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Differences and Similarities Between Guanosine 3′,5′-Cyclic Monophosphate Phosphodiesterase and Adenosine 3′, 5′-Cyclic Monophosphate Phosphodiesterase Activities in Neuroblastoma Cells in Culture (38893)

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An elevation of the intracellular level of adenosine 3′,5′-cyclic monophosphate (cAMP) by prostaglandin E_1, inhibitors of phosphodiesterase or by treatment with analogs of cAMP (dibutyryl cAMP and 8-benzylthio cAMP) in mouse neuroblastoma cells induces many differentiated functions which are characteristic of mature neurons. These include formation of long neurites (1–2), increase in size of soma and nucleus associated with an increase in total RNA and protein contents (3), blockade of cells in G_1-phase of the cell cycle (3), increase in activities of tyrosine hydroxylase (3, 5), choline acetyltransferase (4), and acetylcholinesterase (6, 7), loss of tumorigenicity (3), and increase in sensitivity of adenylate cyclase to catecholamines (8). However, the role of guanosine 3′,5′-cyclic monophosphate (cGMP) in the regulation of expression of these functions in neuroblastoma cells has not been studied. Since an important factor limiting the magnitude and duration of action of cGMP in tissues is the rate of enzymatic destruction of this nucleotide by cGMP phosphodiesterase, the study of the regulation of cGMP phosphodiesterase activity in neuroblastoma cells is important. Recent studies using other cell systems have suggested (9, 10) that cGMP phosphodiesterase may be either specific or more elective for cyclic GMP. Although the regulation of cAMP phosphodiesterase activity in neuroblastoma cells has been studied in some detail (11–13), the existence or the specificity of cGMP phosphodiesterase in neuroblastoma cells is unknown. Therefore, we have compared the properties of cGMP phosphodiesterase with those of cAMP phosphodiesterase in neuroblastoma cells. We now report that cGMP phosphodiesterase in neuroblastoma cells is distinct from cAMP phosphodiesterase, and the activities of these enzymes in part may be differently regulated.

Materials and Methods. The procedures for culturing and maintaining mouse and human neuroblastoma cells were previously described (1, 14). Previously defined mouse clone NBP_2 (15) and human clone IMR-32 (16, 17), which have both tyrosine hydroxylase and choline acetyltransferase activities, were used in this study. Cells (0.25×10^6) were plated in Falcon plastic flasks (75 cm^2) and the medium was first changed at day 2 and then everyday thereafter. At day 4 fresh growth medium was added 1 hr before making the homogenates. The medium added 24 hr earlier had turned acidic by this time. Since the acidic medium is known to change morphology and enzyme activities of mammalian cells (19), it is essential that the fresh growth medium is added for 1 hr before experiment. Cells were washed three times with 0.1 *M* Tris–HCl buffer (pH 7.4), removed by a rubber policeman, and then homogenized in Tris–HCl buffer (0.1 *M*, pH 7.4). The cAMP phosphodiesterase activity in homogenates was assayed by the method of O'Dea *et al.* (20). Multiforms of phosphodiesterase exist in brain (21); some of these are located primarily in particulate whereas others are primarily in supernatant fractions. However, it has been reported (21) that neuroblastoma cells have only one form of phosphodiesterase, having apparently low and high K_m enzymes. The standard reaction mixture of 60 μl included $MgSO_4$, 30 mmoles; snake venom nucleotidase, 20 μg; dithiothreitol, 1.66 mmoles; Tris–HCl buffer, pH 7.4, 25 mmoles; and ^{3}H-cyclic AMP, 17,000 cpm/1.67×10^{-6} moles. The procedure involves the conversion of ^{3}H-cAMP to ^{3}H 5′-AMP by phosphodiesterase. The addition of snake venom 5′-nucleotidase converts ^{3}H 5′-AMP to ^{3}H-adenosine. The reaction mixture was then put on ion-exchange resin-loaded paper (Reeve Angel, SB-2 grade).

The paper was dried and ^{3}H-adenosine was eluted with 0.1 m*M* ammonium formate (pH 7.0). ^{3}H-adenosine activity was counted in a liquid scintillation counter using a dioxane solvent system. The data were expressed as ^{3}H-cAMP hydrolyzed/min/mg protein. Protein was determined by the method of Lowry *et al.* (22) with bovine serum albumin (fraction V) as a standard. The cGMP phosphodiesterase activity in homogenates was also determined by the method of O'Dea *et al.* for cAMP phosphodiesterase (20). ^{3}H-cGMP, 12,000 cpm 1.67×10^{-6} moles was used as a substrate. The data were expressed as ^{3}H-cGMP hydrolyzed/min/mg protein. The effect of 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (R020-1724, Hoffman-La Roche), papaverine (Lilly Co.), and theophylline (Nutritional Biochem. Corp.) on cGMP and cAMP phosphodiesterase activities in homogenates of mouse neuroblastoma cells was studied. The specific activity of cyclic nucleotide phosphodiesterase as a function of protein concentration was determined in homogenates of mouse and human neuroblastoma cells, mouse fibroblasts (L-cells), and adult mouse caudate nucleus. The specific activity and kinetics of cAMP phosphodiesterase as a function of protein concentrations were also studied in cAMP-induced "differentiated" mouse neuroblastoma cells. R020-1724 was used to induce "differentiation." Cells (0.4×10^6) were plated in large Falcon plastic flasks (75 cm^2) and R020-1724 (200 μg/ml) was added 24 hr later. The drug and medium were changed every day and the experiment was performed at 3 days after treatment.

The values of K_m and V_{max} for cAMP phosphodiesterase activity were determined by nonlinear least-squares fitting of the data directly to the Michaelis-Menten equation using Marquardt's procedures (23). In order to have some idea about the nature of molecules which may account for the inhibition of cAMP phosphodiesterase activity, the homogenates containing 10 μg protein/10 μl was boiled for 5 min, or treated with RNase (80 μg RNase/60 μg protein, total volume 60 μl) or DNase (80 μg DNase/60 μg protein, total volume 60 μl) for 30 min at 37° before adding to the reaction mixture (60 μl) containing 3 μg protein. A protein concentration of 10 μg/60 μl was selected because the cAMP phosphodiesterase activity was maximally inhibited at this concentration.

Results. In mouse neuroblastoma cells there are phosphodiesterase activities in both particulate and supernatant fractions which hydrolyze cGMP and cAMP with an apparent K_m of 2–8 μM and with an apparent K_m of 44–222 μM (Table I). These K_m values are consistent with the values reported for brain and other tissues (9, 10), and they are similar to those observed for cAMP phosphodiesterase activity in homogenates of neuroblastoma cells (11).

R020-1724 did not inhibit cGMP phosphodiesterase activity, but markedly inhib-

TABLE I. ACTIVITIES OF cGMP AND cAMP PHOSPHODIESTERASE IN MOUSE NEUROBLASTOMA CELLS IN CULTURE.[a]

Enzymes	Low K_m (μM)	V_{max} of low K_m (pmole/min/μg protein)	High K_m (μM)	V_{max} of high K_m (pmole/min/μg protein)
cGMP Phosphodiesterase (particulate)	1.64	0.33	222.0	0.13
cGMP Phosphodiesterase (supernatant)	3.6	1.07	44	1.46
cAMP Phosphodiesterase (particulate)	4.1	0.90	100	1.9
cAMP Phosphodiesterase (supernatant)	8.0	2.3	80	1.75

[a] The values of K_m and V_{max} for phosphodiesterase activities were determined by least-square linear regression of the double-reciprocal plot. The range of cyclic nucleotides concentrations varied from 0.3 μM to 1000 μM.

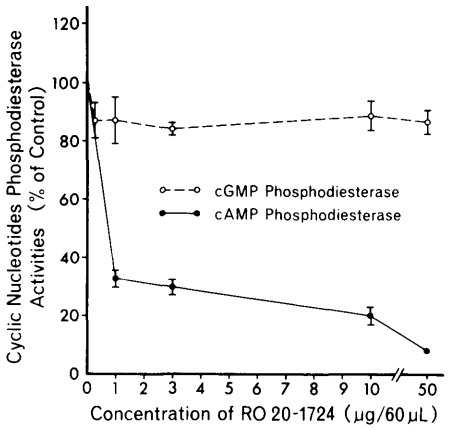

FIG. 1. Effect of 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (R020-1724) on cyclic nucleotides phosphodiesterase activities in homogenates of mouse neuroblastoma cells. The reaction mixture (60 μl) contains 3 μg protein and 17,000 cpm/1.67 × 10^{-6} moles of ^{3}H-cAMP or 12,000 cpm/1.67 × 10^{-6} moles of ^{3}H-cGMP. The enzymes' activities in homogenates were determined in the presence of 30 m*M* magnesium. The basal activities of cAMP phosphodiesterase (178 ± 12 pmole/min/mg protein) and cGMP phosphodiesterase (135 ± 9 pmole/min/mg protein) were considered 100% control values; the enzyme activity in treated cells was expressed as percentage of control. Each value represents an average of six to eight samples. The bar at each point is standard error of the mean.

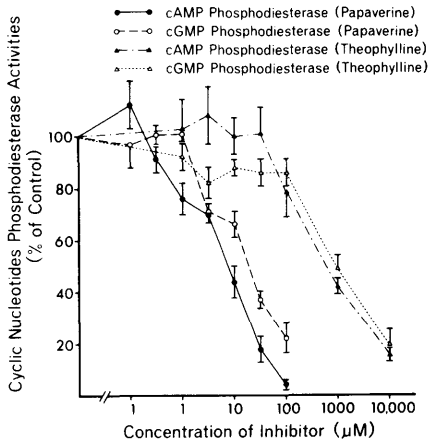

FIG. 2. Effect of papaverine and theophylline on cyclic nucleotides phosphodiesterase activities in homogenates of mouse neuroblastoma cells. The reaction mixture (60 μl) contains 3 μg protein, and 17,000 cpm/1.67 × 10^{-6} moles of ^{3}H-cAMP or 12,000 cpm/1.67 × 10^{-6} moles of ^{3}H-cGMP. The enzymes activities in homogenates was determined in the presence of 30 m*M* Mg. The basal activities of cAMP phosphodiesterase (178 ± 12 pmole/min/mg protein) and cGMP phosphodiesterase (135 ± 9 pmol/min/mg protein) were considered 100% control values; the enzyme activity in treated cells was expressed as percentage of control. Each value represents an average of six to eight samples. The bar at each point is standard error of the mean.

ited cAMP phosphodiesterase activity in mouse neuroblastoma cells (Fig. 1). Since the enzymes were assayed at low substrate concentrations, the above data may mean that the cGMP phosphodiesterase with the low K_m is distinct from the cAMP phosphodiesterase with the low K_m. It has been reported (18) that R020-1724 inhibits rat erythrocyte cGMP phosphodiesterase activity much less than cAMP phosphodiesterase activity.

Papaverine and theophylline inhibited both cGMP and cAMP phosphodiesterase activities to about the same extent (Fig. 2). Papaverine was more potent than theophylline in inhibiting the cyclic nucleotides phosphodiesterase activities. These results are consistent with the observation made on rat brain in which papaverine and theophylline also inhibit both cGMP and cAMP phosphodiesterase activities (24).

The specific activity of cGMP phosphodiesterase in homogenates depends upon the protein concentration (Fig. 3). In mouse and human neuroblastoma cells, and in mouse fibroblasts in culture, the enzyme activity increased at protein concentrations of 1–4 μg/60 μl, reached a plateau up to the concentrations of 10 μg/60 μl, and then decreased. However, in the caudate nucleus the specific activity of cGMP phosphodiesterase decreased as a function of protein concentrations. The specific activity of cGMP phosphodiesterase in all cells at a high protein concentration (50 μg/60 μl) decreased to about the same level. The specific activity of cAMP phosphodiesterase did not increase but rather decreased as a function of protein concentrations in all cells except mouse L-cells (Fig. 4). A maximal inhibition in enzyme activity was achieved at a protein concentration of 10 μg/60 μl. A similar observation was made when the supernatant (100,000*g*, 1 hr) or particulate fraction of malignant and

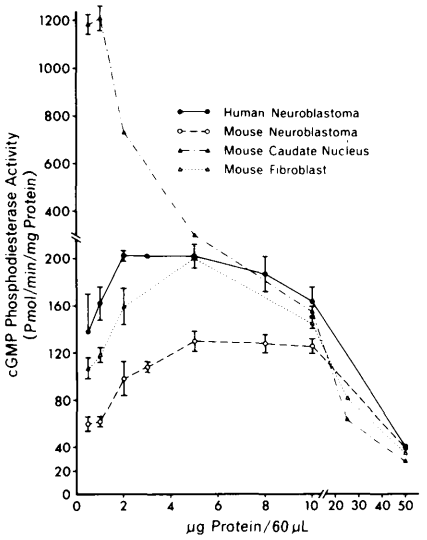

FIG. 3. The specific activity of cyclic GMP phosphodiesterase in homogenates of various cell types as a function of protein concentrations (μg/60 μl of reaction mixture). The reaction mixture (60 μl) contains 12,000 cpm/1.67 × 10–6 moles of ^{3}H-cGMP. The enzyme activity in homogenates was determined in the presence of 30 m*M* magnesium. Each value represents an average of six to eight samples. The bar at each point is standard error of the mean.

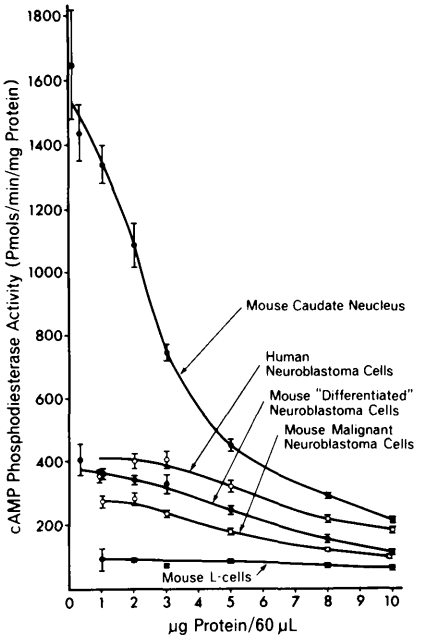

FIG. 4. The specific activity of cAMP phosphodiesterase in homogenates of various cell types as a function of protein concentrations (μg/60 μl of reaction mixture). The reaction mixture (60 μl) contains 17,000 cpm/1.67 × 10–6 moles of ^{3}H cAMP. 4-(3-Butoxy - 4 - methoxybenzyl) - 2 - imidazolidinone, was used to induce "differentiation" in mouse neuroblastoma (NB) cells. The enzyme activity in homogenates was determined in the presence of 30 m*M* magnesium. Each value represents an average of at least six samples. The bar at each point is standard error of the mean.

cAMP-induced "differentiated" neuroblastoma cells was used as a source of enzyme.

Table II shows that at high protein concentrations the K_m values for cAMP phosphodiesterase were increased in mouse malignant neuroblastoma cells. A similar phenomenon was observed in cAMP-induced "differentiated" mouse neuroblastoma cells, human neuroblastoma cells, and in adult mouse caudate nucleus.

When the homogenate containing 10 μg protein/10 μl was boiled and then added (10 μg of protein) to the reaction mixture containing 3 μg protein, the cAMP phosphodiesterase activity was not inhibited (Table III). When the RNase- or DNase-treated homogenate containing 10 μg of protein was added to reaction mixture containing 3 μg protein, the cAMP phosphodiesterase activity was inhibited.

Discussion. The present study shows that the neuroblastoma cells contain phosphodiesterase activities which hydrolyze both cAMP and cAMP with an apparently similar K_m. However, cGMP phosphodiesterase appears to be distinct from cAMP phosphodiesterase. The reasons are as follows: (a) The specific activity of cGMP phosphodiesterase increases at protein concentrations of 1–4 μg/60 μl, reaches a plateau up to the concentrations of 10 μg/60 μl, and then decreases. A maximal inhibition of enzyme activity in all cells was achieved at 50 μg protein/60 μl. The specific activity of cAMP phosphodiesterase decreases as a function of protein concentrations, and a maximal inhibition of enzyme activity in all cells was achieved at 10 μg protein/60 μl. (b) R020-1724 does not inhibit cGMP phosphodiesterase activity, whereas it markedly inhibits cAMP phosphodiesterase activity. (c) The basal level of cGMP phosphodiesterase activity in mouse fibroblasts is about three times greater than that of cAMP phospho-

TABLE II. EFFECT OF PROTEIN CONCENTRATIONS ON THE K_m VALUE OF cAMP PHOSPHODIESTERASE ACTIVITY IN NEUROBLASTOMA CELLS IN CULTURE.[a]

μg Protein/60 μl reaction mixture	K_m (μM)	V_{max} (pmole/min/μg protein)
10-0	11.07 ± 5.1[b]	0.73 ± 0.23
3-0	5.31 ± 2.2	0.73 ± 0.22
1.0	3.83 ± 1.38	1.02 ± 0.17

[a] Each experiment was repeated four times. The K_m and X_{max} values were determined by nonlinear least-squares fitting of the data directly to the Michaelis-Menten equation using Marquardt's procedure (23). The range of cAMP concentrations varied from 0.3 μM to 18 μM.

[b] Standard error of the mean.

TABLE III. EFFECT OF VARIOUS TREATMENTS ON cAMP PHOSPHODIESTERASE ACTIVITY IN MOUSE NEUROBLASTOMA CELLS.[a]

Treatment	cAMP Phosphodiesterase activity (pmole/min/mg protein)
Control homogenate (3 μg protein)	185 ± 13[b]
Control + untreated homogenate (10 μg protein)	86 ± 10
Control + boiled homogenate (10 μg protein)	180 ± 8
Control + DNase-treated homogenate (10 μg protein)	79 ± 8
Control + RNase-treated homogenate (10 μg protein)	88 ± 7

[a] The reaction mixture (60 μl) contains 3 μg of protein plus 10 μg of untreated or treated protein. Each value represents an average of eight samples.

[b] Standard error of the mean.

diesterase activity. The specific activity of cGMP phosphodiesterase in mouse fibroblasts decreases as a function of protein concentrations, whereas the specific activity of cAMP phosphodiesterase does not change under a similar experimental condition.

The decreased levels of cGMP phosphodiesterase and cAMP phosphodiesterase activities at high protein concentrations may be due to the presence of an endogenous inhibitor of these enzymes. An increase in cGMP phosphodiesterase activity at certain protein concentrations may be due to the presence of an endogenous activator of this enzyme. Although the presence of an endogenous activator of cGMP phosphodiesterase (25) and cAMP phosphodiesterase (26) has been demonstrated in mammalian brain, the possibility of existence of endogenous inhibitors of cyclic nucleotides phosphodiesterases in neuroblastoma cells is suggested here. Further experiments are needed to substantiate this point.

R020-1724, which does not inhibit cGMP phosphodiesterase activity, but markedly reduces cAMP phosphodiesterase activity in neuroblastoma cells, induces many differentiated functions which are characteristic of mature neurons (1–8). This indicates that cGMP level may not be involved in the regulation of the expression of "differentiated" functions in neuroblastoma cells. This is further supported by the fact that papaverine which inhibits both cGMP and cAMP phosphodiesterase activities also induces many differentiated functions in neuroblastoma cells (2–4). The addition of exogenous cGMP or dibutyryl cGMP together with prostaglandin E_1 or R020-1724 does not prevent the expression of differentiated functions (4). cGMP or its dibutyryl derivatives do not cause morphological differentiation (4). These data do not rule out the possibility that cGMP may affect the functions other than differentiated ones in neuroblastoma cells. Further work is in progress to elucidate this possibility.

Summary. There are phosphodiesterase activities in both particulate and supernatant fractions which hydrolyze guanosine 3′,5′-cyclic monophosphate (cGMP) and adenosine 3′,5′-cyclic monophosphate (cAMP) with an apparent K_m of 2–8 μM and with an apparent K_m of 44–222 μM. 4-(3-Butoxy-4-methoxybenzyl-2-imidazolidinone (R020-1724) did not inhibit cGMP phosphodiesterase activity in homogenates of mouse neuroblastoma cells, but markedly inhibited cAMP phosphodiesterase activity. Papaverine and theophylline inhibited both cGMP and cAMP phosphodiesterase activities to about the same extent. The former was more

potent than the latter. The specific activity of cGMP phosphodiesterase as a function of protein concentrations first increased and then decreased. The specific activity of cAMP phosphodiesterase decreased under a similar experimental condition.

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