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Induction of Tyrosine Hydroxylase and Dopamine β-Hydroxylase in Cultured Mouse Neuroblastoma by 8Br-cAMP

Involvement of RNA and Protein Synthesis

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SUMMARY

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8Br-cAMP elevated tyrosine hydroxylase (E.C. 1.14.16.2, L-tyrosine, tetrahydropteridine: oxygen oxidoreductase [3-hydroxylating]) and dopamine beta-hydroxylase (E.C. 1.14.17.1, 3,4-dihydroxyphenylethylamine, ascorbate:oxygen oxidoreductase [β-hydroxylating]) activities in neuroblastoma clone NBD-2 from the mouse C-1300 tumor. The increase in activities was associated with a 5–10 fold increase in the V_{max} of both enzymes for both substrate and cofactor. Immunoprecipitation of tyrosine hydroxylase in control and treated cells demonstrated that more immunoprecipitable enzyme was present in the treated samples. Maximal elevation of both tyrosine hydroxylase and dopamine beta-hydroxylase occurred after 48 hr of treatment with 1.0 mM 8Br-cAMP. Actinomycin D (0.1 μg/ml), cycloheximide (2.0 μg/ml) and lysine-deficient medium prevented the 8Br-cAMP elevation of enzyme activity. However, removal of cycloheximide or subsequent addition of lysine (even with simultaneous addition of actinomycin D) allowed the subsequent elevation of enzyme activity. The rate of increase in enzyme activity did not exhibit the lag period typically observed in cells treated only with 8Br-cAMP. These data suggest that cAMP elevates the level of tyrosine hydroxylase and dopamine beta-hydroxylase protein and that an increased production of messenger RNA may be one of the steps necessary for these effects.

INTRODUCTION

In the adrenal medulla and the superior cervical ganglion, stress- and drug-induced increases in sympathetic nervous activity lead to an elevation of tyrosine hydroxylase and dopamine beta-hydroxylase. Further, increases in cAMP[2] levels have been found to precede the increases in these enzymes (1–7). From these studies, the hypothesis

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[2] Abbreviations used, cAMP, 3',5'-cyclic AMP; 8Br-cAMP, 8-bromo-3',5'-cyclic AMP; TH, tyrosine hydroxylase; DBH, dopamine beta-hydroxylase; PBS, phosphate-buffered saline; Me_2PH_4, 6,7-dimethyl-5, 6,7,8-tetrahydropterine.

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has emerged that increased sympathetic nerve activity regulates the production of these catecholaminergic enzymes via the intracellular messenger cAMP. We have demonstrated that elevation of cAMP levels in cultured mouse neuroblastoma cells by cyclic nucleotide phosphodiesterase inhibitors or cAMP analogues produces a marked (8) and specific (9) elevation of TH and DBH. TH and DBH activities were elevated 10–40 fold depending upon the treatment conditions. The induction of enzyme activity was tightly correlated with the ability of the treatments to increase cAMP levels (9). More importantly, little or no changes in monoamine oxidase, catechol *O*-methyltransferase, choline acetyltransferase or acetylcholinesterase activities were observed (9).

The present experiments further examined the elevation of TH and DBH by 8Br-cAMP in neuroblastoma clone NBD-2 (9, 10) and attempted to determine the possible involvement of RNA and protein synthesis therein.

MATERIALS AND METHODS

Culture conditions. Neuroblastoma clone NBD-2 was grown as previously described (10) at 37° in a solution consisting of F-12 media supplemented with newborn calf serum (GIBCO) in an atmosphere of 5% CO_2 and 95% air. Neither antibiotics nor antifungal agents were used. In amino acid deprivation experiments and amino acid incorporation studies, F-12 media was made in the laboratory. Dialyzed newborn calf serum was used in these cases, and media was prepared from chemicals purchased from Sigma Chemical Co.

Cultures were plated at 0.4×10^6 cells per 6 cm diameter culture dish in 5 ml of media. Experimental treatments of the cultured cells were always begun 24 hr after the cells were replated. Culture medium, with or without added drugs, was replaced every 24 hr thereafter. Cells were removed from the culture dish either by scraping with a rubber policeman or by treatment with 0.25% (w/v) Viokase (GIBCO) in PBS (137 mM NaCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl) for 3 min. The action of Viokase was halted by addition of an equal volume of PBS containing 10% newborn calf serum. Cells were centrifuged at 40 × *g* for 5 min, rinsed with PBS, repelleted, and frozen at −70° until assayed.

Enzyme assays. Tyrosine hydroxylase was assayed in neuroblastoma cells after freeze-thawing three times in a dry ice-acetone bath. Triton X-100 (0.01%, v/v, final) was added to aid in the disruption of the cells. TH activity was assayed according to Waymire *et al.* (10, 11) using 100 μM L-[1-^{14}C]tyrosine (10 mCi/mmole), 2.0 mM 6,7-dimethyl-5,6,7,8-tetrahydropterine and 50 mM β-mercaptoethanol (to keep cofactor reduced). Enzyme activity was linear with time for at least 15 min and with cell number to 15×10^6 cells. TH activity, from either control or treated cells, is entirely soluble in both fresh and stored samples.

Dopamine beta-hydroxylase was measured in 30,000 g × 30 min supernatants of 50 mM Tris-HCl (pH 5.5), 0.01% (v/v) Triton X-100 homogenates of $0.1–10 \times 10^6$ neuroblastoma cells. Under these conditions all DBH activity appears in the supernatant. The method of Molinoff *et al.* (12) was employed using 4.0 mM ascorbate, 1.0 mM tyramine and 40 mM fumarate (to maximize the affinity of the enzyme for tyramine [13]). Previous studies established that 1.96 mM $CuSO_4$ provides optimal activity for these conditions in either control (10) or 8Br-cAMP-treated neuroblastoma cells (9). [^{3}H]-Methyl-*S*-adenosyl-L-methionine (90 mCi/mmole, 0.05 μCi/assay) was used as the methyl donor for the phenylethanolamine *N*-methyltransferase (E.C. 2.1.1.28) catalyzed portion of the coupled assay. Samples were routinely assayed at two sample dilutions to assure that enzyme activity was proportional to sample concentration and/or to assess for the presence of inhibitors.

Protein synthesis and RNA synthesis rates. The procedure of Ball *et al.* (14) was used as described previously (9). This involves [^{3}H]leucine incorporation into acid-precipitable material and [^{3}H]uridine incorporation into acid-soluble material to assess, respectively, protein and RNA synthesis rates. Incorporation studies were performed within limits of linearity with respect to both time and amount of tissue.

Measurement of immunoprecipitable tyrosine hydroxylase activity. Antibody specific to TH was used to compare the levels of TH molecules in control and drug-treated neuroblastoma cultures. Antibody, prepared according to Lloyd and Kaufman (15) with TH from bovine adrenal medulla, was a gift from Dr. Tom Lloyd. Immunoprecipitation (16) was performed in 100 μl containing 20 mM K phosphate (pH 7.4), 140 mM KCl, 3 mM $MgCl_2$, 0.2% (v/v) Triton X-100, various amounts of freeze-thawed neuroblastoma supernatant (10,000 × *g* for 15 min) and concentrated control and/or antiserum. The mixtures were incubated at 22–24° for 1 hr and then 4° for 16 hr. The precipitates were removed by centrifugation (16,000 × *g* for 10 min) and the enzyme activity remaining in the supernatant was determined as described above. Control serum was added to each sample as necessary to maintain constant serum concentrations. Recovery of TH activity after incubation in the presence of control serum was ~50% for both control and 8Br-cAMP treated samples. Recovery in the absence of serum was 90% for both control and treated samples.

Drugs. 8Br-cAMP was prepared according to Muneyama *et al.* (17) and purified as described previously (9). Radiochemicals were purchased from New England Nuclear. Actinomycin D and cycloheximide were obtained from Sigma Chemical Co. All other drugs and chemicals were reagent grade or higher purity from commercial sources.

RESULTS

Increased levels of TH and DBH after 8Br-cAMP treatment. Previous studies (9) demonstrated that maximal elevation of TH and DBH activity occurs with a dose of 1 mM 8Br-cAMP and a treatment period of 48 hr. Substrate and cofactor kinetics for TH and DBH from control cells and cells treated (as above) with 8Br-cAMP are presented in Table 1. 8Br-cAMP elevated the V_{max} of both TH and DBH for both substrate and cofactor. The ~10-fold increases in V_{max} are similar to that reported for the effects of dibutyryl cAMP upon TH in the neuroblastoma clone NBP_2 (8). No effects of 8Br-cAMP treatment upon substrate K_m were observed, and 8Br-cAMP did not affect the K_m of DBH for its cofactor, ascorbate. 8Br-cAMP did, however, lower the K_m of TH for pterin cofactor from 1.6 to 1.2 mM.

TABLE 1

Effects of 8Br-cAMP on the kinetic parameters of tyrosine hydroxylase and dopamine beta-hydroxylase

Cells were treated with 1.0 mM 8Br-cAMP for two days. Tyrosine hydroxylase was assayed in freeze-thawed cells dialyzed 24 hr in 15 mM KCl, 1 mM K phosphate (pH 7.0). Dopamine beta-hydroxylase was assayed in 15,000 × *g* for 30 min supernatants of cells homogenized in 0.01% Triton X-100, 20 mM Tris (pH 6.0). Kinetic constants were determined from Eadie-Hofstee plots.

Parameter	Tyrosine hydroxylase		Dopamine β-hydroxylase	
	tyrosine	Me_2PH_4	tyramine	ascorbate
	μM	mM	μM	mM
K_m				
control	78	1.6	530	1.31
8Br-cAMP	77	1.2	570	1.29
	pmole/10^6 cells/hr		*ng octopamine/10^6 cells/hr*	
V_{max}				
control	39	44	24	18
8Br-cAMP	430	470	168	170

The 10-fold increases in V_{max} are several fold higher than that reported for short-term activation of TH, supporting the interpretation that 8Br-cAMP treatment increases the number of enzyme molecules (9). However, the reduction in K_m of TH for pterin cofactor is similar to that seen with short-term activation (18–21) suggesting some alteration in the catalytic activity of TH molecules. Thus, immunoprecipitation studies were performed to assess what portion of the increase in V_{max} might be due to an increase in TH molecules. The results of these studies are illustrated in Fig. 1. 8Br-cAMP treatment (1 mM, 36 hr) shifted the immunoprecipitation curve to the right. Although the curves are not parallel, there is a 4-fold increase in the zero activity intercept; in another experiment, an 8-fold increase was observed. Thus, it appeared that at least some portion of the increased TH activity resulted from an increased number of TH molecules.

Involvement of RNA and protein synthe-

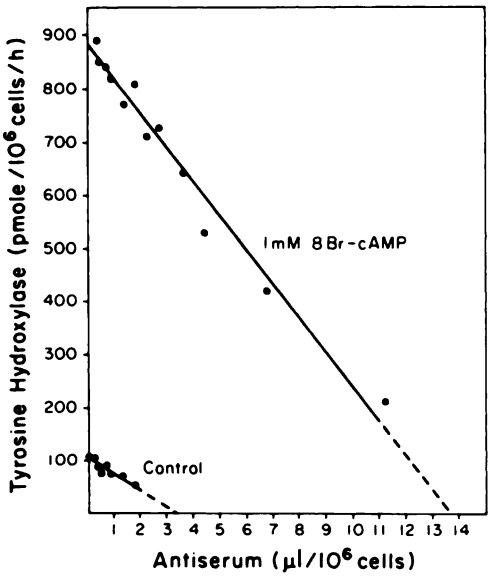

FIG. 1. *Immunoprecipitation of tyrosine hydroxylase in control and 8Br-cAMP treated cultures*

Cells were treated with 1 mM 8Br-cAMP 24 hr after replating. After 36 hr of treatment, samples were collected for immunoprecipitation as described in METHODS, and tyrosine hydroxylase activity remaining in the supernatant after immunoprecipitation was determined. Points are taken from a single experiment. In another similar experiment zero activity intercepts (least squares extrapolation) were 10.3 and 1.4 μl antiserum per 10^6 cells for 8Br-cAMP and control cultures, respectively.

sis in the elevation of TH and DBH by 8Br-cAMP. The relatively slow onset of effect (9, see also below) and the indication of an increase in enzyme molecules (above) suggested the possible involvement of either RNA or protein synthesis in mediating the effects of 8Br-cAMP. Accordingly, conditions that inhibit RNA and protein synthesis were examined for effects upon the changes in enzyme activity elicited by 8Br-cAMP.

First, experiments were conducted to establish conditions that would inhibit RNA and protein synthesis in the NBD-2 cells. Actinomycin D inhibited RNA synthesis by more than 90% at concentrations of 0.1 μg/ml and above (data not presented). And, as shown in Table 2, cycloheximide inhibited protein synthesis by ~90% at either 2 or 5 μg/ml. Omission of lysine from the medium also produced ~90% inhibition (Table 2). Also shown in Table 2, the effects of both cycloheximide and omission of lysine were reversible although addition of lysine appeared to reverse more rapidly the inhibition than did removal of cycloheximide.

The influence of 8Br-cAMP on TH and DBH activity in the presence and absence of 0.1 μg/ml actinomycin D is shown in Fig. 2. 8Br-cAMP (1 mM) elevated TH activity ~10 fold within 48 hr (left), and DBH activity was elevated ~5 fold (right). Actinomycin D completely prevented this effect (Fig. 2, closed squares).

Omission of lysine (Fig. 3) or addition of cycloheximide (Fig. 4) also prevented the increase in enzyme activity produced by 8Br-cAMP.

One possibility raised by these data is that 8Br-cAMP influenced the production of messenger RNA in the cultured NBD-2 cells. At least in the case of TH activity, this is consistent with the slow onset of 8Br-cAMP effect. In the next experiment, we tested the possibility that release from protein synthesis inhibition (by adding lysine, Table 2) might reveal an elevated level

TABLE 2

Effects of cycloheximide and lysine-deficient media on [^{3}H]leucine incorporation in cultured NBD-2 neuroblastoma cells

Cells were exposed to either cycloheximide or lysine-deficient medium for 1 hr in culture prior to a 30 min incubation in the presence of [^{3}H]leucine. To assess reversibility of the inhibition of incorporation, lysine-containing medium was replaced either 15 or 30 min before incorporation was measured. In the case of cycloheximide treatment, medium was removed, cells were rinsed with 5 ml of normal medium, and 5 ml of normal medium was left on the cells for 15 to 30 min before incorporation was measured. Values represent the average of three determinations with 1×10^6 cells per sample. Control incorporation was 17,400 cpm per 10^6 cells.

Treatment	[^{3}H]leucine incorporation (% control)		
	during treatment	15 min posttreatment	30 min posttreatment
Cycloheximide (2 μg/ml)	11	42	78
Cycloheximide (5 μg/ml)	8	38	68
Lysine-deficient medium	8	45	85

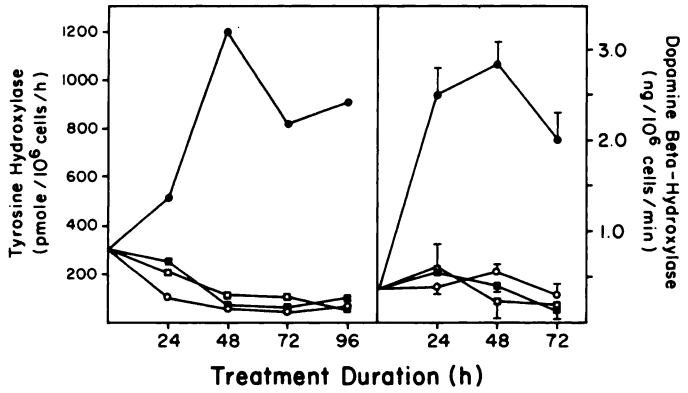

FIG. 2. *Effects of actinomycin D on the elevation of tyrosine hydroxylase and dopamine beta-hydroxylase by 8Br-cAMP*

Twenty-four hours after replating, 8Br-cAMP (1.0 mM) and actinomycin D (0.1 μg/ml) were added from concentrated stock solutions. O—O, no addition; ●—●, 8Br-cAMP; □—□, actinomycin D; ■—■, 8Br-cAMP plus actinomycin D. Tyrosine hydroxylase (left) values represent the average of 2 determinations. Dopamine beta-hydroxylase (right) values represent the mean ± standard error of four determinations.

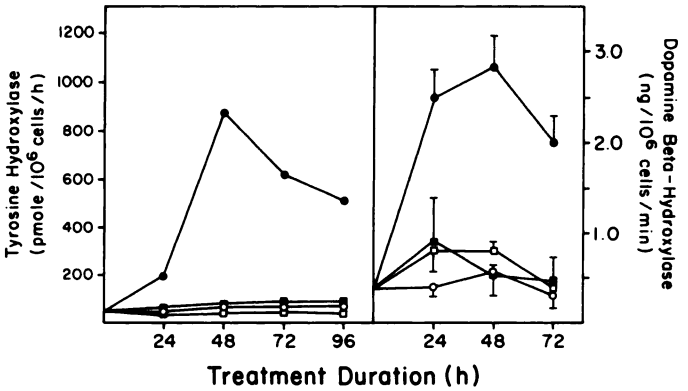

FIG. 3. *Effects of 8Br-cAMP on tyrosine hydroxylase and dopamine beta-hydroxylase in culture medium with or without lysine*

Twenty-four hours after replating, media with or without lysine was replaced and 8Br-cAMP (1.0 mM) was added. O—O, normal media; ●—●, normal media plus 8Br-cAMP; □—□, lysine-deficient media; ■—■, lysine-deficient media plus 8Br-cAMP. All other details as in Fig. 2.

of messenger RNA for TH. Replacement of lysine after 48 hr treatment with 8Br-cAMP produced an increase in TH activity that, during the first 12 hr, was ~3 fold higher than that seen during the first 12 hr of initial 8Br-cAMP treatment in normal medium (Fig. 5, left, closed circles: dashed vs solid line)—even taking into account the slight rebound seen in the absence of 8Br-cAMP (Fig. 5, right). The rate of increase in TH activity with lysine replacement continued for 36 hr with a slope comparable to that seen during the second 24 hr in cells initially treated with 8Br-cAMP. (Removal of cycloheximide also resulted in a subsequent increase in TH activity [data not presented]).

Also presented in Fig. 5, actinomycin D, added to the medium when lysine was replaced, did not prevent the more rapid increase in TH activity produced by lysine replacement. Thus it appeared that some compound did accumulate during 8Br-cAMP treatment in the lysine-deficient medium. It should also be noted that because TH levels were elevated in the presence of

actinomycin D, the likelihood that the effects of actinomycin D presented in Fig. 2 were due to cytotoxicity appears minimal.

DISCUSSION

The results indicate that the interaction of 8Br-cAMP with the NBD-2 neuroblastoma cells to increase TH and DBH activity 1) requires intact protein synthesis machinery and 2) probably involves an increased number of TH and DBH molecules. Further, because the protein synthesis inhibitors block the elevation, an increased synthesis (as opposed to slower degradation) of TH and DBH appears likely.

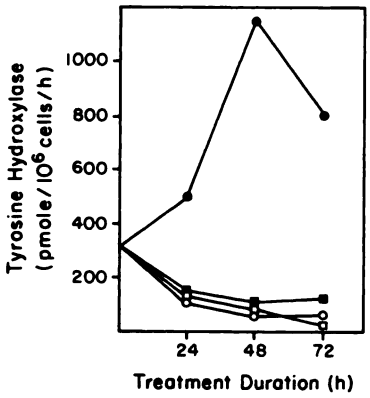

FIG. 4. *Effects of cycloheximide on the elevation of tyrosine hydroxylase by 8Br-cAMP*

Experimental details as in Fig. 2. ○——○, no addition; ●——●, 8Br-cAMP (1.0 mM); □——□, cycloheximide (2.0 μg/ml); ■——■, 8Br-cAMP plus cycloheximide. Averages of four determinations.

Although protein synthesis was necessary for expression of the effects of cAMP, actinomycin D (to block DNA-dependent RNA synthesis) also blocked the effects of cAMP. Since RNA synthesis appears necessary for the cAMP-induced elevation of both TH and DBH, a primary locus for the cAMP effect may be the rate of messenger RNA synthesis for these two enzymes. This is consistent with the ability of protein synthesis inhibitors to block the elevation and with the response of the NBD-2 cells upon return from lysine-deficient to normal media. Normally, there is a lag period of 24 hr in 8Br-cAMP treated cells before TH begins rapidly increasing. Such a lag period and the decrease therein upon lysine replacement support the interpretation that 8Br-cAMP exerted an effect during protein synthesis inhibition that was then more rapidly expressed upon release from the inhibition. Since actinomycin D did not block the increase in TH when lysine was replaced, messenger RNA production is a likely candidate for such an effect.

In addition, the conclusion that the long-term stimulation of TH and DBH activity by 8Br-cAMP results from an alteration in transcription is compatible with results from studies examining transsynaptic induction of TH in the intact animal (22, 23). *In vivo,* actinomycin D blocks the increase in TH activity associated with increased sympathetic nerve activity. In contrast to the present studies, however, the induction

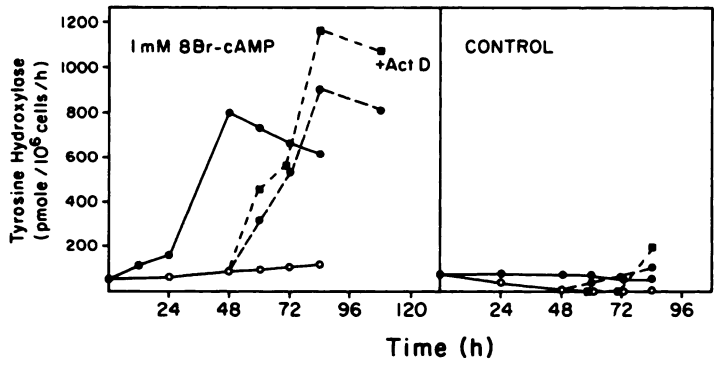

FIG. 5. *Effects of lysine addition on the elevation of tyrosine hydroxylase by 8Br-cAMP*

Twenty-four hours after plating, the cells were placed in medium with (●——●) or without (○——○) lysine and 1 mM 8Br-cAMP was added to half of the samples (left). Forty-eight hours later, some of the cells that were in lysine-deficient medium had lysine added (dashed lines). Half of those cells also had 0.1 μg/ml actinomycin D added (squares). All other details as in Fig. 2. Values represent the mean of four determinations.

of DBH was not blocked (23).

The increase in zero activity intercept produced by 8Br-cAMP (Fig. 1) clearly suggests that 8Br-cAMP increased the number of TH molecules. The difference in slope of the two lines and the difference in K_m for pterin cofactor (Table 1) also suggest that 8Br-cAMP exerted an effect in addition to the increase in TH levels. Similar kinetic changes in the catalytic activity of TH have been observed with cAMP treatment in other tissues (20, 21) and may represent at least a small part of the long-term increases observed in the present study. The K_m alterations in those studies occurred rapidly (minutes versus days) and, as such, may represent a large portion of the increased activity seen at 24 hr in these studies. Such effects would be in addition to an increase in the level of tyrosine hydroxylase at the 48 hr interval.

Recently, Costa and co-workers (24), after finding an apparent elevation of protein kinase catalytic subunits in nuclei of adrenal medulla tissue, hypothesized that cAMP, by activating a protein kinase that phosphorylates a nuclear protein, elevates synthesis of messenger RNA from DNA. Our results are consistent with such an hypothesis.

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