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Induction of Tyrosine Hydroxylase by Cyclic AMP and Glucocorticoids in a Rat Pheochromocytoma Cell Line: Effect of the Inducing Agents Alone or in Combination on the Enzyme Levels and Rate of Synthesis of Tyrosine Hydroxylase

A. WILLIAM TANK, LUCINDA HAM, and PAMELA CURELLA

Department of Pharmacology, University of Colorado Health Sciences Center, Denver, Colorado 80262

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SUMMARY

The enzymatic activity of tyrosine hydroxylase (EC 1.14.16.2) increases in rat pheochromocytoma PC18 cells exposed to either elevated levels of cyclic AMP or glucocorticoids. The cyclic AMP-mediated increase in activity is elicited by cyclic AMP analogs or by compounds which activate adenylate cyclase or inhibit phosphodiesterase. The glucocorticoid-mediated increase is elicited only by glucocorticoid steroid hormones; nonglucocorticoid steroid hormones have no effect on tyrosine hydroxylase. In PC18 cells exposed simultaneously to both cyclic AMP-elevating agents and glucocorticoids, the increase in tyrosine hydroxylase activity is greater than that observed in cells treated with optimal concentrations of either inducing agent alone. Immunochemical titration experiments demonstrate that the increases in tyrosine hydroxylase activity observed in cells treated with the cyclic AMP analog, 8-bromocyclic AMP, and/or the synthetic glucocorticoid, dexamethasone, are due to increases in enzyme protein. Time course studies show that in cells treated with either 8-bromocyclic AMP or dexamethasone, the enzyme level increases slowly to a level 5–7-fold greater than that observed in untreated cells after 4 days of treatment. In cells treated with both of these inducing agents simultaneously, the enzyme level increases to a level 10–12-fold greater than that observed in control cells after 4 days of treatment. This additive increase in activity in cells treated with both inducing agents is observed at all time points. The rates of synthesis and degradation of tyrosine hydroxylase have also been measured in PC18 cells, using an antiserum to tyrosine hydroxylase to rapidly isolate radiolabeled enzyme from cells that have been incubated in the presence of [^{3}H]leucine. The apparent half-life of tyrosine hydroxylase in the PC18 cells is approximately 30 hr. In PC18 cells incubated in the presence of radiolabeled leucine for 60 min, 0.2–0.3% of the total soluble protein synthesized is identified as tyrosine hydroxylase. In cells treated with either 8-bromocyclic AMP or dexamethasone for 24 hr, there is a 6–8-fold increase in the rate of synthesis of the enzyme. In cells treated with both inducing agents simultaneously, there is a 10–12-fold increase in the rate of synthesis; thus, the additive increase in enzyme level observed in cells treated with both inducing agents is paralleled by an additive increase in the rate of synthesis of the enzyme in these cells. Time course studies on the changes in the rate of synthesis of the enzyme demonstrate that the rate of synthesis increases relatively rapidly, approaching a new steady state level after 12 hr of treatment with these inducing agents, and that the additive increase in the rate of synthesis in cells treated with both inducing agents is observed at all time points. Our studies suggest that cyclic AMP and glucocorticoids induce tyrosine hydroxylase in PC18 cells by rapidly increasing its rate of synthesis and that, in the presence of both inducing agents, the rate of synthesis and the enzyme level of tyrosine hydroxylase increase additively.

The level of tyrosine hydroxylase [tyrosine 3-monooxygenase: L-tyrosine, tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating); EC 1.14.16.2], the enzyme which catalyzes the rate-limiting step in the biosynthesis of the catecholamines, increases in the chromaffin cells of the adrenal medulla, in sympathetic ganglia, and in certain catecholaminergic neurons of the brain following prolonged stimulation of these cells by stress or treatment with catecholamine-depleting drugs, like reserpine (1–3). In the periphery this increase can be blocked by surgical transection of the nerve fibers presynaptic to the catecholaminergic cells and by nicotinic receptor antagonists (4, 5). The induction is also blocked by the prior administration of actinomycin D to the animals (6). Recently, we have demonstrated that prolonged stress or reserpine administration

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ABBREVIATIONS: mRNATH, the RNA coding for tyrosine hydroxylase; PBS, phosphate-buffered saline (0.15 M NaCl/0.01 M potassium phosphate, pH 7.4); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; TES, *N*-Tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Bis-Tris, bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane; SDS, sodium dodecyl sulfate.

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increases the level of mRNA coding for tyrosine hydroxylase in rat adrenal glands (7). The evidence cited above supports the hypothesis that prolonged stimulation of catecholaminergic neurons results in the increased transcription of the gene for tyrosine hydroxylase, leading to increased levels of $mRNA^{TH}$ and subsequently increased tyrosine hydroxylase enzyme levels. However, other mechanisms, such as effects on the processing, transport, or stability of the $mRNA^{TH}$ or effects on the rates of synthesis or degradation of the tyrosine hydroxylase enzyme itself cannot be ruled out.

The intracellular messengers which mediate the effects on the levels of tyrosine hydroxylase and $mRNA^{TH}$ have not been unequivocally established. However, two compounds which have been implicated in this response are cyclic AMP and glucocorticoids (8–15). Increases in cyclic AMP and cyclic AMP-dependent protein kinase activity have been correlated in *in vivo* studies with the stress-related, transsynaptic induction of tyrosine hydroxylase in the adrenal medulla (8). Furthermore, cyclic AMP analogs or compounds which elevate intracellular cyclic AMP levels elicit the induction of the enzyme in mouse neuroblastoma cells (9, 10) and in rat pheochromocytoma cells in culture (11). Glucocorticoids have been shown to act as modulators of the transsynaptic induction of tyrosine hydroxylase *in vivo* (12) and to produce increases in the level of the enzyme in organ cultures of rat superior cervical ganglion (13) and in cell cultures of rat pheochromocytoma (11, 14) and mouse neuroblastoma (15). Recently, we have shown that the $mRNA^{TH}$ also increases in response to cyclic AMP and glucocorticoids in a rat pheochromocytoma cell line (11). However, no studies have yet been performed which quantitatively and temporally relate the changes in enzyme activity elicited by these inducing agents with changes in the rate of synthesis of the enzyme or which demonstrate that the increases in $mRNA^{TH}$ elicited by these inducers result in quantitatively equal increases in the rate of synthesis of the enzyme in the intact cells.

During stress, catecholaminergic cells in the periphery may be exposed to increased concentrations of both cyclic AMP and glucocorticoids. Thus, the interaction between these two hormones probably plays a role in the regulation of this enzyme *in vivo*. In mouse neuroblastoma cells simultaneous treatment of the cells with both cyclic AMP analogs and glucocorticoids results in an increase in tyrosine hydroxylase which is greater than the sum of the increases observed in cells treated with either inducing agent alone (15). However, the regulation of the enzyme by these two inducing agents in combination has not been studied in other catecholaminergic model systems, and the mechanisms involved in their combined effects on tyrosine hydroxylase have not been investigated.

In the present report we characterize further the induction of tyrosine hydroxylase produced by either cyclic AMP or glucocorticoids using PC18 cells, a subclonal cell line derived from the rat pheochromocytoma PC12 cells. We demonstrate that the induction is elicited by cyclic AMP analogs or compounds which raise intracellular cyclic AMP levels, and by the glucocorticoid class of steroid hormones. We also show that when cells are incubated in the presence of both cyclic AMP and glucocorticoid, the increase in tyrosine hydroxylase activity is approximately equal to the sum of the increases in activity observed in the presence of either inducing agent alone. Furthermore, we show that the increases in tyrosine hydroxylase activity elicited by these inducing agents alone or in combination are due to increases in enzyme protein, and that these increases are associated with increases in the rate of synthesis of the enzyme. In the accompanying paper (16) we relate the changes in these parameters with the changes in $mRNA^{TH}$ elicited by these inducing agents in the PC18 cells.

Experimental Procedures

Materials

Tissue culture flasks and dishes were obtained from Falcon Plastics Co. (Los Angeles, CA). RPMI 1640 medium was obtained from GIBCO Laboratories (Grand Island, NY). Horse serum was purchased from KC Biologicals, Inc. (Lenexa, KS), and fetal bovine serum was from Sterile Systems, Inc. (Logan, UT). L-[^{14}C]Tyrosine and L-[4,5-^{3}H(N)] leucine were obtained from New England Nuclear Corp. (Boston, MA). D,L-6-methyl-5,6,7,8-tetrahydropterin was purchased from Calbiochem-Behring (San Diego, CA), and catalase was from Boehringer-Engelheim, Ltd. (Elmsford, NY). 8-Bromocyclic AMP, forskolin, cholera toxin, and the steroid hormones were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of the highest purity available from commercial sources.

Antiserum to tyrosine hydroxylase was prepared in rabbits using tyrosine hydroxylase purified from rat pheochromocytoma tumor as an antigen as described elsewhere (17).

Methods

Cell culture conditions. The PC18 cells are a subclone of the rat pheochromocytoma PC12 cell line (18) and were isolated as described elsewhere (see Ref. 24). The cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 50 μg/ml of streptomycin, and 50 units/ml of penicillin. Cultures were maintained at 36° in a water-saturated atmosphere containing 95% air and 5% CO_2. The doubling time of the cells was 25–30 hr.

Induction studies. For induction experiments the cells were subcultured in 60-mm dishes in the medium containing complete serum as described above at a cell density of 2–5 × 10^4 cells/cm^2. The cells were incubated for 12–24 hr and then the medium was removed and replaced with RPMI 1640 medium supplemented with horse serum (10%) and fetal calf serum (5%) that were first dialyzed exhaustively against PBS containing 1 mg/ml of Norit A charcoal. The cells were cultured in the presence of this medium containing dialyzed serum for at least 2 days prior to the addition of the inducing agents. The cells were then treated with the steroid hormones or the cyclic AMP-elevating compounds.

Tyrosine hydroxylase assays. At different times after treatment with the inducing agents, the medium was removed by aspiration and the cells were washed once with 3 ml of ice-cold PBS. The cells were then scraped into ice-cold PBS using a rubber policeman and pelleted by centrifugation at 2,500 × *g* for 5 min. The cell pellets were then frozen at −90°. When tyrosine hydroxylase activity was to be assayed, the cell pellets were homogenized in a buffer containing 20 mM potassium phosphate (pH 7.5), 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, and 50 μg/ml of leupeptin. The homogenate was then centrifuged at 20,000 × *g* for 15 min, and the supernatant was assayed for tyrosine hydroxylase activity. The homogenization liberated greater than 90% of the tyrosine hydroxylase activity into the supernatant. In preliminary experiments we subjected the supernatants to gel filtration using Sephadex G-50 columns as described by Tank *et al.* (17), to remove endogenous catecholamines and other small molecules which might influence the enzyme assay. This step was necessary when assaying tyrosine hydroxylase from the adrenal medulla or other tissues rich in catecholamines, which are inhibitors of the enzyme. However, this step was omitted in subsequent experiments, because the specific activity of the enzyme in PC18 cell supernatants subjected to gel filtration was identical to that measured in untreated PC18 cell supernatants.

The tyrosine hydroxylase enzyme assay was performed by a modifi-

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cation of the coupled decarboxylase assay (19) as modified by Kapatos and Zigmond (20). A 10-μl aliquot of the cell supernatant was added to 15 μl of a reaction mixture containing 0.1 M TES buffer (pH 6.2), 0.1 mM L-[1-^{14}C]tyrosine (specific activity 50–55 Ci/mol), 5 mM ascorbic acid, 1664 units of catalase, and 4 mM D,L-6-methyl-5,6,7,8-tetrahydropterin. This mixture was incubated at 37° for 3 min, and the reaction was terminated by the addition of 5 mM 3-iodotyrosine in 0.1 M Bis-Tris. The [1-^{14}C]dihydroxyphenylalanine produced in the above reaction was decarboxylated by the addition of 3 μl of partially purified aromatic amino acid decarboxylase purified from hog kidney (19) and pyridoxal phosphate. The reaction mixtures were incubated at 37° for 30 min, and the reaction was terminated by the addition of 0.2 ml of 0.8 M perchloric acid. The liberated $^{14}CO_2$ was collected in wells suspended from a rubber septum which capped the tubes. The wells contained 0.2 ml of NCS tissue solubilizer to dissolve the radioactive CO_2. The radioactivity was then measured by liquid scintillation spectrometry. Protein was measured by the method of Bradford (21), using bovine serum albumin as a standard.

Immunotitration studies. The cells were incubated in medium containing dialyzed serum and treated with inducing agents for 4 days, and cell supernatants were prepared as described above. Different volumes of the cell supernatants were mixed with 10 μl of antiserum to tyrosine hydroxylase in a final volume of 100 μl. This mixture was incubated at 4° for 15 min. Then, 50 μl of *Staphylococcus aureus* cells containing Protein A (Ref. 22; Pansorbin obtained from Calbiochem-Behring Corp.), pretreated as described by Kessler (22), was added to the solution, and the mixtures were incubated for another 15 min at 4°. The suspension was centrifuged at 12,000 × *g* for 5 min in a Microfuge, and a 10-μl aliquot of the supernatant was assayed for tyrosine hydroxylase activity.

Isolation of radiolabeled tyrosine hydroxylase by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. Cells were homogenized in the homogenizing buffer described above, supplemented with 1 mg/ml of aprotinin, and the homogenates were centrifuged at 20,000 × *g* for 15 min. Aliquots of cell supernatants containing 100 units of tyrosine hydroxylase activity (1 unit was defined as the amount of enzyme which catalyzed the liberation of 1 pmol of $^{14}CO_2$/min at 30°, when assayed using 0.1 mM tyrosine and 4 mM D,L-6-methyl-5,6,7,8-tetrahydropterin) were mixed with 50 μl of antiserum to tyrosine hydroxylase in the presence of 50 mM potassium phosphate buffer (pH 7.5), 1 mM EDTA, 1 mM EGTA, 50 μg/ml of leupeptin, 0.3 mM phenylmethylsulfonyl fluoride, and 1 mg/ml of aprotinin. This mixture was then allowed to stand at 4° overnight, and the immunoprecipitates were collected and washed as described previously (17). In preliminary experiments this volume of antiserum was shown to completely precipitate 150 units of tyrosine hydroxylase under the above conditions. The washed pellet was dissolved in 50 μl of a solution containing 3% SDS, 5% glycerol, 0.02% bromophenol blue, and 10 mM Tris-HCl (pH 7.8). This solution was placed in a boiling water bath for 10 min. Fifteen μl of 500 mM dithiothreitol were then added to the solutions. The solutions were applied to 10% SDS-polyacrylamide gels and subjected to electrophoresis as described by Rudolph and Krueger (23). After electrophoresis the gels were stained for protein, using Coomassie blue, and destained (17). The protein band corresponding to the M_r = 60,000 subunit of tyrosine hydroxylase was visualized on the gel and cut out with a scalpel. The radioactivity in the tyrosine hydroxylase subunits present in this band was determined by placing the gel pieces containing the enzyme subunits into 1.5 ml of NCS:H_2O (9:1) and heating this suspension for 2 hr at 50° to elute the radiolabeled protein from the gel. A piece of gel which was equal in size to the piece containing the radiolabeled tyrosine hydroxylase, but which did not contain radiolabeled protein, was also cut out as a measurement of background radioactivity in the gel. The cpm in this gel piece (generally 50–70 cpm) were subtracted from the cpm in the gel piece containing the radiolabeled tyrosine hydroxylase to yield an estimate of the cpm incorporated into the enzyme during the 60-min pulse period. Ten ml of scintillation cocktail were then added to this suspension, and the radioactivity was measured using liquid scintillation spectrometry.

Determination of the rate of synthesis of tyrosine hydroxylase. Cells were subcultured at a cell density of 2–5 × 10^4 cells/cm^2, incubated in medium containing dialyzed serum for 2 days, and then treated with inducing agents. At different times the medium was removed and replaced with leucine-free RPMI 1640 medium supplemented with dialyzed serum and 25 μCi/ml of [4,5-^{3}H]leucine (specific activity 60 Ci/mmol). When appropriate, inducing agents were also added to this medium. The cells were incubated in the presence of the labeling medium for 60 min. In preliminary experiments the incorporation of radiolabeled leucine into tyrosine hydroxylase was found to be linear for up to 90 min in the presence or absence of the inducing agents. The medium was removed, and the cells were washed once with ice-cold PBS containing 1 mM leucine. The cells were scraped into ice-cold PBS containing 1 mM leucine with a rubber policeman. The cell suspension was centrifuged at 2500 × *g* for 5 min, and the cell pellet was frozen at −90°. Radiolabeled tyrosine hydroxylase was isolated by immunoprecipitation as described above. Each immunoprecipitation yielded a value representing the cpm [^{3}H]leucine incorporated into 100 units of tyrosine hydroxylase present in a cell supernatant derived from a single dish. For each cell supernatant this value was calculated as the average of two to three immunoprecipitations. The [^{3}H]tyrosine hydroxylase cpm values noted in the tables and figures represent the amount of radioactivity incorporated into the total amount of enzyme present in a dish of cells. This value was calculated by correcting the values for [^{3}H]leucine cpm incorporated into 100 units of tyrosine hydroxylase to the total number of units of the enzyme in each dish of cells.

Incorporation of radiolabeled leucine into total soluble protein was determined by spotting 5 μl of the cell supernatants onto Whatman 3 MM paper disks and then washing the disks sequentially with the following solutions: 5% trichloroacetic acid, boiling 5% trichloroacetic acid, 5% trichloroacetic acid; absolute ethanol, and ether. The paper disks were then placed into scintillation vials, and 1.5 ml of NCS:H_2O (9:1) were added to the vials. The vials were heated for 2 hr at 50°, and then 10 ml of scintillation cocktail were added to the vials. The radioactivity was determined by liquid scintillation spectrometry. Rates of synthesis were expressed as 100 × cpm incorporated into tyrosine hydroxylase per dish/cpm incorporated into total soluble protein per dish.

Determination of the rate of degradation of tyrosine hydroxylase in PC18 cells. Cells were incubated in medium containing dialyzed serum for 2 days. The medium was then removed and replaced with fresh medium containing 10 μM leucine and 100 μCi/ml [4,5-^{3}H]leucine (specific activity 60 Ci/mmol). The cells were incubated in the presence of this labeling medium for 24 hr, at which time the labeling medium was removed and replaced with fresh medium containing 1 mM leucine (unlabeled). At different times this medium was removed, and the cells were washed once with ice-cold PBS containing 1 mM leucine. The cells were then scraped into the same buffer, centrifuged at 2500 × *g* for 5 min, and frozen at −90°. Radiolabeled tyrosine hydroxylase was isolated by immunoprecipitation, and the [^{3}H]leucine cpm incorporated into the enzyme present in each dish of cells were measured as described in earlier sections.

Results

Effect on tyrosine hydroxylase activity of incubation of PC18 cells in the presence of medium containing complete serum, medium containing dialyzed serum, or serum-free medium. Rat pheochromocytoma PC18 cells were isolated as a subclonal cell line derived from the PC12 cell line described by Greene and Tischler (18). This subclonal cell line differed in a number of parameters from the parent PC12 cells (24). The doubling time of the PC18 cells was approximately 25–30 hr, whereas the PC12 cells doubled in 3–4 days. The

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PC18 cells also differed morphologically, in that they adhered tightly to the flask substratum and were flattened and bipolar. For our studies the major advantage of this cell line over the parent PC12 cell line was that both cyclic AMP and glucocorticoids produced large increases in tyrosine hydroxylase activity in the PC18 cells, whereas in the parent PC12 cells dexamethasone produced only a 2-fold increase and cyclic AMP analogs did not produce a statistically significant increase in tyrosine hydroxylase activity (24).

The PC18 cells had relatively high basal tyrosine hydroxylase activity when cultured in medium containing 10% complete horse serum and 5% complete fetal calf serum (see Fig. 1). This basal activity varied considerably (from 2.0 to 7.5 nmol/min × mg of protein), depending upon the lot of serum used and the cell density. Furthermore, in the presence of complete serum, the effects of the synthetic glucocorticoid, dexamethasone, were nonreproducible. Since the variability in basal activity and in response to dexamethasone might be partially due to the presence of glucocorticoids or other factors in the serum, we attempted to perform our experiments in the presence of medium containing either serum that was dialyzed exhaustively against PBS containing 1 mg/ml of charcoal (Norit A) or in the serum-free medium described by Bottenstein and Sato (25). After 2 days of incubation in either of these media, the basal activity of tyrosine hydroxylase decreased to a new steady state level (see Fig. 1). There was no difference in the new steady state level of tyrosine hydroxylase activity achieved in the presence of either serum-free medium or medium containing dialyzed serum. However, in the medium containing dialyzed serum, the cells adhered to the flask bottom and maintained a doubling rate of approximately 25–30 hr for at least 12 days in culture, whereas in the presence of the serum-free medium, the doubling rate decreased markedly, the morphology of the cells changed, and many cells detached from the flask substratum after 3–4 days in culture. Thus, we used the medium containing dialyzed serum in the subsequent induction experiments.

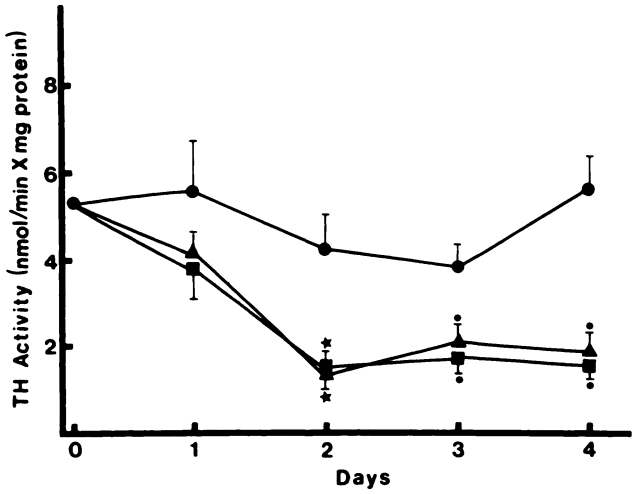

Fig. 1. Effect of different media on tyrosine hydroxylase (*TH*) activity in PC18 cells. Cells were subcultured in medium containing complete serum (10% heat-inactivated horse serum plus 5% fetal bovine serum) and incubated overnight. The medium was then removed and replaced with one of the following media: 1) medium containing complete serum (●); 2) medium containing dialyzed serum (▲; see Experimental Procedures for details of dialysis); or 3) serum-free medium (■; Ref. 25). At different times after this change of medium the cells were harvested and assayed for tyrosine hydroxylase activity. Zero time represents the time at which the medium was replaced with one of the three media described above. The data represent the means ± standard errors from three dishes. *, $p < 0.01$ compared to dishes containing medium supplemented with complete serum; ★, $p < 0.05$ compared to dishes containing medium supplemented with complete serum.

Effect on tyrosine hydroxylase activity of 8-bromocyclic AMP- or cyclic AMP-elevating agents in the presence or absence of dexamethasone in PC18 cells. PC18 cells were cultured in medium containing dialyzed serum for 2 days and then treated with inducing agents for 3–4 days in the same medium. In the experiments described in Table 1A, cells were treated with 10 μM forskolin, 0.5 μg/ml of cholera toxin, or 0.5 mM isobutylmethylxanthine in the presence and absence of dexamethasone. In the absence of the glucocorticoid, these compounds, which are well characterized activators of adenylate cyclase (forskolin and cholera toxin) or inhibitors of phosphodiesterase (isobutylmethylxanthine), produced 2–4-fold increases in tyrosine hydroxylase activity. Dexamethasone by itself elicited a 5-fold elevation of tyrosine hydroxylase activity. In the presence of dexamethasone, forskolin and cholera toxin produced increases in enzyme activity that were greater than the sum of the increases in activity observed in cells treated with either inducing agent alone. Isobutylmethylxanthine plus dexamethasone produced a slightly less than additive increase in enzyme activity.

When cells were treated with the cyclic AMP analog, 8-bromocyclic AMP (1 mM), tyrosine hydroxylase activity increased approximately 6-fold (Table 1B). Experiments in which the concentration of 8-bromocyclic AMP was varied demon-

TABLE 1

Effect of cyclic AMP-elevating agents in the presence and absence of dexamethasone on tyrosine hydroxylase activity in PC18 cells

Cells were incubated in medium containing dialyzed serum for 2 days, at which time the medium was replaced with fresh medium containing dialyzed serum and the indicated compounds at the following concentrations: forskolin, 10 μM; dexamethasone, 1 μM; cholera toxin, 0.5 μg/ml; isobutylmethylxanthine, 0.5 mM; and 8-bromocyclic AMP, 1 mM. Except for the cholera toxin-treated cells, the cells were treated for either 3 days (A) or 4 days (B) with the appropriate drugs and then harvested. Cells were incubated in cholera toxin for 6 hr, at which time the medium was removed and replaced with fresh medium lacking cholera toxin but containing dexamethasone when appropriate, and the cells were then incubated for another 2 days, 18 hr. Tyrosine hydroxylase was assayed in the cell supernatants derived from these cells as described in Experimental Procedures. Data represent means ± standard errors derived from 3–5 dishes in Part A and 10–12 dishes in part B.

	Tyrosine hydroxylase activity	
	nmol CO_2 formed/min × mg protein	-Fold increase
A. Three-day treatment		
Control	2.7 ± 0.53	
Dexamethasone	13. ± 1.6[a]	4.8 ± 0.6
Forskolin	6.1 ± 0.53[a]	2.2 ± 0.2
Forskolin + dexamethasone	24. ± 1.0[a]	8.8 ± 0.3
Choleratoxin	6.3 ± 0.24[a]	2.3 ± 0.1
Choleratoxin + dexamethasone	30. ± 2.4[a]	11. ± 0.9
Isobutylmethylxanthine	12. ± 1.0[a]	4.3 ± 0.4
Isobutylmethylxanthine + dexamethasone	17. ± 1.5[a]	6.1 ± 0.6
B. Four-day treatment		
Control	1.2 ± 0.29	
8-Bromocyclic AMP	6.8 ± 2.2[b]	6.0 ± 0.7
Dexamethasone	6.2 ± 1.3[a]	6.7 ± 1.1
8-Bromocyclic AMP + dexamethasone	9.3 ± 2.5[a]	10.9 ± 2.5

[a] $p < 0.01$ compared to control data.
[b] $p < 0.02$ compared to control data.

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strated that 1 mM 8-bromocyclic AMP was a maximally effective concentration, and that the half-maximal effective concentration was approximately 0.4 mM (data not shown). Furthermore, incubation of the cells in the presence of 1 mM 8-bromocyclic AMP plus forskolin did not produce a significantly greater increase in enzyme activity (data not shown). These data suggest that 1 mM 8-bromocyclic AMP elicited the maximal cyclic AMP-dependent increase in tyrosine hydroxylase activity in the PC18 cells. In the experiments reported in Table 1B, dexamethasone also produced a 6–7-fold increase in tyrosine hydroxylase activity. 8-Bromocyclic AMP plus dexamethasone produced an approximately additive increase in enzyme activity (Table 1B).

Effects of different concentrations of dexamethasone on tyrosine hydroxylase activity in PC18 cells. Cells were treated with concentrations of dexamethasone ranging from 10^{-10} M to 10^{-6} M for 3 days (Fig. 2). Maximal effects on tyrosine hydroxylase activity were observed when the cells were incubated with 10^{-8} M dexamethasone. Concentrations of dexamethasone greater than 10^{-8} M did not produce a significantly greater response. The concentration which produced half-maximal increase in activity was approximately 3×10^{-9} M dexamethasone. When cells were treated with different concentrations of dexamethasone in the presence of 1 mM 8-bromocyclic AMP for 3 days, the maximal response was also observed at 10^{-8} M dexamethasone. Additive effects of 8-bromocyclic AMP and dexamethasone were observed at concentrations of dexamethasone equal to or greater than 10^{-8} M. The response of the PC18 cells to different concentrations of dexamethasone was similar to that observed in mouse neuroblastoma cells treated with dexamethasone (15).

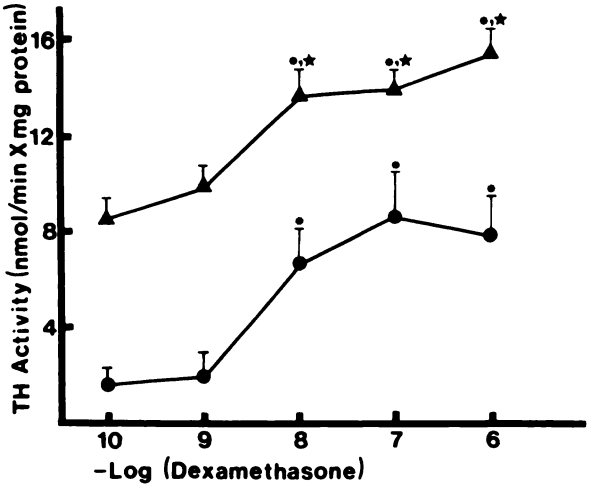

Fig. 2. Effect of different concentrations of dexamethasone in the presence and absence of 8-bromocyclic AMP on tyrosine hydroxylase (*TH*) activity in PC18 cells. Cells were incubated in medium containing dialyzed serum for 2 days, at which time the medium was removed and replaced with fresh medium containing dialyzed serum plus different concentrations of dexamethasone. 8-Bromocyclic AMP (1 mM) was also added to the appropriate dishes. Stock solutions of dexamethasone were prepared using ethanol as a solvent; the concentration of ethanol (0.2%) in the cell medium was constant in all of the dishes. The cells were treated for 3 days with the different concentrations of dexamethasone and then harvested and assayed for tyrosine hydroxylase activity. The enzyme activity in cells incubated in the absence of dexamethasone were as follows: ethanol, 1.7 + 0.41; 8-bromocyclic AMP plus ethanol, 8.5 + 2.0. The data represent the means ± standard errors from four to six dishes. •, $p < 0.01$ compared to control cells treated only with ethanol; ★, $p < 0.05$ compared to cells treated with 8-bromocyclic AMP plus ethanol.

Effects of glucocorticoids and other steroid hormones on tyrosine hydroxylase activity in the PC18 cells. PC18 cells were incubated in the presence of different steroid hormones (all at 10^{-6} M) for 3 days, at which time the cells were harvested and tyrosine hydroxylase activity was assayed in the cell supernatants. Ethanol (0.2%), the vehicle for the steroid hormones, did not produce a significant increase in tyrosine hydroxylase activity in the PC18 cells (Table 2). All of the glucocorticoids tested produced 4–5-fold increases in tyrosine hydroxylase activity (Table 2). Other nonglucocorticoid steroid hormones, such as β-estradiol, progesterone, and testosterone (all at 10^{-6} M) did not produce significant effects on tyrosine hydroxylase activity. These results suggest that only the glucocorticoid class of steroid hormones induces tyrosine hydroxylase in the PC18 cells.

8-Bromocyclic AMP (1 mM) by itself produced a 4–5-fold increase in enzyme activity. The presence of ethanol (0.2%) in the cell medium did not affect the 8-bromocyclic AMP-mediated increase in tyrosine hydroxylase activity. However, in cells incubated in the presence of 8-bromocyclic AMP plus any of the glucocorticoid hormones tested, the increase in enzyme activity was equal to slightly less than the sum of the increases in enzyme activity observed in cells incubated in the presence of either 8-bromocyclic AMP or glucocorticoid alone. In contrast, in cells incubated in the presence of 8-bromocyclic AMP plus the nonglucocorticoid steroid hormones tested, the 8-bromocyclic AMP-mediated increase in enzyme activity was slightly inhibited. These results suggest that only the glucocorticoid class of steroid hormones can interact with cyclic AMP to produce an induction of tyrosine hydroxylase which is greater than that observed with either inducing agent alone.

Immunotitration of tyrosine hydroxylase in PC18 cells treated with 8-bromocyclic AMP and dexamethasone. In order to determine whether the increases in tyrosine hydroxylase activity produced by 8-bromocyclic AMP and dex-

TABLE 2

Effect of different steroid hormones in the presence and absence of 8-bromocyclic AMP (8-BrcAMP) on tyrosine hydroxylase activity in PC18 cells

Cells were incubated in medium containing dialyzed serum for 2 days, at which time the medium was removed and replaced with fresh medium containing dialyzed serum plus the indicated compounds. All steroids were initially dissolved in ethanol at 0.5 mM and added to the incubation medium to produce a final concentration of 0.2% ethanol and 10^{-6} M steroid hormone. Cells were incubated in the presence of the drugs for 3 days, at which time the cells were harvested. Tyrosine hydroxylase activity was assayed in the supernatants derived from these cells. The data represent the means ± standard errors from four to six dishes.

Steroid hormone added	Tyrosine hydroxylase activity			
	In the absence of 8-BrcAMP		In the presence of 8-BrcAMP	
	nmol/min × mg	-Fold increase	nmol/min × mg	-Fold increase
No additions	2.5 ± 0.4		11.6 ± 0.2	4.6 ± 0.1
Ethanol	3.5 ± 0.6	1.4 ± 0.1	12.3 ± 0.2	5.0 ± 0.1
Triamcinolone	13.1 ± 1.0[a]	5.2 ± 0.5	19.7 ± 1.9[b]	7.9 ± 0.8
Corticosterone	12.0 ± 0.7[a]	4.8 ± 0.5	16.6 ± 1.3[b]	6.6 ± 0.5
Hydrocortisone	10.5 ± 0.7[a]	4.2 ± 0.4	17.5 ± 2.6[b]	7.0 ± 1.0
Testosterone	3.0 ± 0.2	1.2 ± 0.1	7.8 ± 1.7	3.1 ± 0.7
β-Estradiol	1.9 ± 0.1	0.7 ± 0.1	7.4 ± 2.4	3.0 ± 0.9
Progesterone	3.2 ± 0.5	1.2 ± 0.1	6.7 ± 1.7[b]	2.7 ± 0.7

[a] $p < 0.01$ compared to untreated control dishes.
[b] $p < 0.05$ compared to dishes treated with 8-bromocyclic AMP alone.

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amethasone were due to increases in enzyme level in the cells or increases in the activity of preexisting enzyme molecules, immunotitration studies were performed on untreated cells or cells treated for 3 days with either 1 mM 8-bromocyclic AMP, 10^{-6} M dexamethasone, or the two inducing agents together. In these experiments the supernatants derived from cells treated with inducing agents were diluted, such that the enzyme activities per μl were identical to that measured in supernatants derived from untreated cells. When increasing amounts of these supernatants were incubated with 10 μl of antiserum to tyrosine hydroxylase (17), and the antibody-antigen complexes precipitated with staphylococcal protein A immunoadsorbent (22), the curves representing the loss of enzyme activity from the different supernatants were identical (Fig. 3). These results demonstrate that the increases in enzyme activity elicited by the inducing agents were due to increases in enzyme protein.

Time courses of the induction of tyrosine hydroxylase enzyme levels in the PC18 cells. Cells were preincubated for 2 days in medium containing dialyzed serum and then treated with either 1 mM 8-bromocyclic AMP, 10^{-6} M dexamethasone, or the two inducing agents together. At different times the cells were harvested, and tyrosine hydroxylase activity was assayed in the cell supernatants. Basal activity of tyrosine hydroxylase remained relatively constant during the 4-day induction period (Figure 4). When the cells were incubated with either 8-bromocyclic AMP or dexamethasone alone, tyrosine hydroxylase activity increased slowly. A significant increase in activity was not observed until after 1 day of treatment with either inducing agent. The rate of increase in enzyme activity was approximately equal for cells treated with either 8-bromocyclic AMP or dexamethasone, reaching a level 6–7-fold higher than that observed in control cells after 4 days of treatment. In preliminary experiments we determined that the induction was maximal after 4 days of treatment (data not shown).

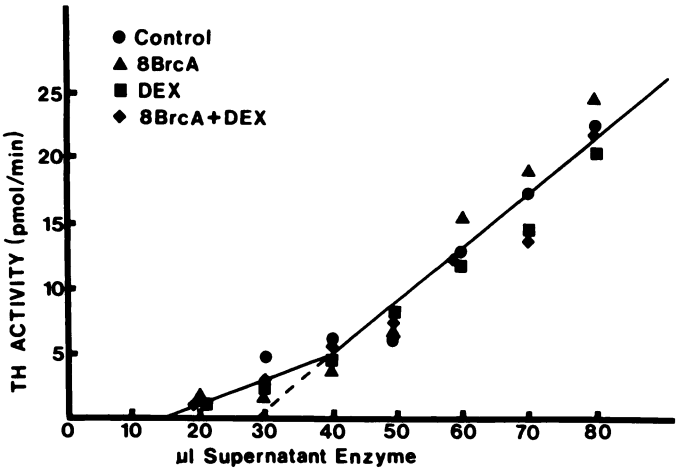

Fig. 3. Immunochemical titration of tyrosine hydroxylase (*TH*) derived from PC18 cells treated with 8-bromocyclic AMP and/or dexamethasone. Cells were incubated for 2 days in the presence of medium containing dialyzed serum, at which time the medium was removed and replaced with fresh medium containing dialyzed serum and the appropriate drugs. Cells were then incubated in the absence and presence of 1 mM 8-bromocyclic AMP (*8BrcA*), 1 μM dexamethasone (*DEX*), or the two inducing agents together for 3 days. The cells were then homogenized and the cell supernatants assayed for tyrosine hydroxylase activity. The enzyme supernatants derived from the treated cells were diluted appropriately to yield the same enzyme activity per μl as measured in the supernatant from the untreated cells. Increasing volumes of enzyme supernatants were then mixed with 10 μl of antiserum to tyrosine hydroxylase for 30 min on ice. The staphylococcal protein A immunoadsorbent (50 μl), prepared as described by Tank and Weiner (15), was added to the solutions, and the mixtures were incubated on ice for 15 min. The immunoadsorbent was then pelleted by centrifugation, and tyrosine hydroxylase activity was assayed in the supernatants. The data were fitted to a straight line by linear regression analysis. The activities of tyrosine hydroxylase, expressed as nmol/min × mg of protein, assayed prior to immunotitration, were as follows: control, 1.2; 8-bromocyclic AMP, 3.8; dexamethasone, 5.2; and 8-bromocyclic AMP plus dexamethasone, 8.6.

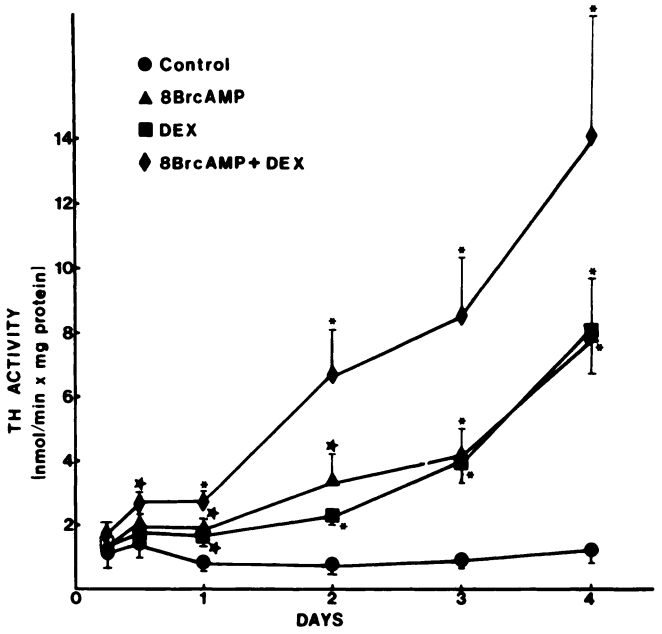

Fig. 4. Time courses of the induction of tyrosine hydroxylase (*TH*) elicited by 8-bromocyclic AMP and/or dexamethasone in PC18 cells. Cells were incubated for 2 days in the presence of medium containing dialyzed serum, at which time the medium was removed and replaced with fresh medium containing dialyzed serum (0 time in the figure) and the appropriate drugs. Cells were then incubated for different periods of time in the absence or presence of 1 mM 8-bromocyclic AMP (*8BrcAMP*), 1 μM dexamethasone (*DEX*), or the two inducing agents together. At the appropriate times cells were harvested and homogenized, and tyrosine hydroxylase was assayed in the cell supernatants. The data represent the means ± standard error from 10–12 dishes. *, $p < 0.01$ compared to control values; ★, $p < 0.05$ compared to control values.

When the cells were incubated with 8-bromocyclic AMP and dexamethasone simultaneously, the increase in tyrosine hydroxylase activity was approximately equal to the sum of the increases in enzyme activity elicited by either inducing agent alone (Fig. 4). This result was observed at all time points.

Isolation of radiolabeled tyrosine hydroxylase from PC18 cell supernatants. In order to measure the rate of synthesis of tyrosine hydroxylase in the PC18 cells, an immunoprecipitation procedure was required to rapidly isolate radiolabeled tyrosine hydroxylase from radiolabeled total, soluble, cellular proteins. Our laboratory has produced a monospecific rabbit antiserum to tyrosine hydroxylase (17), which has precipitating properties. The specificity of this antiserum can be seen in Fig. 5. One major radiolabeled polypeptide band was visualized on an autoradiogram of a polyacrylamide gel, after electrophoretic separation of the proteins present in immunoprecipitates isolated from supernatants derived from differently treated cells, which had been incubated for 60 min in the

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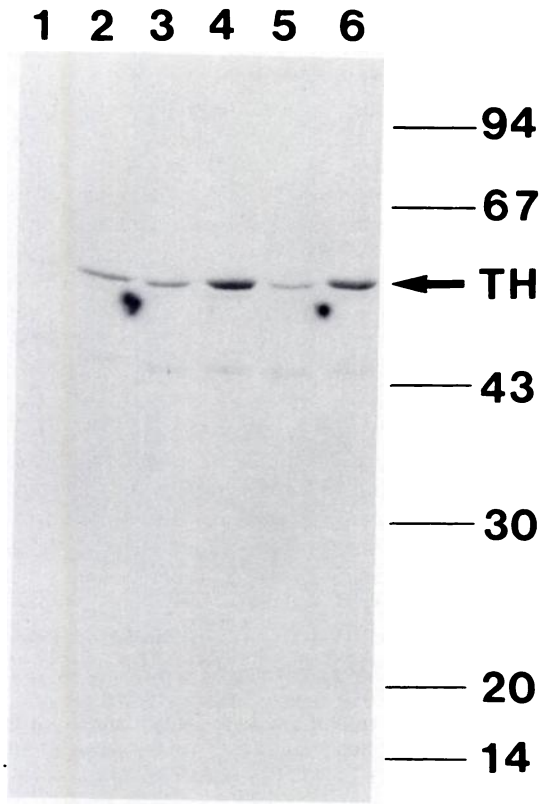

Fig. 5. Autoradiogram of SDS-polyacrylamide gel after electrophoresis of immunoprecipitates from PC18 cell supernatants derived from cells treated with [^{3}H]leucine. Cells were incubated in medium containing dialyzed serum for 2 days, at which time the medium was replaced with fresh medium containing dialyzed serum plus the appropriate drugs. Cells were then incubated for 24 hr (except as noted below) in the absence (*lane 1*) or presence of the following drugs: 1 μM dexamethasone (*lane 2*); 10 μM forskolin (*lane 3*); 1 μM dexamethasone plus 10 μM forskolin (*lane 4*); 0.5 μg/ml cholera toxin (*lane 5*); and 0.5 μg/ml cholera toxin plus 1 μM dexamethasone (*lane 6*). In the experiments involving cholera toxin, cells were incubated in the presence of cholera toxin for 6 hr, at which time the medium was removed and replaced with fresh medium lacking cholera toxin but containing dexamethasone when appropriate, and the cells were incubated for another 18 hr in the absence of cholera toxin. The medium was then replaced with leucine-free medium containing 25 μCi/ml of [^{3}H]leucine, and the cells were incubated for 1 hr. When appropriate, drugs were also added to the labeling medium. The cells were then harvested, and the radiolabeled tyrosine hydroxylase was isolated by immunoprecipitation as described in Experimental Procedures. The immunoprecipitates were then subjected to SDS-polyacrylamide gel electrophoresis. After electrophoresis the gels were stained for proteins, treated with Enhance (New England Nuclear), dried down under heat and vacuum onto Whatman 3MM paper, and exposed to X-ray film for autoradiography. The *numbers* in the right column represent the molecular weights in kilodaltons of the following proteins used as standards for the gel electrophoresis: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa). ←, the R_f of purified tyrosine hydroxylase traced from the stained gel.

presence of [^{3}H]leucine. This band at M_r = 60,000 comigrates with tyrosine hydroxylase purified from rat pheochromocytoma tumor (26) and presumably represents the subunits of the enzyme. A second, fainter band, which we have not characterized, was also seen at times at approximately M_r = 45,000. In preliminary experiments we determined that 50 μl of antiserum completely immunoprecipitated 150 units of tyrosine hydroxylase activity from PC18 cell supernatants. Thus, in the studies on the rate of synthesis and degradation of tyrosine hydroxylase, 50 μl of antiserum were used to precipitate aliquots of cell supernatants containing 100 units of enzyme activity, in order to completely immunoprecipitate all of the enzyme present in that aliquot.

Rate of degradation of tyrosine hydroxylase. The rate of degradation of tyrosine hydroxylase was measured in cells which had been incubated for 2 days in medium containing dialyzed serum, prior to the addition of radiolabeled leucine to the medium. The cells were then incubated in the presence of [^{3}H]leucine for 24 hr, at which time this medium was removed and replaced with fresh medium containing 1 mM unlabeled L-leucine. Radiolabeled tyrosine hydroxylase was isolated from the cells by immunoprecipitation, and the radioactivity in the tyrosine hydroxylase molecules present in each dish of cells was measured as described in Experimental Procedures. In the two experiments described in Fig. 6, the half-life values for the enzyme were found to be 34 hr and 27 hr, and the first order k_d values were calculated as 0.020 hr^{-1} and 0.026 hr^{-1}, respectively. In a third experiment (data not shown), we obtained a half-life of 36 hr and a k_d of 0.019 hr^{-1}. These values represent apparent first order rate constants and half-lives and probably overestimate the half-life values, because we cannot rule out the reutilization of radiolabeled leucine for the new synthesis of tyrosine hydroxylase during the "cold chase" period of the experiment. However, these values do represent an estimate of the rate of degradation of tyrosine hydroxylase in the PC18 cells.

Effects of 8-bromocyclic AMP and dexamethasone on the rate of synthesis of tyrosine hydroxylase in PC18 cells. In order to measure the rate of synthesis of tyrosine hydroxylase, PC18 cells were incubated in the presence or

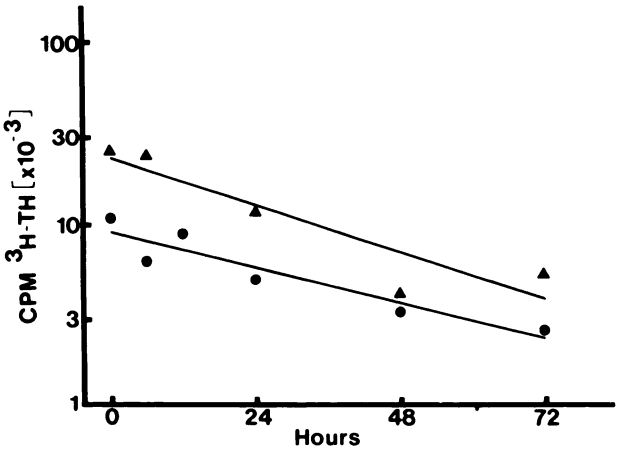

Fig. 6. Rate of degradation of tyrosine hydroxylase (*TH*) in PC18 cells. Cells were incubated in medium containing dialyzed serum for 2 days, at which time the medium was replaced with fresh medium containing dialyzed serum, 10 μM leucine, and 50 μCi/ml of [^{3}H]leucine. The cells were incubated in this labeling medium for 24 hr, at which time the medium was removed and replaced with fresh medium containing dialyzed serum and 1 mM unlabeled leucine (0 time in the figure). At different times the medium was removed, the cells were harvested, and the cpm in the radiolabeled tyrosine hydroxylase were determined as described in Experimental Procedures. The [^{3}H]TH cpm values represent the [^{3}H]leucine incorporated into the total amount of tyrosine hydroxylase present in the cells derived from one dish. Each *data point* in the figure represents the mean value from two dishes. The *two lines* represent two separate experiments. The points were fitted to straight lines by linear regression analysis. The half-life of the enzyme in the two experiments was determined to be 34 hr for experiment 1 (●) and 27 hr for experiment 2 (▲).

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absence of inducing agents for different periods of time and then treated for 1 hr with [4,5-^{3}H]leucine, to label intracellular proteins. Since the half-life of tyrosine hydroxylase in the PC18 cells was determined to be approximately 30 hr (Fig. 6), the pulse-labeling period of 1 hr was short enough to neglect any effect of degradation on the rates of synthesis measurements (27). Tyrosine hydroxylase was then isolated by immunoprecipitation and SDS-polyacrylamide gel electrophoresis, and the cpm of [^{3}H]leucine incorporated into the enzyme during the 1-hr pulse period was determined. The radioactivity incorporated into total soluble protein during the pulse period was also determined. The rate of synthesis of tyrosine hydroxylase was expressed as the radioactivity incorporated into the enzyme divided by the radioactivity incorporated into total soluble protein.

The increases in the rate of synthesis of the enzyme after 24 hr of treatment with a number of different inducing agents is shown in Fig. 5 and Table 3. There was a considerable increase in the incorporation of radiolabeled leucine into tyrosine hydroxylase subunits in cells treated with either dexamethasone, forskolin, or cholera toxin, as depicted in Fig. 5 (compare *lane 1* to *lanes 2*, *3*, and *5*, respectively). In cells treated simultaneously with both dexamethasone plus forskolin, or dexamethasone plus cholera toxin, there was a greater increase in the rate of incorporation of [^{3}H]leucine into the enzyme subunits than was observed in cells treated with these inducing agents alone (*lanes 4* and *6* in Fig. 5).

Experiments using 8-bromocyclic AMP and dexamethasone as inducing agents, in which the rates of synthesis were measured quantitatively, are summarized in Table 3. Incorporation of radiolabeled leucine into total soluble protein was not affected by treatment of the cells with 8-bromocyclic AMP, dexamethasone, or the two inducing agents together. However, there was a dramatic increase in the incorporation of [^{3}H]leucine into tyrosine hydroxylase protein in cells treated for 24 hr with these inducing agents (Table 3). When expressed as the cpm incorporated into tyrosine hydroxylase divided by the cpm incorporated into total soluble protein, the rate of synthesis was stimulated 7–8-fold by either 8-bromocyclic AMP or dexamethasone. In cells treated with both inducing agents, the rate of synthesis increased to a level slightly less than the sum of the increases observed in cells treated with either inducing agent alone.

A time course of the changes in the rate of synthesis of tyrosine hydroxylase is seen in Fig. 7. In cells not treated with inducing agents the rate of synthesis of tyrosine hydroxylase remained relatively constant over time (Figure 7). Approximately 0.2–0.3% of the total soluble protein synthesized represented tyrosine hydroxylase synthesis. When cells were treated with either 8-bromocyclic AMP or dexamethasone, the rate of synthesis of the enzyme increased relatively rapidly to a level approximately 5–6-fold greater than that measured in the control cells after 12 hr of treatment (Fig. 7). The rate of synthesis continued to increase modestly at a slower rate from 12 to 96 hr of treatment with either inducing agent alone. The changes in the rate of synthesis of tyrosine hydroxylase elicited by the inducing agents occurred much more rapidly than the changes in enzyme activity (compare Figs. 5 and 7). In the simultaneous presence of both inducing agents, the rate of synthesis increased rapidly to a new steady state level approximately 9–10-fold greater than that measured in control cells after 12 hr of treatment. The additive increases in the rate of synthesis in cells treated with both inducing agents was observed at all time points up to 2 days of treatment. We cannot yet explain the paradoxical decrease in the rate of synthesis of tyrosine hydroxylase which occurs after 2 days of treatment with both inducing agents.

The increases in the rate of synthesis of tyrosine hydroxylase agreed reasonably well with the increases in tyrosine hydroxylase activity produced after 4 days of treatment with these inducing agents (compare Figs. 4 and 7 and Tables 1B and 3). Also, note that the additive increases in tyrosine hydroxylase activity observed after 4 days of treatment with both 8-bromocyclic AMP plus dexamethasone were paralleled by additive increases in the rate of synthesis of the enzyme after 24 hr of treatment with both inducing agents.

Discussion

Glucocorticoids have been shown to induce tyrosine hydroxylase in rat sympathetic ganglia (12, 13), in rat pheochromocytoma cells (11, 14), and in mouse neuroblastoma cells (15). Cyclic AMP has been shown to induce the enzyme in mouse neuroblastoma cells (9, 10) and in rat pheochromocytoma cells (11). In previous studies on the regulation of tyrosine hydroxylase enzyme levels by cyclic AMP and glucocorticoids, we have used mouse neuroblastoma cells in culture as a model system

TABLE 3

Effect of 8-bromocyclic AMP and dexamethasone on the rate of synthesis of tyrosine hydroxylase (TH) in PC18 cells

Cells were incubated in medium containing dialyzed serum for 2 days, at which time the medium was removed and replaced with fresh medium containing the appropriate drugs. After 24 hr of treatment with these drugs the medium was removed and replaced with leucine-free medium containing dialyzed serum and [^{3}H]-leucine (25 μCi/ml). The appropriate drugs were also added to the labeling medium. The cells were pulse-labeled for 60 min and then washed once with ice-cold PBS containing 1 mM unlabeled leucine. The cells were then harvested and homogenized as described in Experimental Procedures. Radiolabeled tyrosine hydroxylase was isolated by immunoprecipitation followed by SDS-polyacrylamide gel electrophoresis. Radiolabeled total protein was measured as described in Experimental Procedures. The rates of synthesis were calculated as the cpm incorporated into tyrosine hydroxylase protein per dish divided by the cpm incorporated into total soluble protein per dish. The data represent the means ± standard errors from 14–16 dishes.

Treatment	cpm in TH/dish ($\times 10^{-6}$)	cpm in Total protein/dish ($\times 10^{-6}$)	Total mg protein/dish	cpm in TH/cpm in total protein ($\times 10^{5}$)	-Fold increase in rate of synthesis
Control	0.16 ± 0.03	6.4 ± 0.7	0.58 ± 0.04	0.24 ± 0.02	
8-Bromocyclic AMP	1.4 ± 0.14[a]	7.6 ± 0.6	0.64 ± 0.06	1.9 ± 0.15[a]	7.7 ± 0.6
Dexamethasone	1.4 ± 0.15[a]	7.0 ± 0.6	0.62 ± 0.04	1.9 ± 0.11[a]	7.8 ± 0.4
8-Bromocyclic AMP + Dexamethasone	2.2 ± 0.28[a,b]	7.0 ± 0.6	0.63 ± 0.04	3.0 ± 0.2[a,c]	12. ± 0.7

[a] $p < 0.01$ compared to control values.
[b] $p < 0.02$ compared to values derived from cells treated with either inducing agent alone.
[c] $p < 0.01$ compared to values derived from cells treated with either inducing agent alone.

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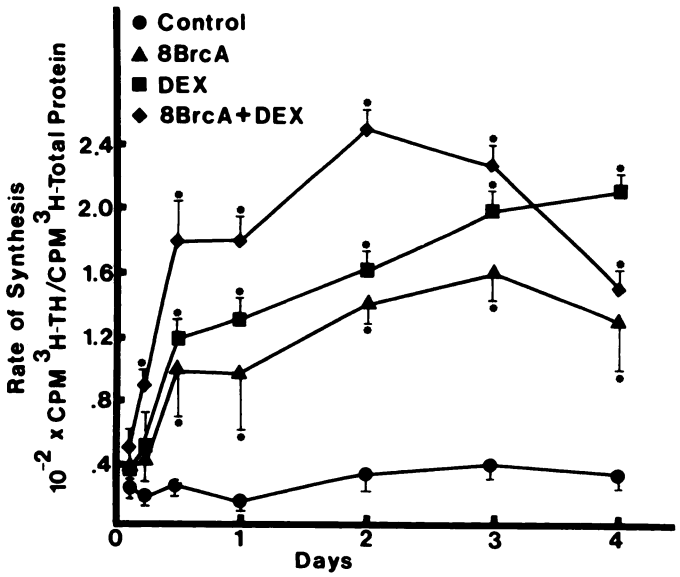

Fig. 7. Time courses of the changes in the rate of synthesis of tyrosine hydroxylase (*TH*) in PC18 cells treated with 8-bromocyclic AMP and/or dexamethasone. Cells were incubated in medium containing dialyzed serum for 2 days, at which time the medium was replaced with fresh medium containing dialyzed serum (0 time in the figure) plus the appropriate drugs. The cells were then incubated in the absence or presence of 1 mM 8-bromocyclic AMP (*8BrcA*), 1 μM dexamethasone (*DEX*), or the two inducing agents together. At the appropriate times the medium was removed and replaced with fresh leucine-free medium containing the appropriate inducing agents and 25 μCi/ml of [^{3}H]leucine. The cells were pulse-labeled for 1 hr and then washed and harvested as described in Experimental Procedures. The cell supernatants were then used to measure the [^{3}H]leucine incorporated into tyrosine hydroxylase or total soluble proteins. The rates of synthesis were calculated as the cpm incorporated into tyrosine hydroxylase protein divided by the cpm incorporated into total soluble protein, all multiplied by 100. The data represent the mean ± standard errors from four dishes. *, $p < 0.01$ compared to control values.

(10, 15). When these cells are incubated with these inducing agents, tyrosine hydroxylase enzyme levels are elevated many-fold. Due to these responses the neuroblastoma cells are a convenient model system to study certain aspects of the induction of tyrosine hydroxylase by cyclic AMP and glucocorticoids. However, these cells are not a good model system to study the intracellular mechanisms involved in the regulation of tyrosine hydroxylase enzyme levels, because the basal level of the enzyme is extremely low. Thus, it is difficult to detect radiolabeled enzyme for rates of synthesis studies or to measure the $mRNA^{TH}$ using either cell-free translations or hybridization techniques.

In the present study we used rat pheochromocytoma PC18 cells, a subclone of the rat pheochromocytoma PC12 cell line described by Greene and Tischler (18). The use of these cells obviates the problem described above for the neuroblastoma cells, in that the PC18 cells possess high basal activity of tyrosine hydroxylase and, when treated with cyclic AMP analogs or glucocorticoids, enzyme activity increases dramatically. Thus, these cells represent an excellent model system to study the intracellular mechanisms involved in the regulation of tyrosine hydroxylase enzyme levels. In this paper we characterize the induction of tyrosine hydroxylase by cyclic AMP and glucocorticoids in this cell line, and demonstrate that the increases in enzyme level are due to increases in the rate of synthesis of the enzyme elicited by these inducing agents. We also demonstrate that when the cells are incubated in the presence of both inducing agents, the increases in the cellular level and the rate of synthesis of the enzyme are approximately equal to the sum of the increases in these parameters measured in cells incubated with either inducing agent alone. In the accompanying paper (16), we measure the effects of these inducing agents alone or in combination on the level of $mRNA^{TH}$ in the PC18 cells.

Tyrosine hydroxylase activity increases 5–7-fold in PC18 cells after treatment with either 8-bromocyclic AMP or dexamethasone. Immunotitration studies show that these increases in enzyme activity are due to increases in enzyme protein, not activation of preexisting enzyme molecules. The cyclic AMP-mediated induction is elicited by cyclic AMP analogs, such as 8-bromocyclic AMP, or compounds which elevate intracellular cyclic AMP by activation of adenylate cyclase, such as forskolin or cholera toxin, or by inhibition of phosphodiesterase, such as isobutylmethylxanthine. The glucocorticoid-mediated induction is elicited only by the glucocorticoid class of steroid hormones. Estrogen, progesterone, and testosterone do not produce an increase in tyrosine hydroxylase activity. These results are in excellent agreement with those reported in other systems, such as superior cervical ganglion organ cultures (28) and mouse neuroblastoma cell cultures (15). It remains to be determined whether the cyclic AMP-dependent and glucocorticoid-dependent responses are mediated by a cyclic AMP-dependent protein kinase and a glucocorticoid receptor, respectively.

Cyclic AMP and glucocorticoids also increase the rate of synthesis of tyrosine hydroxylase in the PC18 cells. Time course studies demonstrate that the rate of synthesis increases to a level 6–8-fold greater than that observed in control cells after 12–24 hr of treatment with either 8-bromocyclic AMP or dexamethasone. In contrast, the enzyme level of tyrosine hydroxylase rises relatively slowly to a level 5–7-fold greater than that observed in control cells after 4 days of treatment with either inducing agent. Thus, even though the increases in the enzyme level and the rate of synthesis of the enzyme are approximately equal in magnitude, the time courses differ dramatically. This result is probably a consequence of the relatively slow rate of degradation of tyrosine hydroxylase in the PC18 cells. The apparent half-life of the enzyme is approximately 30 hr in these cells (Fig. 6). Since the rate at which the level of an enzyme changes from one steady state level to another is approximately equal to four or five half-lives (27), the enzyme activity would be predicted to rise relatively slowly, reaching a new steady state level after 5–6 days. The minor discrepancy between the predicted time to reach a new steady-state (5–6 days) and the observed time to reach a new steady state (4 days) suggests that the apparent half-life may be an overestimation due to reutilization of radiolabeled leucine for synthesis of enzyme during the "cold chase" period of the rate of degradation experiment, or that the half-life of the enzyme has been influenced slightly by treatment of the cells with 8-bromocyclic AMP and/or dexamethasone.

Quantitatively, the increases in tyrosine hydroxylase activity observed after 4 days of treatment with either 8-bromocyclic AMP or dexamethasone alone are approximately equal to the increases in the rate of synthesis of the enzyme after 24 hr of treatment with either inducing agent. These results suggest strongly that cyclic AMP and glucocorticoids induce tyrosine

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hydroxylase by increasing the rate of synthesis of the enzyme. Similar conclusions have been reached by Baetge *et al.* (14), who studied the glucocorticoid-mediated induction of tyrosine hydroxylase in PC12 cells. From the experiments performed in these studies, we cannot conclusively rule out an effect by these inducing agents on the rate of degradation of the enzyme; however, the quantitatively close correlation between the changes in the rate of synthesis of the enzyme and the changes in the level of the enzyme suggest that any effect on degradation rate must be minimal. As described above, there is a slight discrepancy between the predicted and observed rates at which the level of tyrosine hydroxylase reaches a new steady state in cells exposed to 8-bromocyclic AMP and/or dexamethasone. This apparent discrepancy suggests that these inducing agents may slightly increase the rate of degradation of tyrosine hydroxylase. Such an increase might also explain the fact that, in most experiments, the increases in the rate of synthesis of tyrosine hydroxylase are slightly greater than the increases in the enzyme level. However, these conclusions are highly speculative. Further experiments are required to verify an effect of cyclic AMP and/or glucocorticoid on the rate of degradation of tyrosine hydroxylase. The major effect of these inducing agents is on the rate of synthesis of the enzyme.

When the cells are treated with both 8-bromocyclic AMP and dexamethasone simultaneously, tyrosine hydroxylase activity increases to a level that is slightly less than the sum of the increases observed in cells treated with either inducing agent alone. These approximately additive effects are observed at all time points during treatment with these compounds and are observed at 8-bromocyclic AMP and/or dexamethasone concentrations which produce the maximal induction of the enzyme when either inducing agent is employed alone. These results are similar but not identical to those we observe when mouse neuroblastoma cells are exposed simultaneously to both cyclic AMP and glucocorticoids (15). Treatment of the neuroblastoma cells with both inducing agents results in a synergistic effect, instead of an additive effect, on the enzyme induction. During stress, it is likely that catecholaminergic cells are exposed to both glucocorticoids and cyclic AMP; thus, this additive or greater than additive response to the two hormones in these cell lines may represent the physiological response to stress *in vivo*. However, studies on catecholaminergic cells other than transformed cell lines are required to verify this hypothesis.

In the PC18 cells exposed to both inducing agents the additive increases in enzyme level are paralleled by additive increases in the rate of synthesis of the enzyme. As in cells treated with either inducing agent alone, the rate of synthesis increases more rapidly than the enzyme level, but quantitatively the changes in the rate of synthesis are approximately equal to or slightly greater than the changes in the enzyme level.

We have shown in the present study that the increases in tyrosine hydroxylase enzyme level observed in PC18 cells treated with cyclic AMP-elevating agents and glucocorticoids, alone or combined, are due to increases in the rate of synthesis of the enzyme. These increases in the rate of synthesis of tyrosine hydroxylase may be due to increases in the level of $mRNA^{TH}$, since cyclic AMP and glucocorticoids have been shown to elevate $mRNA^{TH}$ in rat pheochromocytoma cells (11, 29). Alternatively, these increases may be due to a combination of effects on the level of $mRNA^{TH}$, the functional activity of the $mRNA^{TH}$, the processing of nuclear precursors to mature $mRNA^{TH}$, the transport of the $mRNA^{TH}$ from the nucleus to the cytoplasm, or to direct effects on the protein translation in the cell. These possibilities are investigated further in the accompanying paper (16).

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Send reprint requests to: Dr. A. W. Tank, Department of Pharmacology, University of Rochester Medical Center, 601 Elmwood Avenue, Rochester, NY 14642.

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