Cyclic 3′,5′-AMP Phosphodiesterase Activity During Cyclic AMP-Induced Differentiation of Neuroblastoma Cells in Culture[1] (37033)

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Cyclic AMP appears to be linked with the "differentiation" of malignant (1–7) and normal (8–9) nerve cells in culture. This cyclic nucleotide may be involved in the regulation of metabolism and function of mammalian nervous tissue (10). Thus an understanding of cyclic AMP metabolism is important to elucidate the mechanism of neural differentiation and functions. An important factor limiting the magnitude and duration of action of cyclic AMP in tissues is the rate of enzymatic destruction of the nucleotide by cyclic 3′,5′-AMP phosphodiesterase (PDE). Of all mammalian tissue studies the brain has the highest PDE activity (11). Therefore, the knowledge of regulation of PDE activity may be important in clarifying the role of cyclic AMP in the differentiation and metabolism of nervous tissue. Here, we report that the PDE activity markedly increases in cyclic AMP-induced "differentiated" mouse neuroblastoma cells in culture. A high level of cyclic AMP appears to be correlated with a high level of PDE.

Materials and Methods. The procedures for culturing neuroblastoma cells and for making the solutions of $N^6O'_2$-dibutyryl adenosine 3′,5′-cyclic monophosphate, 5′-AMP, theophylline and prostaglandin (PG)E_1 (a stimulator of adenylate cyclase), and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (RO 20-1724), an inhibitor of PDE (12), were previously described (1–5). The clone NBA2(1) was used in this study. This clone has a low level of tyrosine hydroxylase (13) and a high level of acetylcholinesterase (4) and catechol-*o*-methyltransferase (14) but lacks choline acetyltransferase; however, tyrosine hydroxylase activity can be stimulated by 10- to 20-fold by sodium butyrate and dibutyryl cyclic AMP (13). To assay the PDE activity, cells (0.5×10^6) were plated in large Falcon plastic flasks (75 cm^2) and dibutyryl cyclic AMP (0.5 m*M*), PGE_1 (10 μg/ml) and RO 20-1724 (200 μg/ml) were added separately 24 hr later. The fresh growth medium and fresh drug solution were added 2 days after treatment, and the enzyme was analyzed 3 days after drug treatment. Since X-irradiation of neuroblastoma cells also causes morphological "differentiation" similar to that produced by dibutyryl cyclic AMP (15), the PDE activity was also measured in X-ray-induced differentiated cells. The radiation factors were described previously (15). Cells were X-irradiated with 600 rads 24 hr after plating, and the enzyme was analyzed 3 days later. The fresh growth medium was changed at Day 2. For enzyme analysis, cells were washed twice with 5 ml cold Tris–buffer (pH 7.4) and then removed by a rubber policeman in 2 ml of buffer and homogenized. The PDE activity was measured according to the method described previously (16). In brief, the procedure involves conversion of ^{3}H-cyclic AMP to ^{3}H-AMP which is then converted to ^{3}H-adenosine by nucleotidase. The activity of ^{3}H-adenosine is counted. The reaction mixture consists of 5 μl 0.10 *M* Tris–buffer (pH 7.4), 5 μl 0.02 *M* $MgSO_4$, 5 μl 0.2 *M* dithiothreitol (DTT), 10 μl nucleotidase (2 mg/ml), 10 μl ^{3}H-cyclic AMP (16,000 cpm/0.17×10^{-5} *M* cAMP) and 15

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TABLE I. Relationship Between Morphological Differentiation and Cyclic 3′,5′-AMP Phosphodiesterase (PDE) Activity in Neuroblastoma Cell Culture.[a]

Treatment	Differentiated cells (% of total cells)	PDE activity (μmole/min/mg protein)
Control (exponential)	9 ± 2	5.6 ± 0.3[b]
Control (confluent)	10 ± 2	4.9 ± 0.5
Dibutyryl cyclic AMP (0.5 m*M*)	51 ± 5	16.1 ± 1.3
Prostaglandin E_1 (10 μg/ml)	72 ± 5	13.5 ± 0.8
RO 20-1724 (200 μg/ml)	71 ± 5	15.9 ± 1.2
Sodium butyrate (0.5 m*M*)	9 ± 3	5.7 ± 0.9
5′-AMP (0.5 m*M*)	10 ± 2	6.4 ± 0.8
3′,5′-cyclic AMP (0.5 m*M*)	8 ± 2	3.6 ± 0.4
600 rads	43 ± 3	6.0 ± 0.7
Theophylline (0.5 m*M*)	7 ± 3	5.8 ± 0.5

[a] Cells (0.5×10^6) were plated in Falson plastic flasks (75 cm^2) and each drug was added individually 24 hr later. Fresh drug and growth medium were added 2 days after treatment and the morphological differentiation and enzyme activity were determined 3 days after treatment according to the method described in the section, Materials and Methods. Each value represents an average of 7–10 samples.

[b] Standard deviation.

μl water. The reaction was started by addition of 10 μl of cellular homogenate. The contents were incubated at 30° for 10 min and the reaction was stopped by boiling the contents for 3 min. The tubes were cooled at 0° and 40 μl of assay volume were spotted on a 17 × 17 mm square of Amberlite SB-2 ion exchange resin loaded paper. The papers were dried and ^{3}H-adenosine was eluted in 1.0 ml of 0.1 m*M* ammonium formate (pH 7.0) in a scintillation vial for 15 min. A dioxane solvent system (10 ml) was added, the paper was removed and the radioactivity of adenosine was counted in a liquid scintillation B spectrometer. Data were expressed as micromoles of ^{3}H-cyclic AMP hydrolyzed per minute per milligram of protein.

Results and Discussion. Table I shows that the morphologically "differentiated" cells induced by dibutyryl cyclic AMP, PGE_1 and RO 20-1724 had PDE activity about threefold higher than those which were relatively undifferentiated. Since the cyclic AMP level in "differentiated" cells increases 3- to 5-fold after treatment with PGE_1 or RO 20-1724 (unpublished data), it appears that during morphological differentiation of mouse neuroblastoma cells both the levels of cyclic AMP and PDE markedly increase. The level of PDE activity in the neuroblastoma cells is not related to the growth rate. This is shown by the fact that sodium butyrate, 5′-AMP and 3′,5′-cyclic AMP which inhibit growth without causing morphological differentiation (1) did not increase the PDE activity. The enzyme activity also did not change when cells were allowed to reach the confluent phase of growth (Table I). Theophylline did not change the level of PDE. This is consistent with the fact that theophylline failed to induce morphological differentiation in neuroblastoma cells (3). This may be due to the fact that theophylline does not inhibit PDE activity in intact cells. X-Ray-induced differentiated cells did not show an increase in the PDE activity. This suggests that the morphological "differentiation" is not always associated with the high level of PDE.

Figure 1 shows that dibutyryl cyclic AMP increased the PDE activity 2 hr after treatment, whereas RO 20-1724 inhibited the PDE activity by about 50% of control. RO 20-1724 is known to inhibit the PDE activity in rat erythrocytes (12); therefore, the in-

TABLE II. Effect of Metabolic Inhibitors on Dibutyryl Cyclic AMP-Induced Cyclic 3′,5′-AMP Phosphodeiesterase (PDE) Activity.[a]

Treatment	PDE activity (μmole/min/mg protein)
Control	5.5 ± 0.6[b]
Control + cycloheximide (5 μg/ml)	1.2 ± 0.2
Control + actinomycin D (5 μg/ml)	5.6 ± 0.3
Dibutyryl cyclic AMP (0.5 m*M*)	10.2 ± 1.6
Dibutyryl cyclic AMP + cycloheximide	6.0 ± 0.9
Dibutyryl cyclic AMP + actinomycin D	11.9 ± 1.5

[a] Cells (0.5×10^6) were plated in the Falcon plastic flasks (75 cm^2). Dibutyryl cyclic AMP was added 24 hr after plating and cycloheximide or actinomycin D was added 24 hr after the addition of dibutyryl cyclic AMP for a period of 6 hr. The PDE activity was determined according to the method described in the section, Materials and Methods. Each value represents an average of 8–10 samples.

[b] Standard deviatioin.

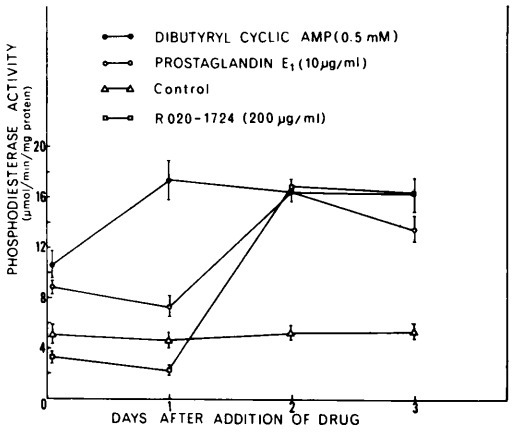

FIG. 1. Neuroblastoma cells (0.5×10^6) were plated in large Falcon plastic flasks (75 cm^2). Dibutyryl cyclic AMP, prostaglandin E_1 and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (RO 20-1724) were added individually for a period of 2 hr, 1, 2, and 3 days. Fresh growth medium and drug solution were added 2 days after treatment with drug. The cyclic AMP phosphodiesterase (PDE) activity was analyzed according to the procedure described in the section, Materials and Methods. Each value represents an average of 8–10 samples. The vertical bars are standard deviations.

hibition of PDE activity in neuroblastoma cells was expected. This compound elevates cyclic AMP level in neuroblastoma cells (unpublished data) within 1 hr of treatment, probably due to an inhibition of PDE; however, the PDE activity did not rise until Day 2. This indicates that an initial rise in the cyclic AMP level does not always lead to an immediate increase in the PDE level. Prostaglandin E_1, also increases the level of cyclic AMP in the neuroblastoma cells (17). This agent increased the PDE activity 2 hr after treatment, but a maximal increase occurred at Day 2. The above data indicate that the intracellular level of cyclic AMP may be one of the important factors in regulating the cyclic AMP phosphodiesterase activity in neuroblastoma cells. In fibroblasts also, a high level of cyclic AMP induces a high level of PDE (18, 19). However, unlike fibroblasts neuroblastoma cells have only one K_m for PDE (20).

Table II shows that cycloheximide, an inhibitor of protein synthesis, reduced the level of PDE activity in control and dibutyryl cyclic AMP treated cells after 6 hr of treatment, whereas actinomycin D, an inhibitor of RNA synthesis, did not. When cells were treated with cycloheximide for 12 hr, many of them were detached from the flask surface. This was not true when cells were incubated in the presence of cycloheximide for 6 hr. The insensitivity of PDE to actinomycin D in control and treated cells was observed for a period of 6–12 hr after the addition of antibiotic. This is in contrast to the observation made on mouse L cells in which dibutyryl cyclic AMP-induced PDE activity is markedly reduced by actinomycin D (18). Therefore, the regulation of PDE activity in the neuroblastoma cells may in part be different from that in the fibroblasts.

Neuroblastoma cells appeared to have no endogenous inhibitor for PDE. This is demonstrated by the experiment in which the specific activity of enzyme did not change when the original homogenate of control cells was diluted by a factor of 4. This was also confirmed by another experiment in which control and dibutyryl cyclic AMP-treated cells were mixed immediately before the enzyme analysis. The specific activity of the enzyme in mixed cells was similar to that which is expected after summing up the individual values. This indicates that an increase in the PDE activity after dibutyryl cyclic AMP treatment is not due to the inhibition of an endogenous inhibitor. A similar finding has been reported with the mouse fibroblasts (18).

This investigation shows that the PDE activity increases during cyclic AMP-induced "differentiation" of neuroblastoma cells in culture. This is probably due to an increase in the level of cyclic AMP. It has been reported that adult mouse cerebrum has three times more PDE and adenylate cyclase than the young ones (21). Therefore a working hypothesis is proposed that during differentiation of neuroblastoma cells the levels of both cyclic AMP and PDE increase, the former probably due to an increase in adenylate cyclase; a reverse process may occur during malignant transformation of nervous tissue.

Summary. The cyclic AMP phosphodiesterase (PDE) activity markedly increased in morphologically "differentiated" mouse

neuroblastoma cells induced by dibutyryl cyclic AMP, prostaglandin E_1 (stimulator of adenylate cyclase) and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (RO 20-1724, an inhibitor of PDE). Initially, RO 20-1724 decreased the PDE activity by about 50% of control, but later it increased the PDE activity. All of the above agents are known to increase the endogenous level of cyclic AMP, and therefore, in the differentiated cells, a high level of PDE correlated well with a high level of cyclic AMP. X-Irradiation which causes morphological "differentiation" similar to that by dibutyryl cyclic AMP did not increase the PDE activity. Sodium butyrate, 3′,5′-cyclic AMP, 5′-AMP and theophylline which inhibit cell division without causing morphological differentiation did not affect significantly the basal level of PDE. Cycloheximide markedly reduced the PDE activity, whereas actinomycin D did not. A working hypothesis is proposed that during "differentiation" of neuroblastoma cells in culture the levels of both cyclic AMP and PDE increase, the former probably due to an increase in adenylate cyclase; a reverse process may occur during malignant transformation of nervous tissues.

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