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Regulation of Tyrosine Hydroxylase Activity in Cultured Mouse Neuroblastoma Cells: Elevation Induced by Analogs of Adenosine 3′:5′-Cyclic Monophosphate

(adrenergic clones/cell morphology/x-irradiation)

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ABSTRACT **Mouse neuroblastoma cells in culture have been used as a model for the study of the mechanism by which activities of tyrosine hydroxylase (EC 1.14.3.a) are regulated in sympathetic tissue. The activity of tyrosine hydroxylase in cultured cells drops to barely detectable activities after 1 week and remains low for months in culture in the uncloned cell line of neuroblastoma. Activity in an adrenergic clone isolated from the uncloned line has about 20% of the activity of the fresh grated tumor cell. N^6, $O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphate causes a concentration and time-dependent increase in enzyme activity in both the cloned and uncloned cell lines. Enzyme activity is elevated by other stable analogs of adenosine 3′:5′-cyclic monophosphate, notably the N^6-monobutyryl, 8-aminomethyl, and 8-methylthio derivatives of the cyclic nucleotide; by the inhibitor of cyclic nucleotide phosphodiesterase, papaverine; and by sodium butyrate. Changes in cell morphology and tyrosine hydroxylase activity are shown not to be necessarily related.**

Cultured mouse neuroblastoma cells provide an excellent preparation for the study at the molecular level of the biochemistry and microanatomy of nervous tissue. Neuroblastoma tumor cells in culture synthesize acetylcholine (1, 2), dopamine, and norepinephrine (4), and these cells have been shown to extend cytoplasmic processes under appropriate conditions (4–7). The morphologically altered cells contain microtubules, neurofilaments, and dense core vesicles (4, 8) and have membranes capable of generating action potentials either spontaneously (9), or in response to acetylcholine (10) or electrical stimulation (9, 10).

Recently, neuroblastoma cultured cells have been used extensively to study the regulation of several of the enzymes involved in metabolism of acetylcholine (1, 2, 11). Little information is available about catecholamine synthesis in cultured neuroblastoma cells other than the observations that the enzymes necessary for metabolism are present (12–14). In this study, we investigated the control, in cultured neuroblastoma cells, of tyrosine hydroxylase (TyrOHase), the enzyme regarded to be the rate-limiting step in the synthesis of catecholamines (15). A preliminary report of this study has been reported in abstract form.‡

Abbreviations: TyrOHase, tyrosine hydroxylase; Me_2PH_4, 6,7-dimethyl-5,6,7,8-tetrahydropterin; DOPA, β-(3,4-dihydroxyphenyl)-L-alanine, dopamine, β-(3,4-dihydroxyphenyl)-ethylamine.

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METHODS

Culture Conditions. The procedures for culturing mouse neuroblastoma have been described (16). In brief, tumor tissue or uncloned cultured cells derived from the neuroblastoma C-1300 were dissociated by treatment with 0.25% Viokase Grand Island Biological Co., Grand Island, N.Y., and the single cells were plated in Falcon plastic dishes or flasks in F12 medium Grand Island Biological Co. supplemented with 10% newborn calf serum without gammaglobulin, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were maintained at 36° in humidified air containing 5% CO_2. The average doubling time of either the uncloned neuroblastoma cells or the cloned line (NBP_2) is about 24 hr. Neuroblastoma cells in culture have acetylcholinesterase activity, but do not contain butyrylcholinesterase (11).

Tyrosine Hydroxylase (EC 1.14.3.a) Assay. Cells were isolated for assay of TyrOHase by treatment with 0.25% Viokase. Viokase treatment was terminated by dilution with an equal volume of F-12 medium. The cells, isolated by centrifugation 700 × *g* for 6 min, were washed twice in tyrosine-free medium, and an aliquot was taken for estimate of cell concentration and, in some cases, protein. TyrOHase was solubilized by treatment with 0.1% Triton X-100 (Beckman), and the activity was measured by a radiochemical technique (17) in which L-[*1*-^{14}C]tyrosine (New England Nuclear Corp.) was enzymatically converted to L-[*1*-^{14}C]dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase. Aromatic L-aminoacid decarboxylase purified from hog kidney, included in the incubation medium, converted all the DOPA formed to dopamine, and the $^{14}CO_2$ was collected in 0.2 ml of NCS$^{(R)}$ solubilizer that was contained in a plastic well (Kontes Co., Vineland, New Jersey), suspended from the rubber septum covering the flask opening. Wells were transferred to toluene scintillation fluid, and the radioactivity was counted by liquid scintillation spectrometry. Preliminary experiments, conducted to establish appropriate conditions for the assay of mouse neuroblastoma TyrOHase, revealed estimated K_m values for tyrosine and 6,7-dimethyl-5,6,7,8-tetrahydropterin (Me_2PH_4) of 0.06 and 0.10 mM, respectively. To assure adequate cofactor concentration throughout the assay, 2 mM Me_2PH_4 was chosen for routine use. The highest concentration of tyrosine that can be used is 0.10 mM, since higher concentrations inhibit the enzyme. Shiman *et al.* (18) have reported a similar observation for bovine adrenal TyrOHase when biopterin was used as cofactor. The assay incubation found to provide optimal activity

TABLE 1. *Tyrosine hydroxylase activity in uncloned mouse neuroblastoma cells after various periods of culturing*

Condition	Tyrosine hydroxylase activity*
Fresh grated tumor cells	57.93 ± 15.20†
Cultured 3 days‡	18.50 ± 4.62
Cultured 100 days‡	1.42 ± 0.51
Cultured 380 days‡	1.38 ± 0.48

* pmol of $^{14}CO_2$ formed in 30 min per 10^6 cells.

† Each value is the average of four separate determinations ± standard error.

‡ Medium was changed every second day.

includes in 0.2 ml: 40 μmol of sodium acetate-HCl (pH 6.1); 0.4 μmol of 6,7-dimethyl-5,6,7,8-tetrahydropterin (Aldrich Chemical Co.); 0.2 μmol of ferrous sulfate; 10 μmol of 2-mercaptoethanol; 0.02 μmol of L-[*1*-^{14}C]tyrosine (10 Ci/mol) (New England Nuclear Corp.), and enzyme from 2.0 to 10.0 × 10^6 neuroblastoma cells. The assay can detect 5.0 pmol of $^{14}CO_2$ formed per 30 min incubation. Values reported represent the average of at least four experiments unless otherwise noted. Results are expressed as pmol of product formed per 30 min per million cells. Activity is not based on protein since this may vary 2-fold during different phases of growth and during treatment with various agents. Protein concentrations after several treatments are given to aid the comparison of our data with that published, which is based on protein concentration. Protein was determined by a modification of the method of Lowry (19). Cell concentration was determined in triplicate, in the Coulter counter.

Morphology. 300–500 Cells were scored for changes in cell size and morphology after various treatments. Cells that form processes greater than 50 μm in length, were termed morphologically differentiated.

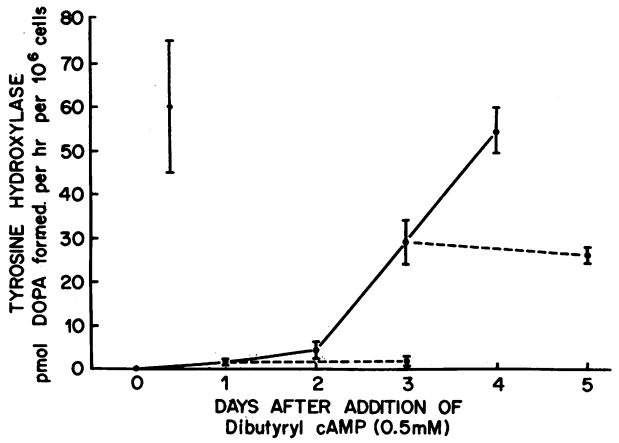

FIG. 1. Tyrosine hydroxylase activity in neuroblastoma tumor freshly removed from mice (*top, left*) and in mouse neuroblastoma cells in culture for at least 100 days after exposure to 0.5 mM N^6, $O^{2'}$-dibutyryl cAMP for various periods of time (*solid line*). In some studies, after 1 or 3 days, the medium was replaced by fresh medium lacking the cyclic nucleotide, and the incubation was continued for two days (*dashed line*).

TABLE 2. *Tyrosine hydroxylase activity in uncloned mouse neuroblastoma cells after various treatments*

Treatment*	Tyrosine hydroxylase activity† 1 Day	3 Days
Serum-free medium	1.4 ± 0.5‡	1.5 ± 0.5
Dibutyryl cAMP (0.5 mM)§	1.1 ± 0.4	32.3 ± 4.8
Dibutyryl cAMP (1.0 mM)	5.0 ± 1.0	50.2 ± 6.5
Dibutyryl cAMP (0.5 mM)† Cycloheximide (5 μg/ml)¶	1.4 ± 0.4	1.8 ± 0.5
Cells in log phase (3 Days after replating)	1.4 ± 0.4	
Cells in confluent phase (6 Days after replating)	1.5 ± 0.4	

* Uncloned cells maintained in culture more than 1 year before treatment.

† Same as in *, Table 1.

‡ Each value is the average of four separate determinations ± standard error.

§ Drugs were added 1 day after replating.

¶ Protein synthesis was inhibited about 90%.

Drug Treatment. Cells were plated at a density of 0.25 × 10^6 cells per 15 ml of medium in 75-mm culture flasks 24 hr before treatment. Growth medium and drug were replaced every second day thereafter.

Radiation. X-irradiation treatments were initiated 24 hr after plating. Radiation factors were 250 kVp and 30 mA, with added filtration of 0.5 mm of Cu and 1.0 mm of Al (hvt = 1.36 mm of Cu). The distance between the target and turntable was 60 cm. Dose rate was 89 rads/min. Cells were x-irradiated at room temperature (22–24°) with a single dose (600 rads) or a fractionated dose (200 rads/day, total 1000 rads).

RESULTS

Tyrosine hydroxylase in primary tumor and in cultured cells

Cells from the primary tumor were dissociated with 0.25% Viokase and the activity was determined. Other cells were placed in culture, and the medium was changed every 3 days. Cells of the primary tumor had high TyrOHase activity (12, 13, 20). The enzyme activity dropped with time in culture, so that by 3 days the activity per million cells was only 25%, and by 7 days the activity was only 2% of that detected in fresh grated tumor cells. Uncloned cells in culture over 1 year maintain this relatively low TyrOHase activity (Table 1).

Modification of tyrosine hydroxylase activity in uncloned cells

Conditions known to induce neurite formation (4–7) and stimulate acetylcholine metabolizing enzymes in cultured neuroblastoma cells (1, 2, 11) were studied for their effect on TyrOHase activity (Table 2). Neither culturing the cells in the absence of newborn calf serum nor growing the cells to a confluent stage is effective in altering the enzyme activity.

TABLE 3. *Tyrosine hydroxylase activity and percent differentiation in cultured mouse neuroblastoma cells 3 days after treatment*

Treatment*	Differentiated cells†	Tyrosine hydroxylase‡
Control	8 ± 2.0§	1.6 ± 0.7
Dibutyryl cAMP (0.5 mM)	45 ± 4.5	98.7 ± 9.5
3′:5′-cAMP (0.5 mM)	8 ± 1.9	1.7 ± 0.8
5′-AMP (0.5 mM)	8 ± 1.8	0.8 ± 0.6
Sodium butyrate (0.5 mM)	9 ± 3.1	36.2 ± 8.1
X-irradiation (single dose—600 rads)	39 ± 5.0	<1.0
X-irradiation (200 rads/day—1000 rads)	27 ± 4.2	<1.0
X-irradiation (single dose—600 rads) plus 0.5 mM dibutyryl-cAMP	44 ± 4.8	111.8 ± 39.9

* Treatment begun 1 day after replating. Cells were maintained in culture over 1 year before treatment.

† Percent of total cells.

‡ Same as in *, Table 1.

§ Each value represents the average of four separate determinations ± standard error.

However, treatment with $N^6,O^{2\prime}$-dibutyryl adenosine-3′:5′-cyclic monophosphate, also known to stimulate neurite formation in cultured neuroblastoma cells (6), produces a dramatic elevation in TyrOHase activity. Addition of 0.5 or 1.0 mM of $N^6,O^{2\prime}$-dibutyryl cAMP (Boehringer Co.) 1 day after replating cells is associated with an increase in activity of TyrOHase 1 or 3 days later; this increase is concentration- and time-dependent (Table 2). Addition of cycloheximide (5 μg/ml) with the $N^6,O^{2\prime}$-dibutyryl cAMP blocks the effect of the dibutyryl cAMP on the enzyme activity. The action of cycloheximide is difficult to interpret, however, since this drug proved to be toxic to the cells. The effect of the dibutyryl cAMP on TyrOHase activity of neuroblastoma cells in culture persists for at least 48 hr after exposure to the cyclic nucleotide (Fig. 1).

Cell morphology and tyrosine hydroxylase activity

The percentage of cultured neuroblastoma cells that change morphologically is similar when cells are grown either in the presence of 0.5 mM dibutyryl cAMP, or in the absence of newborn calf serum, or after x-irradiation (Table 3). With each of these treatments, the increase in cell numbers is markedly reduced (11). Of these treatments, only dibutyryl cAMP altered the TyrOHase activity. Cells subjected to x-irradiation are capable of responding to other stimuli that induce an increase in TyrOHase activity, since addition of dibutyryl cAMP to the irradiated cells causes an elevation in the enzyme activity (Table 3). No change in TyrOHase activity or cell morphology occurs with either 3′:5′-cAMP or 5′-AMP, although cell division is reduced by the cyclic nucleotide. Unexpectedly, sodium butyrate (0.5 mM), a possible breakdown product of dibutyryl cAMP, produces a substantial increase in TyrOHase activity. Sodium butyrate has no apparent effect on cell morphology, although the rate of cell division is markedly reduced by exposure to this substance.

TABLE 4. *Effect of cyclic nucleotides and papaverine on tyrosine hydroxylase activity in cultured mouse neuroblastoma cells*

Addition*	Tyrosine hydroxylase activity†
None (4)‡	1.7 ± 0.3
$N^6,O^{2\prime}$-Dibutyryl-3′:5′-cAMP (3)	100.7 ± 1.6
Papaverine, 0.13 mM, 1 day (2)	5.8 (6.6; 5.0)
Papaverine, 0.13 mM (2)	27.3 (28.6; 26.1)
N^6-Monobutyryl-3′:5′-cAMP (3)	70.2 ± 4.7
8-Aminomethyl-3′:5′-cAMP, 0.3 mM (2)	17.9 (16.0; 19.7)
8-Furfurylamino-3′:5′-cAMP, 0.3 mM (2)	3.8 (3.1; 4.6)

* Unless otherwise specified, additions were made at a final concentration of 0.5 mM and exposure was for 3 days.

† Same as in *, Table 1.

‡ Numbers in parentheses represent number of individual determinations.

Tyrosine hydroxylase, stable cyclic nucleotide analogs, and papaverine

Because of the unexpected stimulation of TyrOHase by sodium butyrate, experiments were conducted to clarify the mechanism of stimulation by dibutyryl cAMP. Incubation of cells in the presence of 0.13 mM papaverine, a compound reported to induce an increase in the concentration of endogenous cyclic nucleotides by inhibition of phosphodiesterase (21), as well as to cause morphological changes similar to those observed with dibutyryl cAMP (5), is associated with a time-dependent increase in TyrOHase activity. N^6-Monobutyryl-3′:5′-cAMP causes morphological changes similar to those induced by dibutyryl cAMP and produces increased TyrOHase activity. Treatment with stable analogs of cAMP that are neither Na^+ salts nor contain a butyrate substituent, notably 8-aminomethyl and 8-furfurylamino cAMP, were also effective in induction of an increase in the enzyme activity (Table 4).

Tyrosine hydroxylase in an adrenergic clone of neuroblastoma

Although working with an uncloned mixed population of neuroblastoma cells permits detection of responses to a particular treatment of any cell type, it presents the possible complication that the rate of replication of the various cell types might be differently influenced by the treatment, and hence, the population of cells might be altered. To eliminate this possibility we have also studied regulation of TyrOHase in a neuroblastoma clone (NBP_2), which is rich in TyrOHase.

Attempts to establish homogeneous lines derived from the neuroblastoma mixed population revealed several clones with TyrOHase activity near that found in the fresh grated tumor (report in preparation). One such clone with high basal TyrOHase activity (20% that of the fresh grated tumor) was used in experiments for determination of the role of cyclic nucleotides and other treatments on regulation of TyrOHase activity (Table 5). As in uncloned neuroblastoma cells, neither growth in serum-free medium nor growth to a steady-state elevates TyrOHase activity. This is in contrast to the report of Amano *et al.* (14) that TyrOHase activity increases as the rate of cell division decreases. Treatment of

TABLE 5. *Tyrosine hydroxylase activity and protein concentrations in mouse neuroblastoma clone NBP_2 after various treatments*

Condition*	Tyrosine hydroxylase†	Protein‡
Control, log phase§	15.1 ± 1.9	1.6 ± 0.2
Control, confluent phase¶	11.2 ± 0.7	—
Serum-free medium‖	17.3 ± 0.4	—
Dibutyryl cAMP, 0.5 mM	473 ± 17	3.11 ± 0.33
8-Methylthio-cAMP, 0.3 mM	587 ± 9	2.95 ± 0.28
Papaverine, 0.13 mM	977 ± 46	3.00 ± 0.23
Sodium butyrate, 0.5 mM	300 ± 12	2.80 ± 0.18

* All drug treatments are started 1 day after replating and continued for 3 days.
† pmol of product formed in 30 min per 10^6 cells.
‡ mg per 10^6 cells.
§ 3 Days after replating.
¶ 6 Days after replating.
‖ Grown in the absence of newborn calf serum for 3 days.

cells for 3 days with 0.5 mM dibutyryl cAMP or 8-methylthio cAMP produced dramatic elevations in TyrOHase activity, as did the inclusion of the phosphodiesterase inhibitor, papaverine, in the growth medium for 3 days. As in the uncloned cell line, sodium butyrate is also effective in increasing TyrOHase activity.

Table 6 presents a comparison of the properties of TyrOHase from control cells and from cloned cells stimulated with dibutyryl cAMP. The Michaelis constants calculated from Lineweaver–Burk plots are similar in both cases. Only the V_{max} for the reaction is altered by treatment with dibutyryl cAMP. TyrOHase from either stimulated or control cells is inhibited by the inclusion of 0.02 M 3-iodotyrosine in the assay. Ferrous iron is apparently bound tightly to the enzyme, since its omission from the assay causes no reduction

TABLE 6. *Properties of tyrosine hydroxylase from control and stimulated mouse neuroblastoma clone NBP_2*

Parameter	Control*	Stimulated†
	mM	
K_m tyrosine‡	0.061	0.059
K_m Me_2PH_4‡	0.099	0.100
	Tyrosine hydroxylase activity§	
V_{max}	17.3	485
V_{max}, 20 mM 3-iodotyrosine added to assay	0.4	12
V_{max}, ferrous iron omitted from assay	17.4	482
V_{max}, 1 mM α,α'-dipyridyl added to assay	3.4	78

* 4 Days after replating.
† Treatment with dibutyryl cAMP started 1 day after replating and continued for 3 days.
‡ Determined from Lineweaver–Burk plots.
§ Same as in †, Table 5.

TABLE 7. *Tyrosine hydroxylase activity in mouse neuroblastoma clone NBP_2. Evaluation of possible interaction between dibutyryl cAMP-treated cells and control cells*

Enzyme source	Tyrosine hydroxylase activity*
Control cells†	15.7
Dibutyryl cAMP-treated cells‡	480.3
Mixture of dibutyryl cAMP-treated§ and control cells	485.4
Control cells + 0.5 mM dibutyryl cAMP in assay medium	15.6

* Same as †, Table 5.
† 4 Days after replating.
‡ Drug treatment started 1 day after replating and continued for 3 days.
§ Equal amounts of enzyme from each source was used.

in enzyme activity, while the inclusion of 1.0 mM α,α'-dipyridyl reduces the enzyme activity by 80%.

The results of the kinetic study suggest that an increase in TyrOHase concentration is responsible for elevation of its activity. Mixing experiments eliminate the possibility that the observed increased activity is due to inactivation of an endogenous inhibitor. The combination of control and dibutyryl cAMP-treated cells yields a TyrOHase activity that approximates the sum of the activities of individually assayed preparations (Table 7). Thus, unless an irreversible inhibitor is implicated, stimulation with dibutyryl cAMP is not due to inactivation of an inhibitor. Dibutyryl cAMP added directly to the assay mixture is also ineffective in changing TyrOHase activity (Table 7).

DISCUSSION

The results obtained in our study indicate that TyrOHase activity in mouse neuroblastoma cells is regulated by a mechanism that is intimately associated with the concentration of cAMP. If the mouse neuroblastoma may be considered as a model for adrenergic nervous tissue, these results suggest that TyrOHase activity, and therefore catecholamine synthesis, may be regulated by some mechanism involving cAMP. This conclusion is supported by results of experiments in which administration of dibutyryl cAMP *in vivo* stimulates TyrOHase activity in adrenal tissue (22).

Regulation of choline acetyltransferase and acetylcholinesterase activity in cultured neuroblastoma cells has been reported to be closely linked with alterations in cell morphology and growth rate (1, 2, 11). Amano *et al.* (14) recently reported that in adrenergic clones TyrOHase activity increases as the growth-rate reaches stationary phase. In our cell line, retardation of growth rate (stationary phase) is not associated with an elevation of TyrOHase activity, and with other treatments (serum-free medium and irradiation) associated with neurite formation, no elevation in TyrOHase activity is observed. Conversely, TyrOHase activity is elevated by treatment with sodium butyrate, which does not produce neurite formation. Treatment with serum-free medium and cytosine arabinoside, which cause inhibition of cell division as well as morphological differentiation, increase

acetylcholinesterase activity without affecting TyrOHase activity (23).

Stimulation of TyrOHase by sodium butyrate was unexpected in these studies. However, it is unlikely that the elevation produced by dibutyryl cAMP is mediated solely by butyric acid, which is formed by hydrolysis of this cyclic nucleotide. Exposure of cells to the more stable analog of cAMP, monobutyryl cAMP, also produces elevation of TyrOHase activity, as does treatment with other stable analogs not containing sodium butyrate. Papaverine stimulation of TyrOHase activity supplies additional evidence that the mechanism of the dibutyryl cAMP-induced elevation is due, at least in part, to the cyclic nucleotide component of the molecule.

The elevation of TyrOHase activity by sodium butyrate, the salt of a short-chain organic acid, may be biologically significant. This compound bears close resemblance to neurobiologically active compounds; such as γ-aminobutyric acid and γ-hydroxybutyric acid. Sodium butyrate could be mimicking the action of one of these naturally occurring substances.

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