compared to 1.38 ± 0.57 pmole per milligram of DNA, $N = 4$, $P = .006$; nuclei, 2.36 ± 0.98 pmole per milligram of DNA compared to 0.95 ± 0.40 pmole per milligram of DNA, $N = 4$, $P = .003$, t-test).

Table 1 shows the effect of DPH and dexamethasone on the generation of 6-ketoprostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), the stable metabolite of prostacyclin, and thromboxane B_2 (TXB_2), the stable metabolite of thromboxane A_2, in A/J female thymocytes. A marked degree of inhibition was produced by both drugs. Dexamethasone and DPH showed an almost identical inhibitory effect over a wide range of concentrations.

In our first study of the association between DPH teratogenicity and the glucocorticoid receptor we demonstrated the blocking of [^{3}H]dexamethasone incorporation into human palatal cells by 0.5 μ*M* DPH (*11*). Salomon and Pratt (*9*), however, were unable to demonstrate direct blocking of [^{3}H]dexamethasone binding to the receptor of embryonic maxillary cytosol of Swiss Webster mice by DPH. Although the method for solubilizing DPH was not given in their report, our present results suggest the possibility that their negative results may be due to the limited solubility of DPH. Burnham *et al.* (*16*) also reported saturable binding of DPH to distinct sites on the membrane fraction of rat brain when they used an alkaline buffer consisting of NaOH, tris, and EDTA for solubilizing DPH.

Usually, ligands that bind to a receptor have similar chemical structures. Although DPH seems to behave as an alternative ligand of the glucocorticoid receptor affecting prostaglandin production, the chemical structure of the drug is quite different from that of glucocorticoids. Similar phenomena, however, have been reported for the estrogen receptor, which binds with diethylstilbestrol, *o,p'*-DDT, and chlordecone, an insecticide (*17*). These compounds have estrogenic properties, but have vastly different chemical structures from estrogen, especially in the case of chlordecone. Thus, it is possible that the drugs of different chemical structure can bind to a hormone receptor and effect a hormone-like response.

Since the binding of DPH is totally adsorbed by DNA-cellulose, and since nuclear incorporation of the [^{3}H]DPH binding protein occurs, it is likely that DPH binds to a glucocorticoid receptor site. This assertion is supported by the fact that dexamethasone and DPH inhibit 6-keto-$PGF_{1\alpha}$ and TXB_2 production to a similar degree, since this is a glucocorticoid receptor–mediated event (*18*).

Further evidence for a common receptor site for DPH and dexamethasone is provided by comparative studies of strains of mice susceptible and resistant to glucocorticoid- and DPH-induced cleft palate. Higher concentrations of glucocorticoid receptors and greater degrees of glucocorticoid-induced inhibition of prostaglandin production were reported in strains sensitive to glucocorticoid-induced cleft palate (*18*). Strains sensitive to DPH-induced cleft palate show significantly higher levels of DPH binding and DPH-induced inhibition of prostaglandin production (*19*).

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Table 1. Inhibition of production of both 6-keto-$PGF_{1\alpha}$ and TXB_2 by drugs in thymocytes from female mice. Thymocytes were suspended in 0.15*M* phosphate buffer containing 0.9 percent NaCl and 0.1 percent gelatin. Portions (2 ml) of the suspension (1×10^7 to 2×10^7 cells) were incubated in the presence of zymosan (250 μg/ml) to stimulate prostaglandin production (*20*) with or without drugs of indicated concentrations at 37°C for 3 hours. The reaction was stopped by adding 10 ml of ethyl acetate. Prostaglandins were extracted with ethyl acetate and were dried under N_2. The residue was dissolved in ethanol, and portions were examined by an RIA procedure. The antibodies used for 6-keto-$PGF_{1\alpha}$ and TXB_2 were specific. The percentage inhibition was calculated as follows: $(A - B)/A \times 100$ percent, where A and B are the amounts of prostaglandins produced, respectively, in the absence and presence of the drug. The control value for 6-keto-$PGF_{1\alpha}$ production was 172 ± 72.5 pg per 10^6 cells, and for TXB_2 was 83.3 ± 13.0 pg per 10^6 cells (five determinations). The difference between two drug effects was not significant.

Drug	Inhibition (%)			
	1 μ*M*	10 μ*M*	50 μ*M*	100 μ*M*
	Inhibition of 6-keto-$PGF_{1\alpha}$			
DPH	13.0 ± 11.0	17.9 ± 8.0	22.2 ± 10.8	37.0 ± 11.8
Dexamethasone	6.6 ± 10.5	10.1 ± 11.8	28.0 ± 11.0	29.5 ± 10.5
	Inhibition of TXB_2			
DPH	2.1 ± 1.9	4.0 ± 6.0	6.8 ± 5.2	13.2 ± 3.0
Dexamethasone	1.2 ± 2.0	5.8 ± 7.0	15.2 ± 11.8	17.0 ± 9.0

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minutes. Then the DNA-cellulose or plain cellulose was washed four times with the same buffer.
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Eukaryotic Transcriptional Regulation and Chromatin-Associated Protein Phosphorylation by Cyclic AMP

Abstract. *Cyclic adenosine monophosphate (AMP) analogs or agents that increase intracellular cyclic AMP rapidly stimulate transcription of the prolactin gene in a line of cultured rat pituitary cells. This effect is correlated with the phosphorylation of a chromatin-associated basic protein designated BRP. These data are consistent with the postulate that increased intracellular cyclic AMP concentrations induce rapid transcriptional effects on specific genes in eukaryotes, mediated by direct or indirect phosphorylation of a specific chromatin-associated protein or proteins.*

Adenosine 3′,5′-monophosphate (cyclic AMP) is an important regulatory molecule serving to control diverse biochemical events in both prokaryotic and eukaryotic organisms. In prokaryotes, it acts as an allosteric effector allowing a specific gene activator protein (termed either CAP or CRP) to bind to specific DNA sequences and thus regulate transcription of several catabolite-sensitive operons (*1*). In eukaryotes, it is believed to act as a "second messenger" through which intracellular events are controlled by external stimuli, including various polypeptide hormones. Both the discovery that cyclic AMP receptor protein is associated with a catalytic subunit in higher eukaryotes (*2*) and studies of cyclic AMP–resistant cell lines (*3*) suggest that the cyclic AMP–dependent protein kinase is responsible for many, and possibly all, of the cyclic AMP–mediated effects. One critical consequence of an increase in cyclic AMP in eukaryotes is the increased biosynthesis of specific proteins, which is invariably associated with an increase in their encoding messenger RNA's (mRNA's) (*4, 5*). Because of the nuclear location of eukaryotic genes, it is necessary to ascertain whether cyclic AMP exerts direct, specific transcriptional effects and how such regulation might occur. Recent advances in recombinant DNA technology allow potential regulation of specific genes by cyclic AMP to be critically assessed.

Cyclic AMP has been shown to overcome the inhibitory effects of a dopamine agonist on prolactin gene transcription in pituitary cells, implying its potential as a gene regulator (*6*). We now report the use of cloned prolactin DNA sequences to demonstrate directly that an elevation of intracellular cyclic AMP is associated with a rapid increase in prolactin gene transcription in a clonal line of rat pituitary cells. We further identify the concomitant phosphorylation of a 23-kilodalton chromatin-bound basic protein. The association of these events suggests that the pathway mediating cyclic AMP regulation may involve phosphorylation of a nuclear protein, which in turn regulates transcription of specific genes.

The cultured clonal cell line (GH) provided a system in which to study the transcriptional regulation of the prolactin and growth hormone genes by both polypeptide and steroid hormones (*7, 8*). Because many of these hormones are thought to act via second messengers, prolactin gene expression was investigated to evaluate potential regulatory effects produced by elevation of intracellular cyclic AMP. Transcription rates were determined in isolated nuclei by elongating nascent RNA transcripts in the presence of [^{32}P]uridine triphosphate. Labeled prolactin RNA transcripts were quantified by hybridization to an immobilized intervening sequence subclone under DNA excess hybridization conditions (*9*), as described (*10*). Based on α-amanitin sensitivity, all the hybridized labeled prolactin RNA products appear to represent polymerase II transcripts.

Elevation of cyclic AMP by addition of the cyclic AMP analogs, 8-bromo–cyclic AMP or dibutyryl cyclic AMP, to cell cultures increased transcription of the prolactin gene four- to fivefold above that in corresponding unstimulated cells (Fig. 1A). A butyrate control was included since butyrate can be generated by metabolism of dibutyryl cyclic AMP (*11*). Addition of forskolin, a diterpene

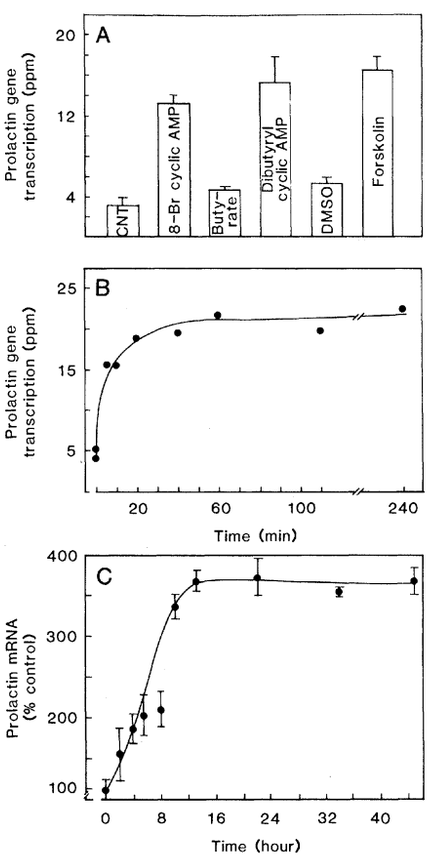

Fig. 1. (A) Effect of elevated cyclic AMP on prolactin gene transcription. Prolactin gene transcription rates were measured by quantifying specific prolactin transcripts as follows. Elongating nascent RNA chains were isolated from nuclei prepared from GH_4 cells that had been incubated with 8-bromo (*8-Br*)–cyclic AMP (2.5 m*M*), butyrate (1 m*M*), dibutyryl cyclic AMP (1 m*M*), forskolin (10^{-6}*M*), or dimethyl sulfoxide (*DMSO*) (0.1 percent as solvent control for forskolin) or in the absence of added agents (*CNT*) for 60 minutes prior to isolation of nuclei. (B) Time course of forskolin (10^{-6}*M*) stimulation of prolactin gene transcription. Forskolin was added in ethanol at a final concentration of 0.5 percent, which was determined to exert no effect on prolactin gene transcription. (C) Time course of prolactin mRNA accumulation following transcriptional stimulation by forskolin (10^{-6}*M*). RNA was prepared by phenol-chloroform extraction and prolactin mRNA was quantified by immobilization of "diazotyzed paper" (DBM) and DNA-excess hybridization with the use of a cloned, nick-translated prolactin complementary DNA probe, as described (*13*). Each point in panels A, B, and C is the average (± standard error of the mean) of triplicate hybridizations per group. Each group consists of four to six plates (3×10^7 cells). The triplicate determination in (B) differed by less than 5 percent. This experiment was representative of six experiments of similar design giving comparable data.

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