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Induction of Tyrosine Hydroxylase by Glucocorticoids in Mouse Neuroblastoma Cells

Enhancement of the Induction by Cyclic AMP

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SUMMARY

The glucocorticoid analogue dexamethasone elicits the induction of tyrosine hydroxylase (TH) in the neuroblastoma cell line NBP_2. This induction requires the continuous presence of dexamethasone in the culture medium and reaches a maximal level after 3 days of continuous treatment. If after 3 days of treatment the medium containing dexamethasone is removed and replaced with fresh medium lacking the glucocorticoid analogue, TH activity remains constant for 1 day and then decreases to control levels with a half-life of 1.6 days. The induction is maximal with concentrations of dexamethasone as low as 10^{-8} M and is also elicited by hydrocortisone and triamcinolone, but not by progesterone, β-estradiol, or testosterone. Furthermore, high concentrations of progesterone, a competitive inhibitor of glucocorticoid-mediated responses, inhibit the induction of TH elicited by dexamethasone. Actinomycin D blocks the dexamethasone-mediated induction of TH, suggesting that RNA synthesis is required for the induction to take place. However, there is no dexamethasone-mediated increase in total RNA synthesis, indicating that the glucocorticoid is apparently increasing the transcription of only a small number of genes. When the cells are treated simultaneously with glucocorticoids plus cyclic AMP analogues or compounds which elevate intracellular cyclic AMP, the increase in TH is greater than the sum of the increases which are elicited in the presence of either inducing agent alone. The observed enhancement of the cyclic AMP-mediated induction by glucocorticoids may provide insight into the intracellular mechanism by which glucocorticoids enhance the nerve growth factor-mediated and/or the stress-induced trans-synaptic induction of TH in peripheral adrenergic neurons.

INTRODUCTION

Tyrosine hydroxylase (EC 1.14.16.2) is the enzyme which catalyzes the rate-limiting step in the biosynthesis of catecholamines. The level of this enzyme in adrenergic neurons is influenced by a number of different compounds or stimuli. The level of TH[2] increases in the chromaffin cells of the adrenal medulla, in peripheral postganglionic adrenergic neurons, and in certain adrenergic neurons of the brain following prolonged activation of these cells by stress or treatment with certain drugs (1–3). In the periphery, this increase apparently requires presynaptic innervation and thus has been termed trans-synaptic induction. The intracellular mechanism that mediates this induction has not been unequivocally established.

The cellular content of TH is also increased by exposure of cells or tissues to cyclic AMP or glucocorticoids. An increase in intracellular cyclic AMP is associated with the subsequent induction of TH in the adrenal medulla and in mouse neuroblastoma cells (4–7). A 1- to 2-hr increase in intracellular cyclic AMP is sufficient to trigger the induction of TH 24–48 hr later (4, 8). Glucocorticoids have been shown to induce TH in organ cultures of the rat SCG and in cell cultures of rat pheochromocytoma (9, 10). With the use of the synthetic glucocorticoid analogue dexamethasone, the glucocorticoid-mediated induction has been shown to be dose-dependent and maximal at a concentration of 10^{-7} M dexamethasone (9, 10). A 4-hr exposure to dexamethasone is reported to produce a maximal induction of the enzyme in these systems.

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[2] The abbreviations used are: TH, tyrosine hydroxylase; SCG, superior cervical ganglion; NGF, nerve growth factor; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; PBS, 0.01 M potassium phosphate-buffered saline (pH 7.4); PGE_1, prostaglandin E_1; PDE, phosphodiesterase.

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In the rat SCG, glucocorticoids have been shown to enhance the trans-synaptic induction of TH elicited by cold stress (11). Furthermore, simultaneous treatment of either the rat SCG or rat pheochromocytoma PC12 cells with glucocorticoids plus NGF elicits an increase in TH activity which is greater than the sum of the increases that occur in the presence of either inducing agent alone (12). Several workers have shown that cyclic AMP levels increase following cold stress or treatment with NGF (4, 13, 14). Thus, it is possible that the effect of glucocorticoids on the NGF-mediated or the stress-related trans-synaptic induction of TH is due to an interaction between the glucocorticoid and cyclic AMP.

In the present study we report that glucocorticoids elicit the induction of TH in cultured mouse neuroblastoma NBP_2 cells. Our results are in reasonable agreement with those of Williams *et al.* (15), who recently reported that glucocorticoids increase TH activity in the same mouse neuroblastoma cell line. We demonstrate in the present report the following additional results: (a) the increase in activity is due to an increase in enzyme protein; (b) the increase in activity is dependent upon the continued exposure of the cells to the glucocorticoid; (c) the increase in activity requires RNA synthesis, but total RNA synthesis is not significantly affected by glucocorticoids; and (d) the simultaneous treatment of these cells with glucocorticoids and cyclic AMP analogues or compounds which elevate intracellular cyclic AMP elicits a greater than additive increase in TH.

EXPERIMENTAL PROCEDURES

Materials

Tissue culture flasks and dishes were obtained from Falcon Plastics Company (Los Angeles, Calif.). Ham's F-12 medium and γ-globulin-free newborn calf serum were obtained from Grand Island Biological Company (Grand Island, N. Y.). [5,6-^{3}H]Uridine and L-[1-^{14}C]tyrosine (51 Ci/mole) were obtained from New England Nuclear Corporation (Boston, Mass.). Ro 20-1724 was a gift from Dr. W. E. Scott, Hoffmann-La Roche, Inc. (Nutley, N. J.). Actinomycin D and 8-bromo-cyclic AMP were purchased from Sigma Chemical Company (St. Louis, Mo.). Pansorbin was purchased from Calbiochem-Behring Corporation (San Diego, Calif.). All other chemicals were of the highest purity available from commercial sources.

Methods

Cell culture conditions. The experiments were performed using mouse neuroblastoma cells, clone NBP_2 (16). Cells were grown in 250-ml Falcon flasks (75 cm^2) in Ham's F-12 medium supplemented with 10% γ-globulin-free newborn calf serum (Grand Island Biological Company), penicillin G (100 units/ml), and streptomycin (100 μg/ml). The cells were maintained in humidified air containing 5% CO_2 at 36° and subcultured before they reached confluency (every 3–4 days). The average doubling time for these cells was 18–22 hr.

Induction studies. For induction experiments, cells were cultured in 50-ml Falcon flasks (25 cm^2 surface area) in the medium described above at an initial cell density of 3×10^5 cells/flask. The cells were treated with dexamethasone and/or the cyclic AMP phosphodiesterase inhibitor Ro 20-1724, and, at various times after the initiation of treatment, cells were harvested by removing the medium and adding 2 ml of 0.25% trypsin/1 mM EDTA. This treatment was terminated by the addition of 2 ml of serum-supplemented medium. An aliquot of this suspension was removed for determination of cell numbers using a Coulter counter. The cells were collected for assay of TH by centrifugation at 700 × *g* for 10 min and then washed once with PBS. The final cell pellet was frozen at −90° in a Revco freezer and thawed rapidly just prior to assay. This pellet, which weighed between 13 and 18 mg, was used for assaying TH activity. We estimated its contribution to the final assay volume at 15 μl.

TH assays. TH activity was assayed by the method of Waymire *et al.* (17) as modified by Zivkovic *et al.* (18). In this assay, L-[1-^{14}C]tyrosine is converted to L-[1-^{14}C] dihydroxyphenylalanine by the action of TH. The generated dihydroxyphenylalanine is then decarboxylated by added aromatic amino acid decarboxylase purified from hog kidney by the method of Waymire *et al.* (17). The reduced pterin cofactor is regenerated by the addition of NADPH and pteridine reductase, purified from sheep liver (19). Catalase (Boehringer-Mannheim) is added to destroy the generated H_2O_2. The concentrations of tyrosine and D,L-6-methyl-5,6,7,8-tetrahydropterin in the assays were 0.10 mM and 1.0 mM, respectively. Triton X-100 (0.1%) was also added to the assay mixture to ensure complete solubilization of TH. The final reaction volume was 0.10 ml. The reaction was allowed to proceed at 37° for 10 min in test tubes capped with a rubber septum. The tubes were then placed on ice and the reaction was terminated by injection of 0.10 ml of 0.8 M perchloric acid into the reaction mixture. The $^{14}CO_2$ liberated was collected for 1 hr at 37° in 0.2 ml of NCS solubilizer contained in a plastic well suspended from the rubber septum. The wells were then transferred to counting vials and the radioactivity was determined by liquid scintillation spectrometry.

Cyclic AMP determinations. Cyclic AMP was assayed by a modification of the method of Harper and Brooker (7, 20).

Determination of the rate of RNA synthesis. The cells were plated in 60 × 15 mm dishes at an initial density of 3×10^5 cells/dish. They were treated 4 days later with dexamethasone and Ro 20-1724. At the designated times, 2 μCi of [5,6-^{3}H]uridine (specific activity 2.2 Ci/mmole) were added to the medium. The [^{3}H]uridine was incubated with the cells at 36° for 45 min. Initial time course studies demonstrated that the incorporation of [^{3}H]uridine into total trichloroacetic acid-precipitable RNA was linear for up to 60 min. The incubation was terminated by aspirating the medium and washing the cells three times with 3 ml of ice-cold PBS. The cells were scraped off the dish with a rubber policeman into 3 ml of ice-cold PBS containing 1 mM unlabeled uridine and transferred to centrifuge tubes. The suspension was centrifuged at 700 × *g* for 10 min. The supernatant was removed, and the cells were lysed with 1 ml of 10% trichloroacetic acid. This suspension was centrifuged at 20,000 × *g* for 15–20 min, and the pellet was washed once with 1 ml of 10% trichloroacetic acid. The pellet was solubilized in 0.75 ml of NCS, transferred to scintillation fluid, and counted. For the determination of unincorporated, labeled uridine,

the trichloroacetic acid supernatant and the trichloroacetic acid wash were combined with the supernatant derived from the final wash of the cells. An aliquot of this solution was transferred to scintillation fluid and counted.

Immunotitration studies. The cells were harvested as described above. After the cell pellet was thawed, a 10% homogenate in 20 mM potassium phosphate buffer (pH 7.6) was prepared. The homogenate was centrifuged at 40,000 × *g* for 30 min, and the supernatant was assayed for TH activity. As previously determined (7), the homogenization liberated approximately 80% of the TH activity into the supernatant whether or not the enzyme was induced. Thus, the supernatant was used for immunotitration studies.

Different volumes of supernatant enzyme were added to 1.5-ml Microfuge tubes containing 10 μl of antiserum (21). A volume of buffer containing 20 mM potassium phosphate (pH 7.6) was added to each tube to produce a final volume of 60 μl. This solution was allowed to stand at room temperature for 30 min. Then, 40 μl of a 10% solution of Pansorbin (*Staphylococcus aureus* cells containing Protein A; ref. 22), pretreated according to the method of Kessler (22), were added to the solution. The tubes were mixed and allowed to stand at room temperature for 15 min. The suspension was centrifuged at 12,000 × *g* for 5 min, and 10 μl of the supernatant were assayed for TH activity.

RESULTS

Effect of dexamethasone on TH activity in neuroblastoma cells. In agreement with our earlier studies (7), basal levels of TH in the NBP_2 cell line increased with time in culture (Fig. 1, top panel). The activity rose approximately 9-fold over the 11 days that the cells were in culture. In addition, we observed changes in the basal activity of TH, depending upon the batch of serum employed; this fact accounts for the differences in basal activity seen in the different experiments. However, the effects of the hormones on TH activity were consistent and independent of the batch of serum used. The rate of cell division is seen in the *inset* to Fig. 1; the cells reached confluency by day 6. Ethanol (34 mM) has no effect on TH activity in NBP_2 cells cultured under these conditions (7).

TH activity in cells treated with 10^{-6} M dexamethasone increased more rapidly than activity in control cells treated only with 34 mM ethanol (Fig. 1, top and bottom panels). The activity in the dexamethasone-treated cells reached a peak after 3 days of treatment to a level approximately 2-fold greater than that observed in control cells. This elevated level was maintained for at least 7 days in cells continuously treated with the glucocorticoid analogue. Removal of dexamethasone on day 3 did not affect enzyme activity for 24 hr. However, after 24 hr, the activity slowly decreased to that seen in control cells. Assuming that first-order kinetics describes this deinduction of the enzyme, the $t_{1/2}$ for this decrease in activity is calculated to be 1.6 days.

Effect of dexamethasone in combination with cyclic AMP-elevating agents on TH activity in neuroblastoma cells. The cells were treated with dexamethasone alone or in combination with either Ro 20-1724 (5×10^{-5} M), PGE_1 (10^{-6} M), PGE_1 (10^{-6} M) plus Ro 20-1724 (5×10^{-5} M), or 8-bromo-cyclic AMP (10^{-3} M). After 3 days of continuous treatment, the cells were harvested and assayed for TH activity. The results are summarized in Table 1. In this set of experiments dexamethasone alone elevated TH activity by 40%. The inhibitor of cyclic AMP phosphodiesterase, Ro 20-1724, at a concentration which was maximal for inducing TH in neuroblastoma cells[3] elicited a 2.7-fold elevation of TH. In combination, Ro 20-1724 plus dexamethasone elicited a 7.2-fold elevation of TH activity. This increase in the presence of both

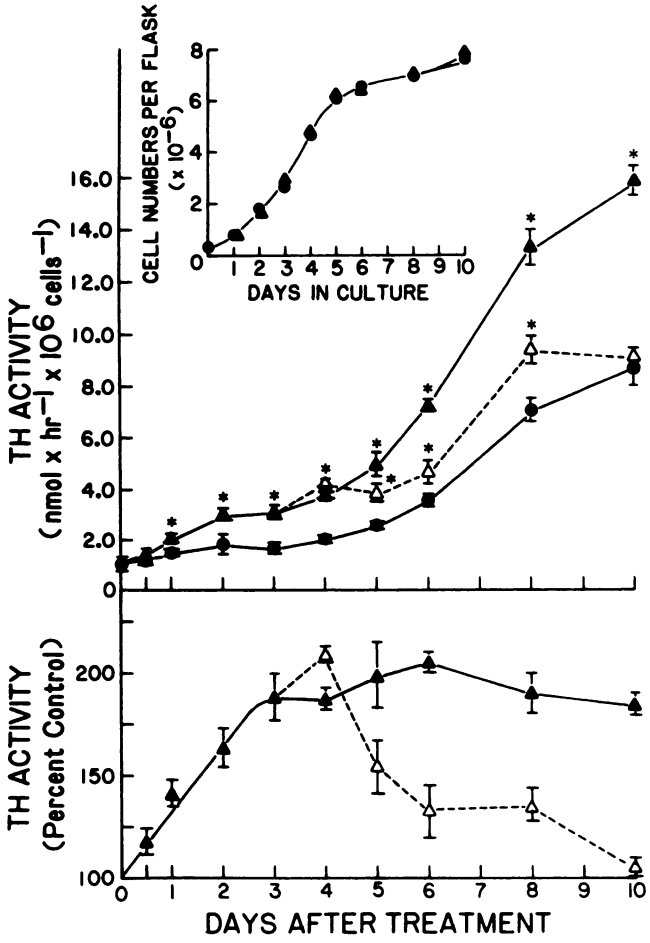

FIG. 1. *Time course of the induction of TH elicited by 10^{-6} M dexamethasone*

The cells were plated at a density of 3×10^5 cells/flask (25 cm^2 surface area) and treated 24 hr after plating. The cells required fresh medium 72 hr after treatment was initiated; thus, the medium (along with dexamethasone when appropriate) was changed daily beginning after 72 hr of treatment. The cells were treated as follows: ●, control (34 mM ethanol); ▲, 10^{-6} M dexamethasone (dissolved in ethanol); △, 10^{-6} M dexamethasone for 3 days. The cells were harvested and assayed for TH activity as described under Methods. Each point represents the mean ± standard error of the mean for three to six flasks. The *inset* shows the rate of cell division for cells treated with 34 mM ethanol (●) or 10^{-6} M dexamethasone (▲). Top panel, activity of TH, expressed as nanomoles × hr^{-1} × 10^6 $cells^{-1}$ versus time. Bottom panel, activity of TH expressed as percentage of control versus time. * $p < 0.05$ for cells treated with dexamethasone compared with cells treated with vehicle alone (34 mM ethanol).

[3] A. W. Tank and N. Weiner, unpublished observations.

TABLE 1

Effect of dexamethasone on TH activity in the presence and absence of either 8-bromo-cyclic AMP or compounds that elevate intracellular cyclic AMP

Cells were plated at a density of 3×10^5 cells/flask (25 cm^2 surface area) and treated with the indicated compounds 24 hr later. All compounds except 8-bromo-cyclic AMP were dissolved in ethanol and added in a volume of 20 μl to the flasks which contained 10 ml of medium. 8-Bromo-cyclic AMP was dissolved directly in medium at the proper concentration. All flasks contained 34 mM ethanol. The cells were harvested after 72 hr of treatment and assayed for TH activity. The values represent the mean ± standard error of the mean of three flasks.

Treatment	TH activity	
	pmoles × $hr^{-1} \times 10^6$ $cells^{-1}$	Fold increase
Control (34 mM ethanol)	514 ± 70	—
Dexamethasone (10^{-6} M)	722 ± 17[a]	1.4 ± 0.1
Ro 20-1724 (5×10^{-5} M)	1372 ± 121[b]	2.7 ± 0.2
Ro 20-1724 (5×10^{-5} M) + dexamethasone (10^{-6} M)	3697 ± 643[b]	7.2 ± 1.3
PGE_1 (10^{-6} M)	1591 ± 79[b]	3.1 ± 0.2
PGE_1 (10^{-6} M) + dexamethasone (10^{-6} M)	6377 ± 870[b]	12.0 ± 1.7
8-Bromo-cyclic AMP (10^{-3} M)	1839 ± 230[b]	3.6 ± 0.5
8-Bromo-cyclic AMP (10^{-3} M) + dexamethasone (10^{-6} M)	3154 ± 447[b]	6.1 ± 0.9
PGE_1 (10^{-6} M) + Ro 20-1724 (5×10^{-5} M)	4193 ± 475[b]	8.2 ± 0.9
PGE_1 (10^{-6} M) + Ro 20-1724 (5×10^{-5} M) + dexamethasone (10^{-6} M)	9380 ± 401[b]	18.0 ± 0.8

[a] $p < 0.05$ for treated cells as compared with controls.
[b] $p < 0.01$ for treated cells as compared with controls.

inducing agents was clearly greater than the sum of the increases observed in the presence of either agent alone. This greater than additive effect was also observed when cells were treated with dexamethasone in combination with either PGE_1 or 8-bromo-cyclic AMP. Simultaneous treatment of the cells with PGE_1 plus Ro 20-1724 elicited a 30-fold increase in cyclic AMP levels (7). This treatment produced the greatest induction of TH, which can reasonably be attributed entirely to elevations in cyclic AMP, that we have observed. Nevertheless, simultaneous treatment with dexamethasone still produced a greater than additive elevation of TH activity in the presence of these two compounds (8.2-fold increase versus 18-fold increase) (Table 1).

Immunotitration studies on neuroblastoma cells treated with dexamethasone in the presence and absence of Ro 20-1724. A fixed concentration of antiserum to TH was mixed with different volumes of supernatant derived from homogenates of neuroblastoma cells treated with 34 mM ethanol (as control), dexamethasone (10^{-6} M), Ro 20-1724 (5×10^{-5} M), or dexamethasone (10^{-6} M) plus Ro 20-1724 (5×10^{-5} M), and TH activity was measured in the supernatants of these mixtures following immunoprecipitation as described under Methods. The point at which the lines generated from these values of TH activity intersect the x-axis provides a relative measurement of the amount of enzyme protein present in the supernatant. As seen in Fig. 2 and Table 2, the increases in enzyme protein in the cells after the different treatments correspond well with the observed increases in the activity of TH. Thus, the increases in activity described in the previous sections appear to represent increases in the intracellular content of the enzyme.

Dose-response curve for dexamethasone. TH activity was measured after 3 days of treatment with different concentrations of dexamethasone. No effect was observed with 10^{-9} M dexamethasone; however, 10^{-8} M dexamethasone elicited a maximal induction of TH (Fig. 3). In the presence of Ro 20-1724, the effect of the glucocorticoid analogue was amplified; however, the concentration-effect relationship for dexamethasone was the same in the presence or absence of the PDE inhibitor; i.e., there was no right or left shift in the curve.

Although it is likely that there are steroids present in the serum, their contribution to the observed dexamethasone-mediated induction of TH apparently is minimal. We base this conclusion on the fact that we obtain a response to the glucocorticoid analogue at a relatively low concentration. If high levels of serum-derived glucocorticoids were present in the medium, we would not expect to observe an increase in TH activity in dexamethasone-treated cells at so low a concentration of the added steroid, because the cells would already be maximally stimulated by the serum-derived glucocorticoids. Likewise, if other steroids which interfere with the glucocorticoid-mediated response were present in large concentrations in the serum, we would not expect to see the dexamethasone-mediated induction of TH at such low concentrations of dexamethasone. The dose-response curve for dexamethasone seen in Fig. 3 is similar to that reported in several other studies on dexamethasone-me-

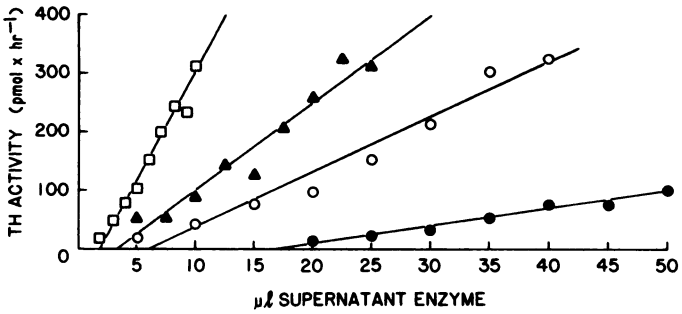

FIG. 2. *Immunotitration of TH isolated from cells treated with dexamethasone in the presence and absence of Ro 20-1724*

Cells were plated at a density of 5×10^6 cells/flask (150 cm^2 surface area) and treated 24 hr later as follows: ●, 34 mM ethanol; ○, 10^{-6} M dexamethasone; ▲, 5×10^{-5} M Ro 20-1724; □, 10^{-6} M dexamethasone plus 5×10^{-5} M Ro 20-1724. Both Ro 20-1724 and dexamethasone were dissolved in ethanol. The cells were harvested after 72 hr of treatment and homogenized, and immunotitrations were performed on the cell supernatants as described under Methods. The data represent the values of TH activity present in the supernatant after centrifugation of the Pansorbin-treated suspension. The data were fit to straight lines by linear regression analysis. The original enzyme activities in the supernatants prior to treatment with antiserum, expressed as picomoles × $hr^{-1} \times 10^6$ $cells^{-1}$, were as follows: control, 603; dexamethasone, 1646; Ro 20-1724, 3011; Ro 20-1724 plus dexamethasone, 7248. There was no loss of activity in enzyme supernatants which were incubated initially for 30 min in the presence of control serum and subsequently treated with Pansorbin.

TABLE 2

Effect of dexamethasone and Ro 20-1724 on TH activity and TH protein levels

The data presented are the same as those seen in Fig. 2. The equivalence points represent the *x*-intercepts of the lines seen in Fig. 2.

Treatment	TH activity (pmoles/hr × 10^3 cells)	Fold increase in activity	Equivalence point (*x*-intercept)	Fold increase in TH protein
Control (34 mM ethanol)	603	—	16.8	—
Dexamethasone (10^{-6} M)	1646	2.7	6.1	2.7
Ro 20-1724 (5×10^{-5} M)	3011	5.0	3.6	4.7
Dexamethasone (10^{-6} M) + Ro 20-1724 (5×10^{-5} M)	7248	12.0	1.7	10.0

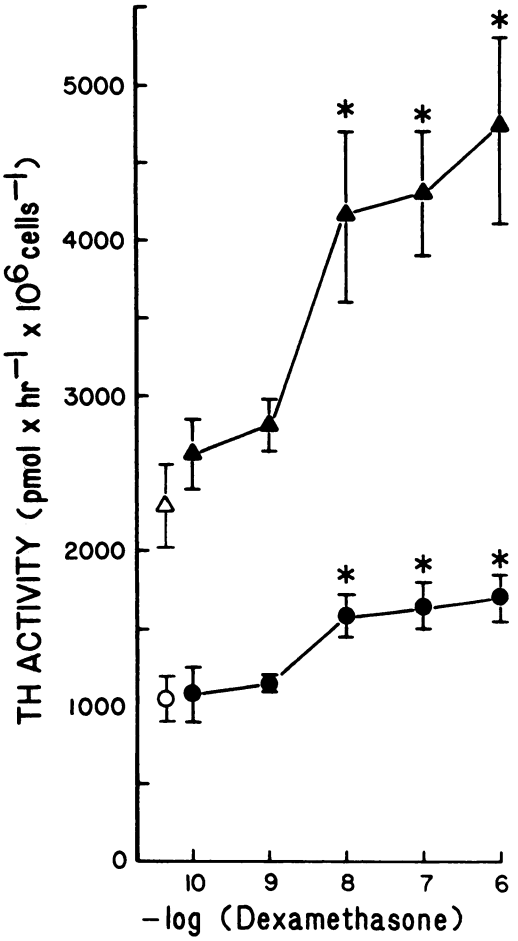

FIG. 3. *Effect of different concentrations of dexamethasone on TH activity in the presence or absence of Ro 20-1724*

The cells were plated at a density of 3×10^5 cells/flask and treated 24 hr later with different concentrations of dexamethasone (dissolved in ethanol) in the presence (▲) or absence (●) of 5×10^{-5} M Ro 20-1724. Cells were harvested and assayed for TH activity 72 hr after treatment was initiated as described under Methods. Each point represents the mean ± standard error of the mean of four to six flasks. The *open symbols* represent the values of TH in cells not treated with dexamethasone. * $p < 0.05$ for cells treated with dexamethasone compared with control cells.

diated induction of enzymes in cells incubated in the presence (10, 23) and absence (24) of serum. Furthermore, using rat pheochromocytoma tumor in organ culture, Lucas and Thoenen (10) have shown that the presence of 5% fetal calf serum has no effect on the dose-response curve for the dexamethasone-mediated induction of TH; this dose-response curve is very similar to that presented in Fig. 3. Even though well-defined culture medium for several cell lines is readily available, we have not yet found a suitable serum-free medium which supports the growth and maintains the adrenergic properties of the NBP_2 cells.

Duration of treatment with dexamethasone required to elicit the induction of TH. In the case of the cyclic AMP-mediated induction of TH, persistent elevation of cyclic AMP elicited a much larger induction of TH than that seen after short-term increases in cyclic AMP (8). However, it was possible to elicit a long-lasting induction of the enzyme (approximately 2-fold after 48 hr) following a 2-hr treatment with Ro 20-1724 or 8-bromo-cyclic AMP (8). We were interested in determining whether prolonged treatment of the cells with dexamethasone was required for the induction of TH and/or whether a short-term treatment of a few hours could trigger a long-lasting response in the cells. We treated cells in different flasks with dexamethasone for 2, 6, 12, 24, and 72 hr and measured TH activity in all the flasks 72 hr after treatment was initiated. The results of this experiment indicate that the continual presence of the glucocorticoid analogue for longer than 24 hr was necessary in order to demonstrate the induction at 72 hr (Fig. 4). A similar dependence on the presence of dexamethasone was seen when Ro 20-1724 was present in the medium for the entire 72 hr (Fig. 4).

Effect of different steroids on TH activity. To determine whether the induction of TH was elicited by only the glucocorticoid class of steroid hormones, we treated the cells with different steroids for 3 days and measured TH activity. The glucocorticoid compounds, hydrocortisone and triamcinolone (both at 10^{-6} M), elicited the induction of TH (Table 3). Greater than additive responses were observed when these compounds were added along with Ro 20-1724 (Table 3). Treatment of the cells with progesterone, testosterone, and estradiol (all at 10^{-6} M) did not significantly elevate TH activity in the presence or absence of Ro 20-1724. Progesterone is also a known competitive inhibitor of glucocorticoid-mediated responses (25). This inhibition is due to its properties as an antagonist of the cytosolic glucocorticoid receptor (25). A 100-fold excess of progesterone completely blocked the dexamethasone-mediated induction of TH in the NBP_2 cells (Table 3). This blockade of the glucocorticoid-mediated effect occurred in the presence or absence of Ro 20-1724. However, progesterone did not significantly affect the cyclic AMP-mediated induction of TH elicited by Ro 20-1724 (Table 3). These results demonstrate that the induction of TH by dexamethasone is not a nonspecific steroid effect, is elicited only by the

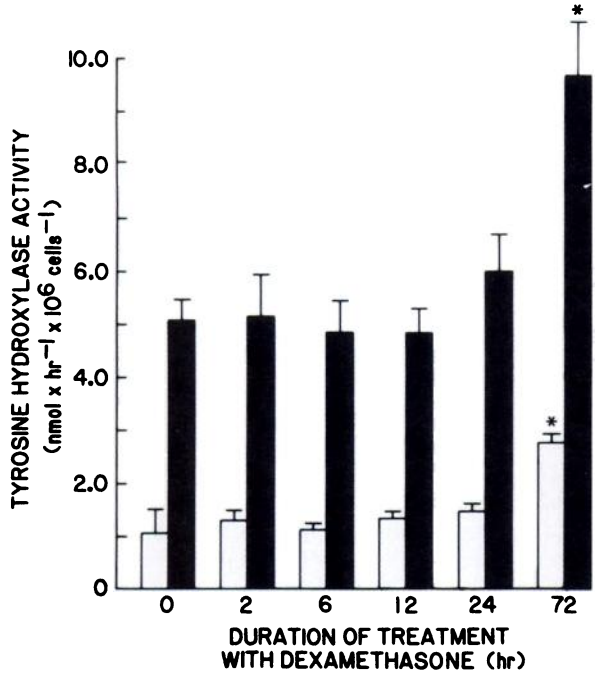

FIG. 4. *Dependence of the induction of TH on the duration of treatment with dexamethasone in the presence or absence of Ro 20-1724*

Cells were plated at a density of 3×10^5 cells/flask and treated 24 hr later with 10^{-6} M dexamethasone in the presence (*solid bars*) or absence (*open bars*) of 5×10^{-5} M Ro 20-1724. The medium was removed from flasks at the designated times and replaced with fresh medium lacking dexamethasone. When appropriate, Ro 20-1724 was added to the fresh medium in order to maintain elevated cyclic AMP levels throughout the experiment. The medium was also replaced in control flasks at each time point. All of the cells were harvested after 72 hr of treatment and assayed as described under Methods. Each point represents the mean ± standard error of the mean of five flasks. * $p < 0.01$ for cells treated with dexamethasone compared with those cells not treated with dexamethasone.

glucocorticoid class of steroids, and most likely is due to the interaction of the glucocorticoid with the cytosolic glucocorticoid receptor (25, 26).

Effect of dexamethasone on total RNA synthesis. We tested the effect of dexamethasone on the rate of incorporation of [^{3}H]uridine into total trichloroacetic acid-precipitable RNA (Table 4). The amount of radioactivity recovered in the trichloroacetic acid pellets after a 30-min [^{3}H]uridine pulse varied dramatically depending upon the length of time between treatment of the cells with inducing agents or control vehicle and the time of the addition of [^{3}H]uridine to the medium. However, dexamethasone in the presence or absence of Ro 20-1724 did not consistently increase the incorporation of [^{3}H]uridine into trichloroacetic acid-precipitable RNA. The radioactivity recovered in the trichloroacetic acid-soluble fraction, representing unincorporated [^{3}H]uridine, was also unaffected by dexamethasone or Ro 20-1724 (data not shown). Therefore, the induction of TH by dexamethasone does not appear to be a consequence of an increase in total cellular RNA synthesis, but is most likely due to an effect of dexamethasone on a few select genes.

Effect of actinomycin D on the dexamethasone-mediated induction of TH. In preliminary studies in which the effects of actinomycin D on the cyclic AMP-mediated induction of TH were examined, we observed that, when cells which were cultured for 4 days prior to treatment were used, the rate of the induction of TH by cyclic AMP was maximized over a 12-hr period, and, during this time, the addition of actinomycin D produced minimal cellular toxicity.[3] In these studies we also showed that actinomycin D at a concentration of 5 μg/ml inhibited RNA synthesis by greater than 95% within 1 hr after its addition to the medium. There was no effect on cell morphology until 12 hr after treatment. Thus, we used the same experimental protocol to test the effect of actino-

TABLE 3

Effect of different steroids on TH activity in the presence and absence of Ro 20-1724

Cells were plated at a density of 3×10^5 cells/flask and treated 24 hr later with the indicated compounds. All of the steroids and Ro 20-1724 were dissolved in ethanol, and 20 μl of the concentrated solution were added to 10 ml of medium, resulting in a final concentration of 34 mM ethanol in the medium. All flasks contained this concentration of ethanol. All steroids were present at a final concentration of 10^{-6} M, unless otherwise indicated, and Ro 20-1724 was present in a final concentration of 5×10^{-5} M. TH activity was measured after 72 hr of treatment as described under Methods. The values are expressed as means ± standard error of the mean.

Steroid	TH activity					
	Without Ro 20-1724			With Ro 20-1724		
	N^a	pmoles/hr × 10^6 cells	Fold increase	N	pmoles/hr × 10^6 cells	Fold increase
None	9	1431 ± 266	—	9	3692 ± 531	2.6 ± 0.4
Dexamethasone	9	2899 ± 100[b]	2.0 ± 0.1	9	7308 ± 954[b]	5.1 ± 0.7
Hydrocortisone	9	2700 ± 105[b]	2.1 ± 0.2	6	6730 ± 1081[c]	4.7 ± 0.7
Triamcinolone	9	3284 ± 230[b]	2.1 ± 0.1	6	8760 ± 1341[b]	6.1 ± 0.9
Progesterone	9	1593 ± 84	1.1 ± 0.1	9	3524 ± 470	2.5 ± 0.3
β-Estradiol	9	1680 ± 85	1.2 ± 0.1	6	3271 ± 296	2.3 ± 0.2
Testosterone	9	1676 ± 72	1.2 ± 0.1	6	3627 ± 332	2.5 ± 0.2
Dexamethasone (10^{-7} M)	6	2983 ± 156[b]	2.1 ± 0.1	6	6468 ± 576[b]	4.5 ± 0.4
Progesterone (10^{-5} M)	6	1380 ± 67	1.0 ± 0.1	6	3072 ± 213	2.2 ± 0.4
Dexamethasone (10^{-7} M) + progesterone (10^{-5} M)	6	1744 ± 145	1.2 ± 0.1	6	3317 ± 268	2.3 ± 0.2

[a] Number of flasks used for each condition.
[b] $p < 0.01$.
[c] $p < 0.05$ for cells treated with steroids as compared with cells not treated with steroids.

TABLE 4

Effect of dexamethasone on [^{3}H]uridine incorporation into total trichloroacetic acid-precipitable RNA in the presence and absence of Ro 20-1724

Cells were plated at a density of 3×10^5 cells into flasks containing 15 ml of medium. The medium was removed 4 days later and replaced with 10 ml of fresh medium. Cells were treated at this time with 34 mM ethanol, 10^{-6} M dexamethasone, 5×10^{-5} M Ro 20-1724, or both dexamethasone and Ro 20-1724. Both dexamethasone and Ro 20-1724 were dissolved in ethanol, and all flasks contained a final concentration of 34 mM ethanol. The cells were treated for the times indicated before the addition of [^{3}H]uridine to the medium. The incorporation studies were performed as described under Methods. The values are expressed as the mean ± standard error of the mean of three flasks.

Duration of treatment	cpm recovered in pellet/10^6 cells			
	Control	Dexamethasone	Ro 20-1724	Dexamethasone plus Ro 20-1724
hr				
0.5	4568 ± 64	4105 ± 254	4176 ± 166	3682 ± 241[a]
1.0	5069 ± 282	5279 ± 467	4883 ± 463	4889 ± 181
2.0	6154 ± 201	5363 ± 94[a]	4951 ± 279[a]	5005 ± 887
4.0	9748 ± 318	8178 ± 293[a]	9348 ± 514	7986 ± 388[a]
8.0	1947 ± 29	2209 ± 69[a]	2200 ± 80[a]	2031 ± 144
12.0	2736 ± 240	2747 ± 135	2870 ± 70	2719 ± 151
24.0	2074 ± 29	2403 ± 122	1988 ± 133	1942 ± 79
48.0	1682 ± 61	2034 ± 150	2043 ± 163	2040 ± 90

[a] $p < 0.05$ for cells treated with dexamethasone and/or Ro 20-1724 as compared with controls.

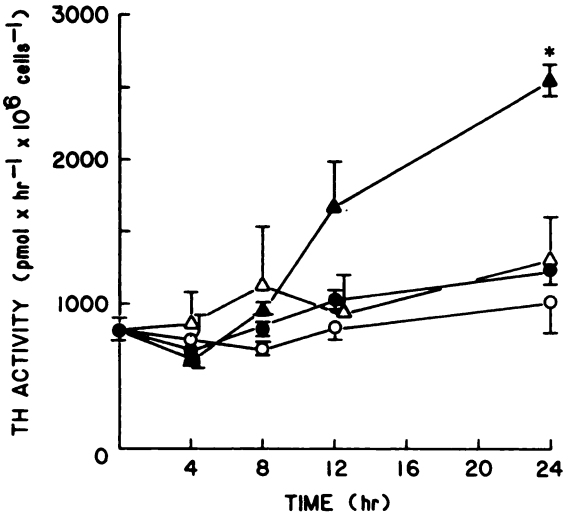

FIG. 5. *Effect of actinomycin D on the dexamethasone-mediated induction of TH*

Cells were plated at a density of 3×10^5 cells/flask (25 cm^2 surface area) containing 15 ml of medium. The medium was removed 4 days later and replaced with 10 ml of fresh medium. Cells were treated at this time with the following: ●, 34 mM ethanol; ○, actinomycin D (5 μg/ml) plus 34 mM ethanol; ▲, 10^{-6} M dexamethasone (dissolved in ethanol); △, 10^{-6} M dexamethasone plus actinomycin D (5 μg/ml). The cells were harvested at the designated times and assayed for TH activity as described under Methods. Each point represents the mean ± standard error of the mean of three or four flasks.

mycin D on the dexamethasone-mediated induction of TH.

No increase in TH activity was observed for 8 hr after treatment of 4-day-old cultures with dexamethasone (Fig. 5). Between 8 and 24 hr, TH activity rose to a level approximately 2-fold greater than that observed in control cells. When actinomycin D was added to the medium simultaneously with dexamethasone, the increase in TH activity normally observed at 12 and 24 hr was completely abolished. These results suggest that the dexamethasone-mediated induction of TH requires the synthesis of an RNA species. This result is consistent with the generally accepted mechanism described for steroid-mediated induction of other proteins (26).

Effect of dexamethasone on cyclic AMP levels in NBP$_2$ cells. Cyclic AMP was measured in the cells following treatment with dexamethasone, Ro 20-1724, or both compounds combined (Table 5). Dexamethasone had no measurable effect on intracellular cyclic AMP levels. The PDE inhibitor Ro 20-1724 increased cyclic AMP, as shown previously (7). Even in the presence of the PDE inhibitor, however, dexamethasone did not elevate cyclic AMP above the level observed in the presence of Ro 20-1724 alone.

DISCUSSION

Glucocorticoids have been shown to elevate TH activity in the rat SCG, in rat pheochromocytoma cells, and in mouse neuroblastoma cells (9–11, 15). In the present study, we observed that glucocorticoids induce TH in the mouse neuroblastoma cell line, NBP$_2$, and that this induction is enhanced by simultaneous treatment with cyclic AMP analogues or compounds which elevate intracellular cyclic AMP.

The glucocorticoid analogue dexamethasone elevates TH activity approximately 1.5- to 2-fold in the neuroblastoma cells. Immunotitration studies indicate that this increase in activity is due to an increase in enzyme protein. The dexamethasone-mediated induction of TH is dependent upon the continual presence of the glucocorticoid in the culture medium. A maximal induction is achieved after 3 days of continuous treatment. We have previously observed analogous results with cyclic AMP; a maximal induction of TH is not achieved unless cyclic AMP is continuously elevated in neuroblastoma cells for at least 5 days (7).

If after 3 days of treatment the medium containing the glucocorticoid analogue is removed and replaced with fresh medium lacking dexamethasone, TH activity remains constant for 1 day and then slowly decreases to control levels, with a half-life of approximately 1.6 days.

TABLE 5

Effect of dexamethasone on cyclic AMP levels in NBP_2 cells in the presence and absence of Ro 20-1724

Cells were plated at a density of 5×10^5 cells/dish (60 mm) and treated 24 hr later with the indicated compounds. All flasks contained a final concentration of 34 mM ethanol. Cells were harvested by aspiration of the medium and the immediate addition of 2 ml of 5% trichloroacetic acid to the dish. The cells were scraped off the dish with a rubber policeman. The suspension was centrifuged, and the supernatant was assayed for cyclic AMP as described under Methods. The values are expressed as the mean ± standard error of the mean of three dishes.

Treatment	Cyclic AMP at the specified time after treatment was begun (pmoles/10^6 cells)			
	15 min	1 hr	12 hr	24 hr
Control (34 mM ethanol)	10.9 ± 0.9	12.2 ± 1.1	14.8 ± 1.6	8.9 ± 0.2
Dexamethasone (10^{-6} M)	11.8 ± 2.4	12.8 ± 1.0	14.2 ± 1.6	8.8 ± 0.3
Ro 20-1724 (5×10^{-5} M)	13.4 ± 0.2	19.9 ± 2.2[a]	18.6 ± 1.3	27.0 ± 5.0[a]
Dexamethasone (10^{-6} M) + Ro 20-1724 (5×10^{-5} M)	13.4 ± 0.6	13.9 ± 0.2	23.0 ± 2.2[a]	27.1 ± 3.7[a]

[a] $p < 0.05$ for treated cells as compared with controls.

Interestingly, this kinetics of deinduction differs slightly from the kinetic data observed following the removal (after 3 days of treatment) of the PDE inhibitor Ro 20-1724 and the subsequent decrease in cyclic AMP to basal levels.[3] In the latter case TH activity remains constant for 2 days and then decreases, with a half-life of 2.3 days.

Another difference between the cyclic AMP-mediated induction and the glucocorticoid-mediated induction of TH is the extent of induction observed following short-term treatment of the cells with the inducing agents. Even though cyclic AMP must be elevated for 5 days to achieve maximal induction of TH, we have shown that a 1- to 2-hr elevation of cyclic AMP triggers a limited induction which can be observed 48 hr following the increase in cyclic AMP (8). However, short-term treatment of the cells with dexamethasone does not trigger a significant, long-lasting induction of TH. The glucocorticoid must be continuously present in the medium for the induction to proceed. These results are in contrast to the studies of other investigators (9, 10), who have shown that a 4-hr treatment with dexamethasone elicits a maximal induction of TH in the SCG and in pheochromocytoma cells.

The action of steroid hormones is generally believed to be mediated by the interaction of the steroid with a cytosolic receptor (26). This hormone-receptor complex is then translocated to the nucleus, where it binds to the chromatin and presumably increases the rate of transcription of a few select genes. Our results are consistent with the above model for steroid action. We cannot unequivocally state that a cytosolic glucocorticoid receptor is involved in the induction of TH; however, the response to dexamethasone is specific to the glucocorticoid class of steroids, and it can be blocked by progesterone, a known antagonist of the glucocorticoid receptor (25). Actinomycin D totally blocks the dexamethasone-mediated induction of TH, suggesting that RNA synthesis is required for the induction to occur. However, total RNA synthesis is not elevated by dexamethasone in the presence or absence of Ro 20-1724, suggesting that the glucocorticoid causes an increase in the rate of transcription of only a small number of genes in the neuroblastoma cells. We have no evidence as yet that an increased rate of transcription of the gene coding for TH occurs following dexamethasone treatment; however, a recent report suggests that there is an increase in functional mRNA for TH after dexamethasone treatment of PC12 cells (27).

When neuroblastoma cells are treated with dexamethasone plus agents that elevate cyclic AMP, the resulting increase in TH is greater than the sum of the increases elicited by either inducing agent independently. An enhancement of the glucocorticoid-mediated induction of TH has been described in two other situations. Drugs which directly or indirectly cause a prolonged stimulation of nicotinic receptors on the adrenergic cell bodies of the SCG amplify the glucocorticoid-mediated induction of TH (9, 11). In addition, the glucocorticoid-mediated induction of TH is enhanced in SCG organ cultures when NGF is also present (12). It is possible that the effects of prolonged nerve stimulation and NGF are mediated by cyclic AMP, and that the enhancement of the glucocorticoid-mediated induction of TH by these treatments is analogous to that described in the present study. Cyclic AMP is elevated in the adrenal medulla following stimulation of this organ with nicotinic agonists (4). A number of recent reports indicate that cyclic AMP is elevated following treatment of target cells with NGF (13, 14). Furthermore, analogues of cyclic AMP mimic some of the effects of NGF in certain model systems (28, 29). However, the effects of nerve stimulation and NGF on cyclic AMP remain controversial. Otten *et al.* (30) have shown that cyclic AMP is not elevated in the SCG following long-term stress. Furthermore, other investigators have concluded that the actions of NGF are not mediated by cyclic AMP (31, 32).

The enhancement of the cyclic AMP-mediated induction of TH by glucocorticoids in neuroblastoma cells is most likely not caused by a greater increase in cyclic AMP elicited by the glucocorticoid. In neuroblastoma cells, dexamethasone does not elevate cyclic AMP, even in the presence of a PDE inhibitor. Furthermore, in the presence of PGE_1 plus Ro 20-1724, cyclic AMP levels are increased 30-fold (7) and TH activity is increased 8-fold after 3 days of treatment. This induction is the greatest that we have observed with agents that presumably act by elevating cyclic AMP. In contrast, cyclic AMP levels are increased only 2- to 3-fold in the presence of Ro 20-1724 alone (Table 5; ref. 7), yet TH activity is increased approximately 3-fold after 3 days of treatment. The relative changes in cyclic AMP levels and TH activity in cells treated with Ro 20-1724 alone or with PGE_1 plus Ro 20-1724 suggest that the 30-fold increase in cyclic

AMP that occurs in cells treated with PGE_1 plus Ro 20-1724 maximally stimulates the cyclic AMP-mediated induction mechanism. Yet dexamethasone further enhances the cyclic AMP-mediated induction when added along with PGE_1 plus Ro 20-1724. In addition, the kinetics of deinduction and the effects of short-term treatment of the cells with either inducing agent might be expected to be identical, if they were operating through a common mechanism. This does not appear to be the case.

A greater than additive response to the simultaneous presence of dexamethasone and elevated cyclic AMP has been observed for the induction of several other enzymes (33). However, the mechanism(s) behind this effect is not understood. Both inducing agents have been shown to increase functional mRNA levels for several proteins (33). Thus, different sites of action for glucocorticoids and cyclic AMP have been postulated to account for the fact that each compound acts to increase mRNA levels and, in combination, they act synergistically to increase enzyme protein. According to this hypothesis, glucocorticoids apparently act to increase the rate of transcription of the gene coding for a protein, whereas cyclic AMP acts either to increase the rate of the post-transcriptional processing of the mRNA or to decrease the rate of degradation of the mRNA coding for the protein (33, 34). It has also been shown that cyclic AMP increases the rate of translation of mRNA for tyrosine aminotransferase (35). Alternatively, one inducing agent may regulate the initial effector of the other inducing agent. It has been shown that steroid hormones influence the level of Type I cyclic AMP-dependent protein kinase in various tissues (36). Conversely, it is possible that cyclic AMP may regulate the level of the glucocorticoid receptor (33). Further study is required to elucidate the mechanism(s) behind the interaction of cyclic AMP and glucocorticoids in the induction of TH in neuroblastoma cells.

Williams *et al.* (15) recently reported that TH activity increases in NBP_2 cells in response to treatment with glucocorticoids. However, we observed that the induction is maximal with the use of 10^{-8} M dexamethasone, whereas in the study by Williams *et al.* (15), 25 μM dexamethasone was required to elicit a significant increase in TH activity, and even 100 μM dexamethasone did not produce a maximal induction. The latter workers (15) observed that, even at these high concentrations of steroid hormone, the effect on TH activity is specific to the glucocorticoid class of steroids. Nevertheless, in most studies concerning glucocorticoid-mediated enzyme induction, the effective concentration of glucocorticoid has been found to be in the range of 10^{-9}–10^{-7} M (9, 10, 23–27, 34, 35).

In summary, we have shown that glucocorticoids elicit an induction of TH in neuroblastoma cells. This induction is dependent upon the continual presence of the glucocorticoid in the culture medium. Only the glucocorticoid class of steroids elicits the induction. Although RNA synthesis appears to be required for the induction to occur, no effect of the glucocorticoid is observed on total RNA synthesis. When the cells are treated simultaneously with dexamethasone plus cyclic AMP analogues or agents which elevate intracellular cyclic AMP, a greater than additive induction of TH is observed. This interaction between glucocorticoids and cyclic AMP may be analogous to the glucocorticoid-mediated enhancement of the NGF-mediated and/or stress-related transsynaptic induction of TH in other tissues.

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