BINDING OF CYCLIC NUCLEOTIDES WITH SOLUBLE PROTEINS INCREASES IN "DIFFERENTIATED" NEUROBLASTOMA CELLS IN CULTURE

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SUMMARY: The binding of cAMP with soluble proteins from mouse neuroblastoma (NB) cells (100,00 X g supernatant) increased by about 2-fold after treatment with Prostaglandin E_1 (PGE_1) or 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (RO20-1724). This increase was due to an increase in the level of binding proteins, and was not observed in 5'-adenosine monophosphate-,x-irradiated- or 6-thioguanine-treated NB cells. The electrophoretic characteristics of cAMP binding proteins of "differentiated" and malignant NB cells were identical. There were two binding peaks, but the extent of binding at each peak was relatively high in "differentiated" NB cells. The binding of cAMP with soluble proteins from rat glial and mouse L-cells did not significantly change after treatment with PGE_1 or RO20-1724. cAMP and cGMP bind with same proteins, but cAMP has about 10-fold higher binding affinity than cGMP.

INTRODUCTION

An elevation of the intracellular level of adenosine 3':5'-cyclic monophosphate (cAMP) in neuroblastoma (NB) cells by prostaglandin E_1 (PGE_1), inhibitors of cAMP phosphodiesterase or by analogs of cAMP, irreversibly induces many differentiated functions which are characteristic of mature neurons (1-4). Dibutyryl cAMP (5) also induces neurites, changes membrane glycopeptides and increases neural enzymes in other NB clones, and in hybrids (NB X glial). The irreversibility of cAMP-induced "differentiation" in NB cells is in contrast to that observed in glial (6) and sarcoma (7) cells, in which cAMP-effects are reversible soon after the removal of drug. These findings suggest that the "differentiated" NB cells develop a permanent mechanism(s) for protecting the formed cAMP from hydrolysis, whereas the nonneural tumor cells do not. We now report that the binding of cAMP with soluble proteins increases by 2-fold in

cAMP-induced "differentiated" mouse NB cells, whereas it does not change in PGE_1- or RO20-1724-treated rat glial cells and mouse L-cells. cAMP and cGMP bind with the same proteins, but cAMP has about 10-fold higher binding affinity than cGMP.

MATERIALS AND METHODS

Neuroblastoma Cell Culture: Procedures for culturing and maintaining the mouse NB cells were described (1). Previously defined (2) clone NBP_2 was used in this study. The rat glial cells (C-6) and mouse L-cells were grown under conditions of those described for mouse NB cells. Differentiation was induced by treating NB cells with 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (RO20-1724), a specific inhibitor of cAMP phosphodiesterase (8), or with PGE_1, a stimulator of adenylate cyclase activity (2). Cells (0.25×10^6 for control, 0.4×10^6 for RO20-1724-treatment, 0.3×10^6 for PGE_1-treatment, 0.5×10^6 for 5'-AMP-treatment, 1×10^6 for x-irradiation- and 6-thioguanine-treatment), were plated in Falcon plastic flasks (75 cm^2), and RO20-1724 (200 µg/ml), PGE_1 (10 µg/ml), 5'-AMP (0.25 mM), 6-thioguanine (0.5 µM) and x-irradiation (600 rads) were given separately 24 hrs after plating. The procedure for irradiating the NB cells has been previously described (2). The number of cells plated for various treatments was different because the growth rate was differently affected by different agents. The drug and medium were changed daily. Control cultures were treated identically except that an equivalent volume of solvent without drug was added.

Binding of (^3H) cAMP With Soluble Proteins: Cells (3 days after treatment) were removed from the flask surface by Viokase solution (0.25%), washed three times with growth medium and one time with 0.05 M Tris-HCL buffer (pH 7.4) containing 0.25 M sucrose, 3 mM $MgCl_2$ and 4 mM 2-mercaptoethanol. Cells were homogenized in five volumes of the same buffer. Particulate materials were removed by centrifugation 100,000 X g for 90 min. The protein was determined according to the method of Lowry et al. (9). The reaction mixture contained 33.3 mM

sodium acetate buffer (pH 4.0), 10 mM theophylline, 6.7 nM (^{3}H) cAMP (sp. act. 27.5 Ci/mM), or 33.3 nM (^{3}H) cGMP (sp. act. 21 Ci/mM), 100 µg of soluble proteins, and water to a total volume of 0.3 ml. After incubation for 1 hr at 0°, the reaction mixtures were diluted with 0.7 ml of 20 mM potassium phosphate buffer (pH 6), incubated for an additional 10 min at 0°, filtered through a Millipore filter (0.45 µ), presoaked with potassium phosphate buffer and washed three times with a total of 15 ml of the same buffer. The filters were placed into counting vials containing 1 ml of 2-ethoxyethanol and then 10 ml of dioxane cocktail (one liter dioxane contains 60 g naphthalene, 6 g PPO and 0.05 g POPOP) was added. Radioactivity was counted in a liquid scintillation counter.

Polyacrylamide Gel Electrophoresis of Soluble Proteins of Neuroblastoma Cells: To ascertain the electrophoresis characteristics of the cAMP binding proteins, soluble proteins were separated by disc gel electrophoresis (10). Separations were carried out for 3 hrs at 2 mamp/tube. After electrophoresis the gels were divided into 2 mm segments using a Gilson gel fractionator. Fractions were eluted with 0.3 ml of homogenizing buffer. Each fraction was collected directly into vials having 0.6 ml of reaction mixture, and was assayed for bound (^{3}H) cAMP as described above. Electrophoretic separations, gel fractionation and incubation were carried out at 0 to 4°.

RESULTS AND DISCUSSION

Treatment of NB cells with agents (PGE_1 and RO20-1724) which induce many differentiated functions by increasing the intracellular level of cAMP (1-5), stimulated cAMP binding by about 2-fold (Table 1). In both malignant and "differentiated" NB cells, two peaks of binding proteins were observed and the magnitude of each of these peaks increased in "differentiated" cells (Fig. 1). The binding was completely prevented if the soluble proteins were boiled for 5 min, and it was reduced to about 15% if the soluble proteins were treated with protease (10 µg protease/100 µg protein for 1 hr at 0°C). The increase

Table 1. Binding of cAMP with Soluble Proteins of Neuroblastoma Cells After Various Treatment.

Treatment of Cells	Bound cAMP (pmol/mg protein)
Control	1.83 ± 0.13
Prostaglandin E_1 (10 μg/ml)	3.13 ± 0.12
RO20-1724 (200 μg/ml)	3.83 ± 0.27
5'-adenosine monophosphate (0.25 mM)	1.48 ± 0.10
6-thioguanine (0.5 μM)	1.98 ± 0.20
x-irradiation (600 rads)	2.03 ± 0.18

Cells were treated with various agents 24 hrs after plating. The soluble proteins (100,000 X g supernatant) were prepared 3 days after treatment. The incubation condition has been described in the section of Materials and Methods. Each value represents an average of six samples ± SD.

in cAMP binding in "differentiated" cells may reflect an increased level of binding proteins rather than the appearance of new binding proteins. An increase in cAMP binding occurred 24 hrs after treatment of NB cells with PGE_1 and was completely blocked by cycloheximide (unpublished observation).

The addition of unlabeled cAMP (0.33 μM) to the reaction mixture almost completely prevented the binding of (^{3}H) cAMP, whereas the addition of much higher levels of 5'-AMP (2 μM) did not significantly affect the binding. The (^{3}H) cAMP binding capacity of the soluble protein from "differentiated" cells and malignant cells was determined before and after separation of free cAMP by chromatography on Sephadex G-25 columns. The amount of bound (^{3}H) cAMP was identical in both cases suggesting that the endogenous free cAMP was too low to significantly affect the results.

Neither the inhibition of cell division nor the increase in total protein is sufficient to increase the level of cAMP binding proteins in NB cells. This is shown by the fact that 5'-AMP, x-irradiation and 6-thioguanine (6-TG) inhibit cell division (2) and the amount of protein in x-irradiated- and 6-TG-treated

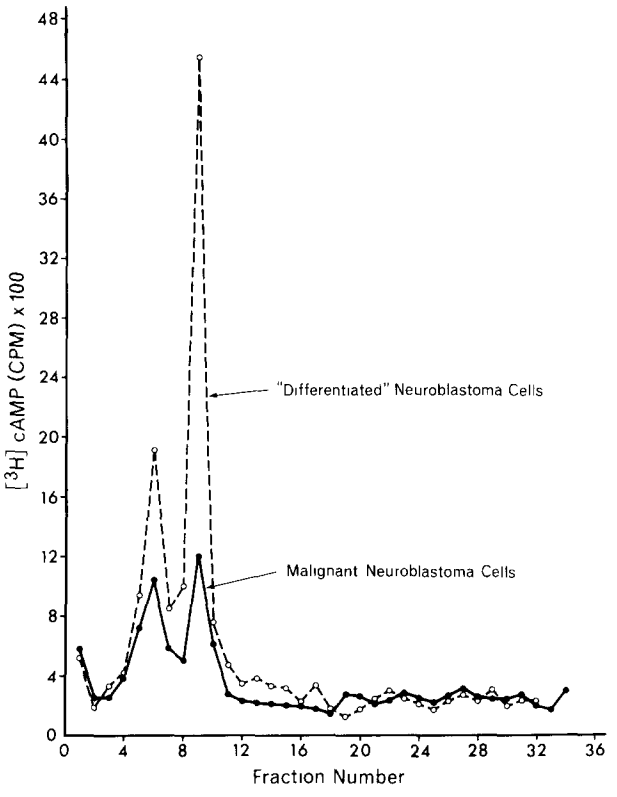

Fig. 1. - Binding of (^{3}H) cAMP with protein fractions obtained after polyacrylamide gel electrophoresis. The soluble proteins of malignant and "differentiated" neuroblastoma cells were applied on the gel. RO20-1724 was used to induce differentiation. The incubation condition has been described in the section of Materials and Methods. The experiments were repeated twice.

NB cells increases (about 3-fold) to an extent similar to that observed in cAMP-induced "differentiated" NB cells (2). However, the level of binding proteins does not change in cells treated with any of the above agents (Table 1).

The binding of (^{3}H) cAMP with soluble proteins of PGE_1- or RO20-1724-treated glial and L-cells was similar to that observed in untreated cells (Table 2). The increased amount of binding proteins in "differentiated" NB cells may in part account for the existence of high cAMP level (2) in the presence of high cAMP phosphodiesterase activity (3), since the protein bound cAMP is protected from hydrolysis. Glial and L-cells after treatment with PGE_1

Table 2. Binding of cAMP with Soluble Proteins of Glial and L-Cells After Various Treatment.

Cell Type	Treatment	Bound cAMP (pmol/mg protein)
Rat glial cells	Control	1.88 ± 0.30
Rat glial cells	Prostaglandin E_1 (10 µg/ml)	1.56 ± 0.40
Rat glial cells	RO20-1724 (200 µg/ml)	1.77 ± 0.25
Mouse L-cells	Control	0.80 ± 0.02
Mouse L-cells	Prostaglandin E_1 (10 µg/ml)	0.89 ± 0.10
Mouse L-cells	RO20-1724 (200 µg/ml)	0.91 ± 0.03

Cells were treated with various agents 24 hrs after plating. The soluble proteins (100,000 X g supernatant) were prepared 3 days after treatment. The incubation condition has been described in the section of Materials and Methods. Each value represents an average of six samples ± SD.

or RO20-1724, do not increase the level of binding proteins. Since the cAMP-effects are irreversible in NB cells, whereas they are reversible in nonneural tumor cells, the irreversibility or reversibility of cAMP-effects on mammalian cells appears to correlate with the increase or lack of increase in the level of cAMP binding proteins. Further work is needed to substantiate this.

The present study suggests that cAMP may regulate the level of its own binding proteins in NB cells. For example, PGE_1 and RO20-1724 which increase the cAMP level (2), elevates the level of binding proteins. However, x-irradiation, 6-thioguanine, and 5'-AMP which do not increase the cAMP level (2-3) do not change the amount of binding proteins. In glial and L-cells cAMP either requires a second factor in order to increase the level of binding protein or has no regulatory role.

If our present findings could be applicable to in vivo conditions, the rational for developing new therapeutic approaches for the treatment of neuro-

blastoma and nonneural tumors may be different. For example, an elevation of the intracellular level of cAMP may be of therapeutic value for neuroblastoma tumors, whereas an elevation of the intracellular level of cAMP binding proteins may be of therapeutic value for nonneural tumors.

cAMP and cGMP bind with the same proteins, but cAMP has about 10-fold higher binding affinity than cGMP. This is substantiated by the following observations: a) the concentration of cGMP required to saturate the soluble protein is about 330 nM, whereas the concentration of cAMP required to saturate the soluble protein is about 33 nM; b) the addition of 33 nM of nonradioactive cAMP into the reaction mixture reduces the binding of both (^{3}H) cAMP and (^{3}H) GMP by 50%, whereas a concentration of 330 nM of nonradioactive cGMP is needed to produce a similar inhibition in binding; and c) the pattern of binding of (^{3}H) cAMP and (^{3}H) cGMP with the protein fractions (obtained after polyacrylamide gel electrophoresis) was similar.

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