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SPECIFICITY OF THE ACTION OF cAMP AGONISTS IN THE INDUCTION OF R_I cAMP-BINDING PROTEIN IN MOUSE NEUROBLASTOMA CELLS

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Abstract—Differentiation in the mouse neuroblastoma cells is induced by cAMP and is characterized by neurite extension and increased acetylcholinesterase, cAMP-phosphodiesterase, and R_I cAMP-binding activities. To gain a better understanding of the regulation of expression and the possible function of the R_I cAMP-binding protein in neuroblastoma cell differentiation, we evaluated the specificity of action of cAMP analogues and agents that increased intracellular cAMP concentration in the induction of the 47,000-dalton R_I protein. The amount of R_I in cell extracts was quantitated by the photoactivated incorporation of 8-N_3-[^{32}P]cAMP into the 47,000-dalton R_I and by ELISA and Western blot techniques. Our results showed that dibutyryl cAMP, forskolin, prostaglandin E_1, 3-isobutyl-1-methyl xanthine, and papavarine gave a 2- to 4-fold increase in the R_I cAMP-binding protein coincident with the expression of various morphological and biochemical differentiation phenotypes in the mouse neuroblastoma cells. However, the effects of 8-bromo-cAMP were different. 8-Bromo-cAMP effectively promoted neurite extension and increased acetylcholinesterase and cAMP-phosphodiesterase activities; however, there was no concomitant increase in the R_I cAMP-binding protein. The result raises interesting questions concerning the coupling of expression of the various differentiation phenotypes in the mouse neuroblastoma cells.

We have been interested in the mechanism of cAMP action in the control of neuroblastoma cell differentiation. Our previous studies have demonstrated the induction of a 47,000-dalton R_I cAMP-binding protein coincident with neurite extension and increased acetylcholinesterase activity in neuroblastoma cells prompted to differentiate by treatment with dibutyryl cAMP or serum withdrawal [1–3]. We further showed that, while the 47,000 dalton R_I protein in neuroblastoma cells is similar, if not identical, to the 47,000-dalton regulatory subunit of the type I cAMP-dependent protein kinase, the R_I in neuroblastoma cells is not associated with the catalytic subunit of the kinase and, hence, is unlikely to be involved in regulating the catalytic subunit activity [2].

These results suggest differential regulation of expression of the regulatory and catalytic subunit proteins of the cAMP-dependent protein kinase in neuroblastoma cells. The function of the free R_I cAMP-binding protein, particularly as it relates to the action of cAMP in the control of neuroblastoma cell differentiation, is an important but as yet unresolved problem.

A survey of the literature revealed that most of the studies reporting an increase in the R_I cAMP-binding protein in neuroblastoma cells were done by treating cells with dibutyryl cAMP [2, 4–6]. In view of the considerable biological effects of sodium butyrate, including its profound effects in inducing morphological and biochemical differentiation of erythroleukemic cells [7] and its effect in hyperacetylating histone [8], and the possibility that sodium butyrate may be present in cells treated with dibutyryl cAMP (either as a trace contaminant of the compound or as a metabolite), we evaluated the effects of analogues of cAMP and agents that increase intracellular cAMP level (either through activation of adenyl cyclase or inhibition of phosphodiesterase) in the control of neuroblastoma cell differentiation, particularly the induction of the R_I cAMP-binding protein. Our goal is to determine the specificity of effects of cAMP in regulating the expression of the R_I cAMP-binding protein, and through this we hope to gain a better understanding of the regulation of expression and the function of the R_I cAMP-binding protein in neuroblastoma cell differentiation.

MATERIALS AND METHODS

All tissue culture supplies were from the Gibco Co., Grand Island, NY. 8-N_3-[^{32}P]cAMP and [^{3}H]cAMP were from ICN, Irvine, CA. Dibutyryl cAMP, sodium butyrate, 3-isobutyl-1-methyl xanthine, papavarine, and histone II AS were from the Sigma Chemical Co., St. Louis, MO. 8-Bromo-cAMP either was purchased from the Sigma Chemical Co. or was synthesized by bromination of cAMP according to the methods of Muneyama *et al.* [9].

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Forskolin was obtained from Calbiochem, San Diego, CA. Prostaglandin E_1 (PGE_1) was a gift from the laboratory of Dr. A. Tashjian, Harvard School of Public Health. Sterile stock solutions of sodium butyrate (100 mM), dibutyryl cAMP (100 mM), and 8-bromo-cAMP (100 mM) were prepared in Dulbecco's modified Eagle's medium; stock solutions of forskolin (20 mM), PGE_1 (28 mM), and 3-isobutyl-1-methyl xanthine (200 mM) were prepared in dimethyl sulfoxide (DMSO), ethanol, and methanol respectively.

Cell growth and differentiation. Seed cultures of the NS-20 and N-18 mouse neuroblastoma cell lines were obtained from the laboratory of Dr. M. Nirenberg, NIH. The cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were plated at a density of $1–2 \times 10^5$ cells per 100 mm plate and were cultured under standard conditions [1, 2, 10].

To test the effects of analogues of cAMP and activators of adenyl cyclase and inhibitors of phosphodiesterase, these agents were added to designated plates of neuroblastoma cell cultures to a final concentration as indicated at 2–24 hr after plating the cells. The effects of DMSO, ethanol, and methanol, vehicles used to dissolve forskolin, PGE_1, and 3-isobutyl-1-methyl xanthine, respectively, were also tested; these solvents, at concentrations of 0.1% (DMSO), 0.1% (ethanol), and 0.5% (methanol), had no effect on any of the variables measured.

Cells were cultured in the absence and presence of these agents until they reached an early stationary phase of growth (this translated to a culture period of 4–5 days when cells were plated at a density of $1–2 \times 10^5$ cells per 100 mm dish). It should be noted that the "cAMP-elevating" agents used in this study slowed the growth rate (increased doubling time) and reduced the saturation density of the neuroblastoma cells during the logarithmic and plateau phases of cell growth, respectively; this result is consistent with our previous observation of the effects of dibutyryl cAMP and serum deprivation on growth of the N-18 mouse neuroblastoma cells [1, 2]. Representative fields of the early stationary phase cell cultures were photographed to determine the fraction of cells bearing neurites (defined as processes with a length twice the diameter of the neuroblastoma soma); the cells were then harvested and homogenized in a 10 mM Tris (pH 7.4) buffer containing 1 mM EDTA (approximately 0.2 to 0.4 ml of buffer per 100 mm plate of cells). A 150,000 *g* supernatant fraction (cytosol) obtained from the cell homogenate was dialyzed overnight against 1000× volume of the Tris–EDTA buffer, and used to assay for the amount of R_I cAMP-binding protein, cAMP-phosphodiesterase and acetylcholinesterase activities.

Quantitation of R_I by ELISA. An antiserum was raised against the 47,000-dalton regulatory subunit protein of type I cAMP-dependent protein kinase from bovine skeletal muscle [2]. This antiserum was used to assay for the amount of R_I in neuroblastoma cell extracts in the "ELISA" and "Western blot" procedures. The amount of R_I present in cell extracts was quantitated by its inhibition of binding of the anti-R_I antibody to microtiter wells pre-coated with purified R_I. Briefly, the wells of a 96-well microtiter plate were coated with 10 ng of purified R_I (4°, overnight) and then blocked with 1% bovine serum albumin in a buffer containing 125 mM sodium borate (pH 8.2), 140 mM NaCl, 0.05% Tween 20, and 0.05% Triton X-100 (BTT; borate–Tween–Triton) (1 hr at 37°). The microtiter plate was then washed five times with the BTT buffer and set aside until use. Extracts of the neuroblastoma cells or known amounts of the purified R_I protein were incubated with a 1:500 to 1:800 dilution of the antibody against R_I for overnight at room temperature on a platform shaker. Because the antibody had a lower affinity for the R_I protein with bound cAMP, all samples (cell extracts and purified R_I) were pretreated with 5 M urea for 30 min at 30° to remove bound cAMP. The samples were then diluted to reduce the urea concentration to 0.5 M prior to carrying out the ELISA assay. A reduced binding of the antibody to R_I with bound cAMP has been shown previously by studies from other laboratories [11, 12].

The incubation mixtures of the cell extracts (or purified R_I) and antibody were then carefully transferred to individual wells of the microtiter plate precoated with R_I and were incubated at 37° for 1.5 to 2 hr. Then the incubation mixture was removed and the microtiter plate was rinsed five times with the BTT buffer. A 100-μl aliquot of a 1:1000 diluted goat anti-rabbit IgG-horseradish peroxidase conjugate (BioRad) was added to individual wells and incubated at 37° for 1 hr. After pouring off the solution and rinsing the plate five times with BTT buffer, 100-μl aliquots of the substrate solution of peroxidase (6 mg of *o*-phenylenediamine and 6 μl of 50% H_2O_2 in 10 ml of 0.1 M citrate phosphate buffer, pH 5) was added to each well and incubated at room temperature for 30–60 min. The reaction was stopped by the addition of 40 μl of 4 N H_2SO_4, and the optical density at 490 nm was quantitated spectrophotometrically with an MR 700 miniplate reader (Dynatech). The amount of cytosol protein needed to give a 50% inhibition of the binding of antibody to microtiter well coated with R_I was used to estimate the concentration of R_I in that sample; a competition curve generated with known amounts of purified R_I in solution against R_I coated on the microtiter well was used as the standard.

Western blot quantitation of the amount of R_I cAMP-binding protein in neuroblastoma cell extracts. The amount of R_I in cell extracts was quantitated by a modification of the "Western blot" technique previously described [12, 13]. Briefly, aliquots of cytosol preparations from neuroblastoma cells, containing 150–300 μg cytosol protein, and known amounts of the purified R_I protein from bovine skeletal muscle were applied to sample wells of a sodium dodecyl sulfate (SDS)-polyacrylamide (5–15%) slab gel. Upon completion of the electrophoresis procedure, proteins were electrophoretically transferred to a sheet of nitrocellulose membrane (0.22 μm pore size) at a constant voltage of 6 V/cm in a transfer buffer of 25 mM Tris base, 192 mM glycine, and 20% methanol for 12–16 hr at room temperature. The nitrocellulose membrane was then incubated in phosphate-buffered saline (PBS) containing 5% bovine serum albumin, 0.25% gelatin, and 0.01% NaN_3

(from here on referred to as the blocking medium) overnight at 4° to saturate non-specific protein binding sites. The membrane was then probed with a 1:50 to 1:100 dilution of the antiserum against the R_I protein in the blocking medium that contained 0.05% Tween 20 and 0.3% Triton X-100 for overnight at 4°. The membrane was then washed overnight with several changes of PBS containing 0.05% Tween 20 and 0.3% Triton X-100. Antigen–antibody complex on the nitrocellulose membrane was probed and quantitated by incubating with 1 μCi of ^{125}I-labeled protein A in the blocking medium containing 0.05% Tween 20 and 0.3% Triton X-100 for 5 hr at room temperature. The nitrocellulose membrane was then washed extensively in PBS containing 0.05% Tween 20 and 0.3% Triton X-100, air dried, and processed for autoradiography. The amount of radioactivity associated with the R_I protein was quantitated by cutting out portions of the nitrocellulose membrane containing the radioactive bands and counting in a gamma counter. The amount of radioactivity associated with a known quantity of the purified R_I protein was used as a standard to determine the amount of R_I in cell extracts.

Other methods. The photoactivated incorporation of 8-N_3-[^{32}P]cAMP was done using 100–200 μg of dialyzed cytosol protein and 1 μM 8-N_3-[^{32}P]cAMP (sp. act. 4–8 Ci/mmol). Samples were incubated at 4° for 30 min followed by a 30-min incubation at room temperature on a platform shaker (this was to facilitate the exchange of 8-N_3-[^{32}P]cAMP with ligands bound to proteins). Samples were then photolyzed and subjected to analysis by SDS–polyacrylamide gel electrophoresis and autoradiography according to procedures previously described [1, 2]. cAMP-phosphodiesterase and acetylcholinesterase activities were assayed according to methods previously described [1, 2, 10].

RESULTS

All experiments in this study were carried out using both the NS-20 and N-18 mouse neuroblastoma cell lines. While there were differences in the specific activity of the R_I cAMP-binding protein, cAMP-phosphodiesterase, and acetylcholinesterase activities in extracts obtained from the two cell lines (as an example, the amount of R_I, expressed as pmol of 8-N_3-[^{32}P]cAMP incorporated into the 47,000-dalton band per mg cytosol protein, was three to eight times higher for the NS-20 cells than the N-18 cells), qualitatively similar results were obtained for the two cell lines. Furthermore, qualitatively similar results on R_I cAMP-binding protein, phospho-

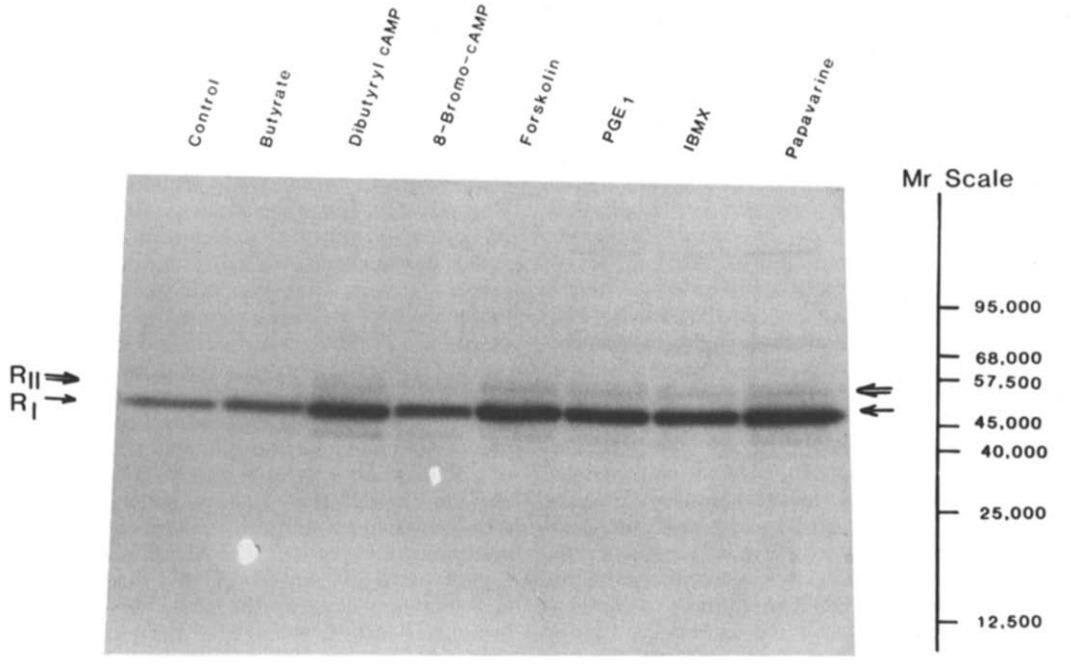

Fig. 1. Autoradiogram illustrating the incorporation of 8-N_3-[^{32}P]cAMP into cytosol proteins of control neuroblastoma cells and cells treated with sodium butyrate (1 mM), dibutyryl cAMP (1 mM), 8-bromo-cAMP (1 mM), forskolin (20 μM), PGE_1 (28 μM), 3-isobutyl-1-methyl xanthine (1 mM), and papavarine (0.5 mM). The NS-20 mouse neuroblastoma cell line was cultured under standard conditions as described in the text. Cells were plated at a density of approximately 1×10^5 cells per 100 mm plate. The test agents were added 4 hr after seeding the cells to a final concentration as indicated. Cells were cultured for 5 days in the absence and presence of the test agents, and the early stationary phase cell cultures were harvested. A 150,000 *g* supernatant fraction was prepared and used to assay for the incorporation of 8-N_3-[^{32}P]cAMP, which was done under standard conditions using 200 μg cytosol protein and 1 μM 8-N_3-[^{32}P]cAMP. Samples were analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography. The positions on the gel of the 52,000–54,000-dalton R_{II} and the 47,000-dalton R_I protein are indicated by arrows.

Table 1. Quantitation of the 52,000–54,000-dalton R_{II} and the 47,000-dalton R_I in neuroblastoma cell extracts by the photoactivated incorporation of 8-N_3-[^{32}P]cAMP

Conditions for cell culturing	Photoactivated incorporation of 8-N_3-[^{32}P]cAMP	
	R_{II} (52–54 kD) (pmol/mg cytosol protein)	R_I (47 kD) (pmol/mg cytosol protein)
Control	1.9 ± 1.8 (8)	7.4 ± 3.2 (14)
Butyrate (1 mM)	1.5 ± 1.3 (4)	8.1 ± 2.3 (6)
Dibutyryl cAMP (1 mM)	2.0 ± 1.4 (9)	18.8 ± 7.4 (11)
8-Bromo-cAMP (1 mM)	2.0 ± 1.7 (5)	6.9 ± 4.5 (10)
Forskolin (20 μM)	1.8 ± 1.0 (8)	24.2 ± 14 (10)
PGE_1 (28 μM)	3.2 ± 1.4 (4)	28.9 ± 9.9 (7)
3-Isobutyryl-1-methyl xanthine (1 mM)	3.3 ± 2.5 (3)	19.8 ± 11 (6)
Papavarine (0.5 mM)	2.5 ± 0.5 (2)	25.65 ± 9.3 (4)

Cells were plated at a density of 1–2 × 10^5 per 100 mm plate, and the test agents were added 2–24 hr after plating. Cells were cultured in the absence and presence of the test agents for a period of 4–5 days. Groups of six 100-mm plates of cells were used for each of the conditions. Aliquots of the cytosol, containing 100–200 μg protein, were used to assay for the incorporation of 8-N_3-[^{32}P]cAMP according to methods described in the text. Portions of the dried gel containing the radioactive bands of R_{II} and R_I were excised and solubilized in H_2O_2, and the amount of radioactivity was quantitated by liquid scintillation spectrometry. Results are presented as means ± SD; the number of times that the determination was made is given in parentheses.

diesterase, and acetylcholinesterase activities were obtained with cell homogenate, and the 150,000 *g* pellet (membrane) and supernatant (cytosol) fractions of the homogenate. To avoid redundancy, only results obtained with cytosol preparations of the NS-20 cell line are presented in the report.

Figure 1 is an autoradiogram illustrating the incorporation of 8-N_3-[^{32}P]cAMP into cytosol proteins of control NS-20 neuroblastoma cells and cells treated with the various pharmacological agents as indicated. The levels of the 52,000–54,000-dalton R_{II} and the 47,000-dalton R_I, quantitated by the amount of radioactivity incorporated, are presented in Table 1. Results showed that dibutyryl cAMP increased by 2- to 3-fold the amount of 8-N_3-[^{32}P]cAMP incorporated into R_I. This effect of dibutyryl cAMP can be reproduced by other agents which increase cellular cAMP concentration either through activation of adenyl cyclase or inhibition of phosphodiesterase. Thus, forskolin and PGE_1, activators of adenyl cyclase, and 3-isobutyl-1-methyl xanthine and papavarine, inhibitors of phosphodiesterase, increased the amount of 8-N_3-[^{32}P]cAMP incorporated into the R_I protein by 2- to 4-fold over that of the untreated control. Butyrate, a possible metabolite of dibutyryl cAMP, was without effect at the 1 mM concentration tested. Of particular interest is the observation that treatment of the neuroblastoma cells with 1 mM 8-bromo-cAMP did not give an increase in incorporation of 8-N_3-[^{32}P]cAMP into the 47,000-dalton R_I protein.

To affirm results obtained with the photoactivated incorporation of 8-N_3-[^{32}P]cAMP, we also quantitated the amount of R_I with the antiserum against the purified bovine skeletal muscle R_I protein using the ELISA and Western blot procedures. Results in Fig. 2 showed that the amount of R_I quantitated by the ELISA technique in extracts of the control, dibutyryl cAMP-, 8-bromo-cAMP-, and PGE_1-treated NS-20 neuroblastoma cells was 0.09% (i.e. 19 pmol/mg protein), 0.22% (47 pmol/mg), 0.07% (14 pmol/mg), and 0.31% (65 pmol/mg) of total cytosol protein respectively. The reason as to why high concentrations of cell extracts did not, whereas the purified R_I protein did, inhibit completely the binding of the anti-R_I antibody to microtiter wells coated with the purified R_I protein is not known. One possibility may be antigenic difference of the R_I protein from bovine skeletal muscle and mouse neuroblastoma cells.

Results on immuno-Western blot quantitation of the amount of R_I, as shown in Fig. 3, gave values of 0.11% (23 pmol/mg), 0.23% (49 pmol/mg), and 0.076% (16 pmol/mg) of total cytosol protein for the control, dibutyryl-cAMP- and 8-bromo-cAMP-treated neuroblastoma cell extracts respectively. The immunoblot in Fig. 3 also demonstrated the presence of a 40,000-dalton protein recognized by the anti-R_I antibody in extracts of the control and 8-bromo-cAMP-treated (but not dibutyryl cAMP-treated) neuroblastoma cells. Several lines of evidence suggest that this 40,000-dalton protein may be a degradative product of the native 47,000-dalton R_I protein. Thus: (i) storage of the purified 47,000-dalton R_I protein resulted in its conversion to a 40,000-dalton species, as determined by SDS–polyacrylamide gel electrophoresis and protein staining of the gel. Since the 40,000-dalton species was not present in the original purified R_I preparation, it was presumed that the 40,000-dalton band was a degradative product of R_I. A similar observation was reported previously [14]. (ii) Incubation of cell extracts at 30° resulted in a time-dependent conversion of the 47,000-dalton R_I to a 40,000-dalton band as determined either by the incorporation of 8-N_3-[^{32}P]cAMP or Western blotting. (iii) Mild treat-

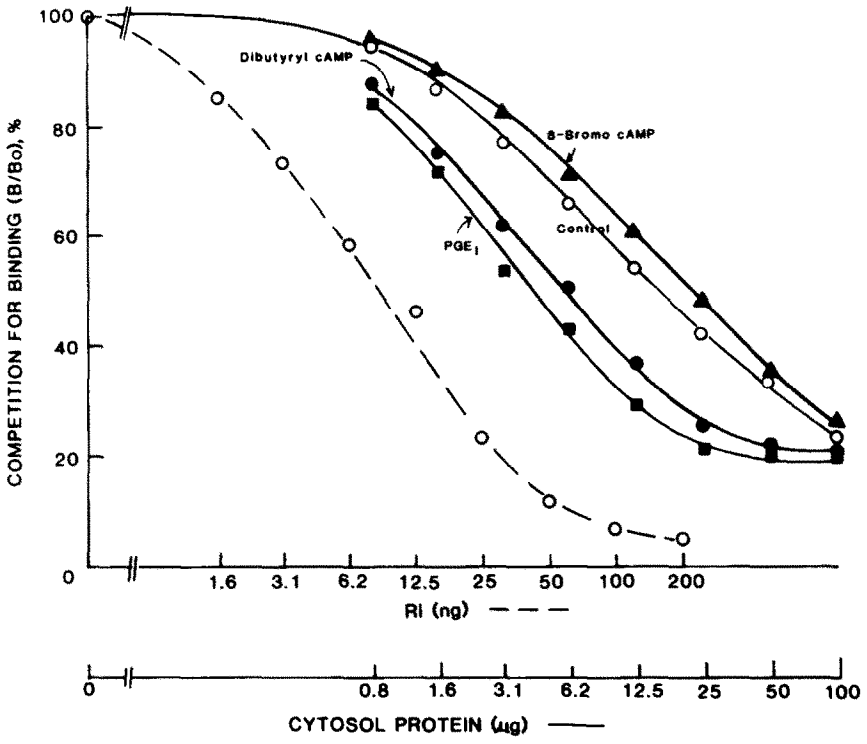

Fig. 2. ELISA quantitation of the amount of R_I in cytosol preparations of control neuroblastoma cells and cells treated with 1 mM dibutyryl cAMP, 1 mM 8-bromo-cAMP, and 28 μM PGE_1. Cells were plated at a density of approximately 2×10^5 cells per 100 mm dish. The test agents were added 4 hr after plating the cells, and cells were cultured in the absence and presence of the test agents for 4 days. Cells were harvested and fractionated to yield cytosol preparations. Aliquots of the cytosol containing various amounts of proteins as indicated were incubated with 100 μl of a 1:500 dilution of the antibody against the R_I protein. A parallel series of tubes containing known amounts of the purified R_I was used to generate a standard curve. At the end of the incubation period, the reaction mixtures were transferred into individual wells of a 96-well Immunolon plate, each well precoated with 10 ng of the purified R_I protein. Results illustrate the inhibition of binding of the antibody to microtiter wells by R_I in cell extracts or purified R_I in solution. The amounts of cytosol protein and the purified R_I protein needed to give a 50% inhibition of binding of antibody to the microtiter well were used to calculate the amount of R_I in cell extracts. Using this method, the amount of R_I in extracts of the control, dibutyryl cAMP-, 8-bromo-cAMP-, and PGE_1-treated NS-20 neuroblastoma cells were 0.09, 0.22, 0.07 and 0.31% of total cytosol protein respectively.

ment of the purified R_I protein or cell extracts with trypsin [1:200 to 1:400 (w:w) ratio of trypsin to substrate protein] resulted in a rapid conversion of the 47,000-dalton R_I to a 40,000-dalton protein which, upon further incubation, broke down into lower molecular weight species. (iv) It is a well known fact that regulatory subunits of cAMP-dependent protein kinases are unusually labile to proteolytic degradation and that many of the proteolytic fragments retain the biological activity to bind cAMP

Table 2. Effects of dibutyryl cAMP, 8-bromo-cAMP, and PGE_1 in regulating neurite outgrowth, acetylcholinesterase, cAMP-phosphodiesterase, and R_I cAMP-binding activities in NS-20 mouse neuroblastoma cells

Culture conditions	Neurite outgrowth (% cells having neurites)	AChE (nmol/min/mg)	cAMP-PDE (pmol/min/mg)	R_I (pmol/mg)
Control	5 ± 2 (5)	7.0 ± 5 (2)	65 ± 7 (5)	5.4 ± 1.3 (4)
Dibutyryl cAMP (1 mM)	85 ± 10 (3)	18.4 ± 8 (2)	142 ± 18 (3)	17.8 ± 2.2 (3)
8-Bromo-cAMP (1 mM)	89 ± 8 (5)	20.5 ± 2.1 (2)	127 ± 29 (5)	4.9 ± 1.4 (4)
PGE_1 (28 μM)	90 ± 5 (3)	16.0 ± 3.2 (2)	166 ± 65 (3)	20.5 ± 6.0 (3)

Conditions for cell culturing are as described in legend of Table 1. Representative fields of the cell cultures at an early stationary phase of growth were photographed, and the number of cells having neurites was scored. The remaining plates of cells were harvested, and the cytosol fraction was used to assay for acetylcholinesterase, cAMP-phosphodiesterase, and R_I cAMP-binding protein according to methods described in the text. Results are expressed as means ± SD; the number of times that the measurement was made is given in parentheses.

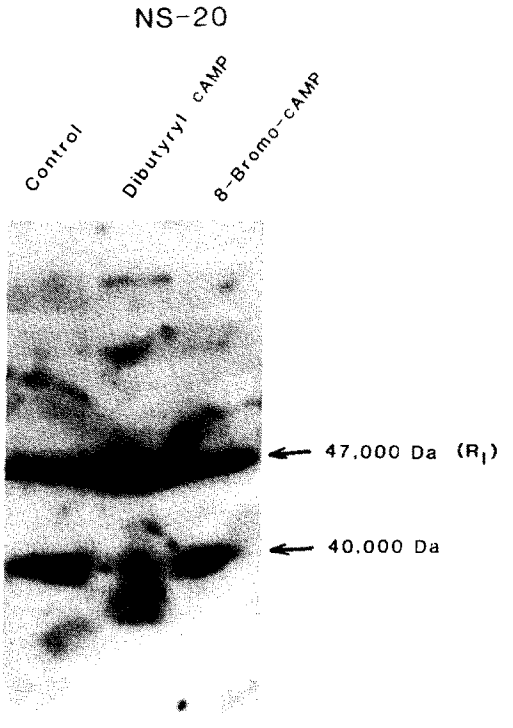

Fig. 3. Western blot of R_I in neuroblastoma cell extracts. Cells were plated at a density of approximately 1.5×10^5 cells per 100 mm dish. Cells were cultured in the absence and presence of the test agents for 5 days prior to harvesting. Aliquots of cytosol preparations, containing 150 μg protein, from control and 1 mM dibutyryl cAMP- and 1 mM 8-bromo-cAMP-treated NS-20 neuroblastoma cells were subjected to SDS–polyacrylamide gel electrophoresis. Proteins on the slab gel were then electrophoretically transferred onto a sheet of nitrocellulose membrane (0.22 μm pore size). The membrane was probed with the antiserum against the 47,000-dalton regulatory subunit of the type I cAMP-dependent protein kinase from bovine skeletal muscle, followed by incubation with 1 μCi of ^{125}I-labeled protein A. The positions on the gel of the 47,000-dalton R_I and a 40,000-dalton degradative product of the R_I protein are indicated.

[14–16]. For example, it was reported that the regulatory subunit of the type I cAMP-dependent protein kinase from porcine skeletal muscle was labile to proteolysis such that in addition to the dimeric regulatory subunit having a molecular weight of 92,500 a small cAMP-binding protein of M_r 35,000 was yielded in the purification procedure [14, 15]. Endogenous proteolytic degradation of the type II kinase regulatory subunit protein was observed upon aging of crude and to some extent purified protein preparation [15, 16]. The endogenous proteolytic cleavage of regulatory subunit proteins was stimulated by Ca^{2+}, and could be minimized by using chelating agents [15, 16].

Whether the occurrence of the 40,000-dalton protein in neuroblastoma cell extracts represents the result of endogenous (physiological) turnover of R_I or an artifact of cell homogenization and fractionation is not known; suffice to say that we added 1 mM EDTA to the homogenization buffer to minimize proteolytic degradation of the regulatory subunit proteins in our manipulations. It should also be noted that simply mixing cytosols of neuroblastoma cells or the purified R_I protein and cytosol at 4° prior to analysis resulted in an additive level of the 47,000-dalton (and 40,000-dalton, if any) protein. This result indicates the absence from the cytosol of substances that would rapidly convert the 47,000-dalton R_I to the 40,000-dalton protein, or the presence of substances that might interfere with the detection of these proteins.

It is noteworthy that, while the three methods of quantitating R_I (photoaffinity labeling, ELISA, and Western blotting) gave similar information concerning the rank order of R_I concentration in various cell extracts, the ELISA and Western blot methods consistently gave higher estimates of R_I concentration than the photoaffinity labeling method. This result suggests that the incorporation of 8-N_3-[^{32}P]cAMP is useful in giving relative, but not absolute, measures of the R_I protein in cell extracts.

The observation that 8-bromo-cAMP did not induce the R_I cAMP-binding protein raises questions concerning the role of R_I in neuroblastoma cell differentiation, and the coupling of the induction of R_I cAMP-binding protein and the other differentiation phenotypes (such as neurite extension and increased acetylcholinesterase activity). For this, we evaluated the effects of dibutyryl cAMP, 8-bromo-cAMP and PGE_1 in promoting neurite outgrowth, induction of acetylcholinesterase, cAMP-phosphodiesterase and R_I cAMP-binding protein. Results in Table 2 showed that the addition of 8-bromo-cAMP to NS-20 cells promoted neurite outgrowth and increased acetylcholinesterase and cAMP-phosphodiesterase activities without a concomitant increase in the R_I cAMP-binding protein. The effects of dibutyryl cAMP and PGE_1 are consistent with those of our previous results; these agents gave concomitant increases in neurite extension, acetylcholinesterase, cAMP-phosphodiesterase and R_I cAMP-binding activities. Results similar to that obtained with dibutyryl cAMP were obtained using 3-isobutyl-1-methyl xanthine (data not shown).

DISCUSSION

The functional role of the R_I cAMP-binding protein in the differentiation of neuroblastoma cells is an issue of relevance and significance to the mode of action of cAMP in the control of neuroblastoma cell differentiation. One possibility is that the induction of the R_I cAMP-binding protein plays a pivotal role in the action of cAMP in evoking and in consolidating the various differentiation functions. The alternative is that the induction of R_I and the concurrent differentiation process is merely coincidental. The possibility exists that the increased expression of the R_I cAMP-binding protein may serve a function similar to that of the increased cAMP-phosphodiesterase, both buffer the cell against a persistant stimulation by cAMP (or its analogue). The observation of induction of cAMP-phosphodiesterase by agents that elevate cAMP concentration in a number of different experimental systems [17–21] is consistent with this hypothesis.

The biochemical basis as to why 8-bromo-cAMP did not, whereas other "cAMP-elevating" agents did, increase the steady-state level of the R_I cAMP-binding protein in neuroblastoma cells remains to be determined. One possibility may be, with the exception of 8-bromo-cAMP, that all the other agents stabilize R_I against intracellular degradation; this will be the subject of another study. The fact that 8-bromo-cAMP can evoke neurite extension and increase acetylcholinesterase activity without a concomitant increase in the R_I-cAMP-binding protein suggests that the increase in R_I is perhaps not an absolute requirement for the expression of these differentiation phenotypes observed under this experimental condition.

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