Differential Effects of Dibutyryl Cyclic Adenosine Monophosphate and Retinoic Acid on the Growth, Differentiation, and Cyclic Adenosine Monophosphate-binding Protein of Murine Neuroblastoma Cells

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ABSTRACT

Dibutyryl cyclic adenosine 3'-5'-monophosphate (Bt2cAMP) and β-all-trans retinoic acid (RA) have been shown separately, and in some cases in combination, to modulate the growth, differentiation, and cAMP-dependent protein kinase (PK-A) activity of various tumor cells. The effects of Bt2cAMP and RA on a cholinergic clone (S20) of C1300 mouse neuroblastoma cells were explored in the present study. Treatment of these cells with 1 mM Bt2cAMP for 3 or more days resulted in 93% inhibition of cell proliferation in monolayer cultures and in 98% inhibition of colony formation in semisolid medium (0.5% agarose). In contrast, treatment of the cells with 1 or 10 µM RA had no inhibitory effects on cell proliferation in monolayer cultures but enhanced colony formation in agarose by up to 130%. The growth of cells treated with a combination of Bt2cAMP and RA was inhibited, although less so than with Bt2cAMP alone. Cells treated with Bt2cAMP alone or Bt2cAMP and RA extended long, neurite-like, cellular processes indicative of differentiation, whereas only a few untreated or RA-treated cells produced such extensions. The amount of [3H]cAMP-binding protein increased gradually up to 2-fold during a 3-day treatment with Bt2cAMP; in contrast it decreased by nearly 2-fold during RA treatment. These changes occurred in the level of the type I regulatory subunit (RI) of PK-A as determined by photoaffinity labeling with 8-azidoadenosine cyclic 3'-5'-[32P]monophosphate. The increase in RI following Bt2cAMP treatment was corroborated by DEAE-cellulose chromatography. This analysis also demonstrated that type I PK-A is the predominant kinase in the untreated S20 cells and that RI exists as a free subunit in Bt2cAMP-treated cells. The activity of PK-A decreased by about 20% following treatment with either Bt2cAMP or RA and by 45% following treatment with a combination of both agents. These results suggest that the distinct effects of Bt2cAMP and RA on the anchorage-independent growth of S20 cells may be related to their opposite effects on the level of RI.

INTRODUCTION

cAMP3 has been implicated in the regulation of the growth and differentiation of various normal and malignant cells (1–4). In the specific case of mouse neuroblastoma cells it has been shown that Bt2cAMP induces morphological and biochemical differentiation in vitro (5–9). The mechanism by which cAMP modulates these fundamental cellular processes is not fully understood. However, it has been proposed that most, if not all, of the effects of cAMP are mediated by PK-A of which there are two forms, type I and type II, in many mammalian tissues (10–12). The enzymes are complexes containing two catalytic subunits and two regulatory subunits each. The catalytic subunits of type I and type II PK-A are identical, but the regulatory subunits are different (13–17). Prashad et al. (18–21) have shown that Bt2cAMP induces cAMP-binding protein in mouse neuroblastoma cells and suggested that this binding protein might be a biochemical marker of neuroblastoma differentiation (22).

Retinoids have also been shown to modulate the growth and differentiation of a variety of untransformed, transformed, and tumor cells in vitro (23–25). Among the cells that are affected by retinoids are several murine (26–29) and human neuroblastoma cells (30, 31). Retinoic acid inhibited the growth of these cells and stimulated their morphological differentiation.

The present study was undertaken to explore the relationships between the effects of Bt2cAMP and RA on the growth, differentiation, and protein kinase activity, in a cholinergic clone (S20) of mouse neuroblastoma cells.

MATERIALS AND METHODS

Cell Culture. The cholinergic clone S20 of C1300 mouse neuroblasto
toma (45, 46) was used. The cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C in a humidified incubator with an atmosphere of 6% CO2:94% air.

Treatment with Bt2cAMP and RA. Bt2cAMP (Sigma Chemical Co., St. Louis, MO) was dissolved in Dulbecco's modified Eagle's medium and kept as a stock solution of 10 mM. RA (BASF Aktiengesellschaft, Ludwigshafen, Federal Republic of Germany) was dissolved in DMSO and kept at –70°C as a stock solution of 10 mM concentration. Bt2cAMP was added to regular growth medium to a final concentration of 1 mM, and RA was added to a final concentration of either 1 or 10 µM. The control cultures received the appropriate amounts of the solvent DMSO (either 0.01% or 0.1%, respectively). At these concentrations DMSO itself had no effect on the neuroblastoma cells.

Assays for Inhibition of Cell Proliferation and Colony Formation. To analyze the effects of Bt2cAMP and RA on the growth of the S20 neuroblastoma cells in monolayer cultures (anchorage-dependent growth) the cells were seeded in regular growth medium in 35-mm diameter tissue culture dishes and incubated at 37°C for either 5 or 24 h for cell attachment. The medium was removed by aspiration and replaced with a fresh medium supplemented with 1 mM Bt2cAMP or 1 or 10 µM RA or both compounds. The cells were incubated at 37°C for 72 h and then the media were replaced with similar fresh media. After 6 days the cells were detached by treatment with 0.25% trypsin in 2 mM EDTA for 5 to 10 min so as to obtain single-cell suspensions; the cells were counted using an electronic particle counter (Model ZBI, Coulter Electronics). Samples were also tested for viability by exclusion of 0.1% Trypan blue using a hemacytometer.
To analyze the effects of Bt2cAMP and RA on the ability of the S20 cells to form colonies in semisolid medium (anchorage-independent growth) the cells were suspended in 0.5% low-temperature gelling agarose (Sea Plaque, FMC Corp., Rockland, ME) dissolved in regular growth medium or in medium supplemented with 1 mM Bt2cAMP or 1 or 10 μM RA. Samples of 1 ml of the cell suspension in agarose were placed on top of a precast layer of semisolid 1% agarose in regular medium in 35-mm dishes. The cell-containing layer was allowed to gel for 10 min at 4°C and then the dishes were transferred to a 37°C incubator. After 72 h the cultures were refed by placing 1 ml of regular growth medium without or with the appropriate agents on top of the cell-containing agarose layer. This medium was replaced with similar fresh medium after an additional 72 h. After 7 days of growth the colonies were counted under the x40 magnification of an inverted phase microscope.

Preparation of Cell Extracts. Untreated or treated cells were detached by scraping with a rubber policeman and homogenized in a buffer (buffer A) consisting of 50 mM Tris-HCl, pH 7.5, 0.25 mM sucrose, 3 mM MgCl₂, 4 mM 2-mercaptoethanol, 50 mM benzamidine-HCl, and 1 mM phenylmethylsulfonylfluoride for assays of cAMP binding and protein kinase activity and for photoaffinity labeling. Samples were homogenized with a Dounce homogenizer. The homogenates were centrifuged at 12,000 × g for 10 min and the supernatant, containing the soluble proteins, was collected and designated cytoplasmic extract. Protein concentration was determined by the method of Lowry et al. (47), using bovine serum albumin as a standard.

Assay for Protein Kinase. PK-A activity in the cytoplasmic extracts was determined by the method of Prashad et al. (9). Twenty micrograms of cell extract protein were mixed with a buffer consisting of 50 mM sodium acetate, pH 4.0, 1 mM dithiothreitol, 4 mM EDTA, and 20 nM (2 μCi) of 8-8N³[32P]cAMP in a final volume of 100 μl. The reactions were conducted in the absence and presence of 10 μM cAMP (Sigma Chemical Co., St. Louis, MO). Reaction mixtures were incubated at 30°C for 10 min, and then each mixture was spotted on phosphocellulose paper (P81, Whatman) and processed as previously described (48). In some assays the histone was replaced by kemptide (Sigma Chemical Co.) which was added at 4 μg assay (100 μM). There was no significant difference between results obtained with histone and with kemptide.

Assay for cAMP Binding. cAMP-binding activity (indicative of the amount of the regulatory subunit of PK-A) was measured in the cytoplasmic extracts by the method of Gilman (49). Twenty-five μg of cell extract protein were mixed with a buffer consisting of 50 mM sodium acetate, pH 4.0, 1 mM dithiothreitol, 10 μM [γ-32P]ATP (2000 cpm/mmol; ICN, Irvine, CA), and 1 mg/ml of histone H2B (Worthington Biochemical Corp.) in a final volume of 100 μl. Analyses of the effects of the two agents on the anchorage-dependent growth of S20 cells revealed that Bt2cAMP almost completely suppressed colony formation in agarose, whereas RA treatment stimulated colony formation significantly at 1 μM (Fig. 2 and Table 1). Observations of cultures 24 h after seeding cells in agarose in the presence of RA revealed no

RESULTS

Effects of Bt2cAMP and RA on Cell Morphology and Growth. S20 neuroblastoma cells extended short neurite-like processes (up to twice the cell diameter) when grown in regular growth medium. These processes were apparent mainly in areas where cell density was low (Fig. 1A). A very similar behavior was observed in cells grown in the presence of 1 μM RA (Fig. 1C). In contrast, cells grown in the presence of 1 mM Bt2cAMP extended numerous, much longer processes (Fig. 1B). Many of these processes were branched and some were thicker than those elaborated by untreated or RA-treated cells. Cells treated with both Bt2cAMP and RA were clustered in aggregates and the cells in the periphery of such aggregates extended long processes (Fig. 1D), as did cells treated with Bt2cAMP alone. The stimulation of this morphologic differentiation by Bt2cAMP was observed as soon as 48 h of treatment. Cells treated with Bt2cAMP alone or with RA also appeared to have a larger diameter and to be more spread on the substratum. Their tendency to be detached by trypsinization was much lower than that of control cells or cells exposed to RA alone (data not shown). Observations of control and treated cultures (Fig. 1) and quantitative analyses of cell numbers in such cultures (Table 1) clearly indicated that Bt2cAMP treatment resulted in a marked inhibition of cell proliferation in monolayer cultures whereas RA had only a small effect. The growth inhibitory effect of Bt2cAMP became apparent after 3 to 4 days (data not shown). There were no floating cells or debris in Bt2cAMP-treated cultures and cell viability was greater than 95% indicating that Bt2cAMP was cytostatic and not cytotoxic. When the cells were grown in the presence of both Bt2cAMP and RA their growth was also inhibited but less so than by Bt2cAMP alone (Table 1).

Analyses of the effects of the two agents on the anchorage-independent growth of S20 cells revealed that Bt2cAMP almost completely suppressed colony formation in agarose, whereas RA treatment stimulated colony formation significantly at 1 μM (Fig. 2 and Table 1). Observations of cultures 24 h after seeding cells in agarose in the presence of RA revealed no
increase in the proportion of cell clumps as compared with untreated cultures. The size distribution of colonies observed under the microscope after 7 days of growth was similar in untreated and in RA-treated cultures. These findings indicate that RA treatment increases the proportion of cells capable of forming colonies rather than enhance cell adhesion to form cell clusters that can be counted as colonies. Interestingly, the growth inhibitory effect of Bt2cAMP superceded the stimulatory effect of RA however, cells exposed to both compounds together were able to form small colonies (Fig. 2D and Table 1) and their number was significantly higher than in cultures exposed only to Bt2cAMP.

Effects of Bt2cAMP and RA on cAMP-dependent Protein Kinase and cAMP-binding Proteins. Treatment of the S20 neuroblastoma cells with either Bt2cAMP or RA decreased the PK-A activity measured in the presence of cAMP by about 20% whereas treatment with both compounds combined decreased the activity by 45% (Fig. 3). However, because the intracellular level of cAMP is increased in Bt2cAMP treated cells (9), PK-A activity, measured in the absence of exogenous cAMP in the assay, was nearly 4-fold higher in cells treated with Bt2cAMP and about 2.5-fold higher in cells treated with both RA and Bt2cAMP than in untreated cells or in cells treated with RA only (Fig. 3).

There are two classes of soluble cAMP-dependent protein kinases in mammalian tissues, and they are designated type I and type II (12). The two forms of the enzyme possess identical catalytic subunits with a molecular weight of 40,000 and are distinguished by having different types of regulatory subunits, a type I (RI) or a type II (RII) subunit with M, 48,000 and 55,000, respectively (13, 14, 50). The activation of PK-A by cAMP is initiated by the binding of cAMP to the regulatory subunits of the holoenzyme which leads to the release of active catalytic subunits from the complex. To evaluate the amounts of the catalytic subunit in cells treated with Bt2cAMP or RA we used immunoblotting techniques. These revealed that the amount of the catalytic subunit decreased by 10, 30, and 20% in cells treated with Bt2cAMP, RA, or a combination of both agents. The lack of a linear correlation between the decrease caused by these agents in PK-A activity and in the amount of
the catalytic subunit might represent differences between levels of enzymatically active protein and immunoreactive protein. In any event, both types of analysis indicated a decrease in enzymatic activity following treatment with either Bt2cAMP or RA.

Next we analyzed the effect of these agents on the binding of \[^3H\]cAMP to cell extracts. Fig. 4A demonstrates that cAMP binding increased in control cultures during a 3-day growth while the cells were proliferating exponentially. The cause for this is not known. Treatment with Bt2cAMP and with RA exerted opposite effects on cAMP binding. Whereas Bt2cAMP caused an increase of up to 200% in binding, treatment with RA caused a decrease to 50% of the binding in control cells (Fig. 4A). The binding of cAMP in cells treated for 3 days with the combination of Bt2cAMP and RA was higher than in the RA-treated or the untreated cells but lower than in cells treated with Bt2cAMP alone (Fig. 4B). The addition of RA at concentrations of up to 10 \(\mu M\), directly into extracts of either untreated control cells or Bt2cAMP-treated cells before the \[^3H\]cAMP binding assay caused no reduction. This observation suggests that RA has to be present for over 24 h in intact cells to exert its effects. The increase in cAMP-binding protein in cells treated with Bt2cAMP was not merely a result of growth inhibition because a similar inhibition of cell proliferation by treatment with 100 \(\mu M\) dexamethasone or by lowering the serum concentration to 1%, was not accompanied by any significant changes in RI levels (data not shown).

To determine whether the effect of Bt2cAMP was on RI or on RII, cytoplasmic proteins extracted from untreated or treated cells were incubated with 8-N3[^32P]cAMP and the regulatory subunits were photoaffinity labeled, separated by SDS-PAGE, and identified by autoradiography (Fig. 5). The major labeled protein in extracts of untreated cells, as well as in extracts of cells treated with either Bt2cAMP or RA, migrated in the gels as RI (Fig. 3, A, C, and E). A minor cAMP-binding protein comprising about 7% of the RI level was observed on the gels as RII (Fig. 3, A, C, and E). That the binding of 8-N3[^32P]cAMP was specific was indicated by the ability of excess cAMP to block the labeling of RI and RII (Fig. 5, B, D, and F). Compared with controls, the amount of RI in Bt2cAMP-treated cells increased nearly 2-fold whereas it decreased by 2-fold in RA-treated cells. The time course of these opposite effects of Bt2cAMP and RA that is presented in Fig. 4B, demonstrates that although the level of RI in untreated cells increases for up to 3 days of growth, the levels of RI in Bt2cAMP-treated cells are higher and in RA-treated cells lower than in the untreated cells. There is a remarkable similarity between the effects of Bt2cAMP and RA on the cAMP-binding as measured by \[^3H\]cAMP-binding and by photoaffinity labeling with 8-N3[^32P]cAMP (compare A and B in Fig. 4).

Since Bt2cAMP treatment increases the intracellular cAMP level and leads to direct activation of PK-A by release of free catalytic subunit we chromatographed extracts of untreated and of Bt2cAMP-treated cells over DEAE-cellulose to separate and assay, with and without exogenous cAMP, for free catalytic subunit and for the type I and type II enzymes. As shown in Fig. 6 (top) type I PK-A is the major enzyme in control cells where it is present at a level 15 times higher than the type II enzyme. Likewise, the major cAMP-binding protein is RI. Both the PK-A type I and RI were eluted with 0.08 M KCl. About 30% of the total PK-A activity in the extract of control cells did not bind to the DEAE-cellulose column and was eluted before the salt gradient was applied (Fig. 6). In contrast, chromatography of extracts from Bt2cAMP-treated cells resulted in a different elution pattern (Fig. 6, bottom). All the protein kinase activity was washed with the buffer before application of the salt gradient. This activity is probably associated with the free catalytic subunit because it was similar in the absence and in the presence of exogenous cAMP. No further PK-A activity was eluted by the salt gradient. The total PK-A activity measured in the fractions of control cells in the presence of cAMP was 3365 pmol[^32P] label as compared to 3255 pmol[^32P] label in Bt2cAMP-treated cell extract fractions. Thus, only a small decrease in the catalytic subunit was observed in Bt2cAMP-treated cells as indicated also by the immunoblotting results described above. The salt gradient eluted free RI from extracts of Bt2cAMP-treated cells and the amount of this protein, estimated by binding of \[^3H\]cAMP, was more than twice the amount found in the corresponding fractions eluted from extracts of control cells in accordance with the results obtained with unfraccionated cell extracts (Fig. 4).

DISCUSSION

The major finding reported here is that Bt2cAMP and RA exert opposite effects on the anchorage-independent growth and on the level of the cAMP-binding protein RI of S20 neuroblastoma cells. Bt2cAMP inhibited both anchorage-dependent and anchorage-independent growth of the S20 cells, and increased the level of RI, whereas RA had no effect on their anchorage-dependent growth, stimulated anchorage-independent growth, and decreased the RI level. In addition, Bt2cAMP stimulated the morphological differentiation of these neuroblastoma cells, whereas RA had no significant effect. Cells treated with both Bt2cAMP and RA together were growth inhibited and had a higher cAMP-binding capacity than untreated cells, although less than in cells treated with Bt2cAMP alone, suggesting that RA only partially reduced the effects of Bt2cAMP.

The growth inhibitory effects of high cellular cAMP concen-
concentrations, achieved by treatment of cells with analogues of cAMP (e.g., Bt2cAMP) or phosphodiesterase inhibitors, have been observed in various cells (2, 3, 29). Previous studies with other mouse neuroblastoma cells (5) have demonstrated that Bt2cAMP can inhibit proliferation and induce morphological and biochemical differentiation. However, the present report is the first to show that Bt2cAMP can suppress the ability of mouse neuroblastoma cells to form colonies in soft agarose which is often used as a criterion for cell transformation because this property is exhibited almost exclusively by transformed and tumorigenic cells (54). Its suppression by Bt2cAMP might be another indication for enhanced differentiation, which is accompanied by decreased expression of properties associated with the transformed phenotype. Bt2cAMP also decreased the expression of N-ras and N-myc protooncogenes in S20 cells.4

Retinoic acid has been shown to inhibit the anchorage-dependent growth and the anchorage-independent growth of several human neuroblastoma cell lines (30, 31). In some of these cells RA also induced morphological differentiation (30, 31) and suppressed the expression of the N-myc oncogene (55). Earlier studies described the ability of RA to inhibit the proliferation of mouse neuroblastoma cells of line C1300 (27) and clone N18 (26, 28) and NB2a (29). In contrast, our studies demonstrated that RA not only failed to inhibit the anchorage-dependent proliferation of clone S20 of C1300 neuroblastoma cells but enhanced their ability to form colonies in agarose. Such findings are not unprecedented since we have previously found that clones and variants of B16 melanoma cells exhibit differential responsiveness to the growth inhibitory effects of RA (56). Among six human melanoma cell lines two were inhibited by RA, three were unaffected, and one was stimulated (57). However, none of these cell lines was stimulated to form colonies in agarose (58). A combination of epidermal growth factor and RA has been shown to enhance the ability of normal rat kidney (NRK) cell line 536-SA6 to form colonies in soft agar, but RA alone had no effect on the anchorage-independent growth of these cells (59). It is possible that RA potentiates the action of an endogenous transforming growth factor in the S20 cells and that this factor enhances colony formation in agarose. Other growth stimulatory effects of retinoids have been observed in several cell types (60).

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4 N. Prashad, manuscript in preparation.
Bt₂cAMP AND RA EFFECTS ON NEUROBLASTOMA CELLS

The concurrent exposure of cells to RA and Bt₂cAMP has enhanced the effect of RA on the differentiation of F-9 murine embryonal carcinoma cells (32) and U-937 human histiocytic lymphoma cells (35). Furthermore, the myeloid leukemic cell lines HL-60 and U-937, which are not responsive to agents known to increase intracellular cAMP levels (e.g., prostaglandin E₂ and cholera toxin), were rendered responsive by pretreatment with a low dose of RA, a dose that by itself was not sufficient to induce differentiation (36). Thus, RA must have primed the cells to respond to an increase in cAMP levels. A similar conclusion was drawn from results of studies with F-9 embryonal carcinoma cells (28, 32). However, the induction of transglutaminase in HL-60 cells and in murine peritoneal macrophages required the simultaneous presence of both RA and Bt₂cAMP; it was proposed that the primary effect of Bt₂cAMP was to increase the sensitivity of the cells to RA (37, 38). Our studies with mouse neuroblastoma cells indicate that RA and Bt₂cAMP exert opposite effects on anchorage-independent growth and that the inhibitory effects of Bt₂cAMP are reduced in the presence of RA. An analogous result was reported recently for neonatal mouse keratinocytes in which 8-bromo cAMP stimulated cell proliferation, but a retinoid inhibited cell proliferation and inhibited the stimulatory effects of the cAMP analogue (61).

The mechanism by which Bt₂cAMP suppresses growth and promotes differentiation of the S20 neuroblastoma cells is not known. It has been shown that exogenously added Bt₂cAMP increases the intracellular cAMP level in neuroblastoma cells (9, 18) and induces the synthesis of cAMP-binding proteins (18-21, 62-65). Most effects of cAMP are thought to be mediated by cAMP-dependent protein kinases (66). Changes in the level and ratio of PK-A type I and type II have been related to cell proliferation, differentiation, and malignancy (15, 22, 39, 40, 42-44, 67, 68). However, the treatment of S20 neuroblastoma cells with Bt₂cAMP, which resulted in enhanced differentiation, did not increase PK-A activity as measured in the presence of cAMP. The increase in protein kinase activity in Bt₂cAMP-treated cells as measured in the absence of cAMP in...
and then with 200 ml of a KCl gradient (0-0.3 M in buffer B). Fractions containing the 12,000 x g supernatant, containing 20 mg protein, was applied in buffer B, containing Bt2cAMP. The cells were then scraped into buffer B and treated cells. S20 neuroblastoma cells were grown for 3 days in the absence or in the presence of 1 min Bt2cAMP. The cells were then harvested and analyzed for [3H]cAMP binding and PK-A activity in the presence and absence of 10 U姆cAMP. Arrows, the beginning of the salt gradient; RI/C, and RII/C, location were PK-A type 1 and II are eluted from this column, respectively.

The assay is probably the result of a Bt2cAMP-induced increase in cellular cAMP as described before (9, 18).

Treatment of mouse melanoma cells with RA inhibited cell proliferation and enhanced PK-A activity (39). The effect of RA was limited to PK-A type I since an increase in the amount of the cytosolic RI regulatory subunit was observed in the treated cells (40). RA treatment of F9 mouse embryonal carcinoma cells induced their differentiation into parietal endoderm cells and increased the amounts of cytosolic RI and RII regulatory subunits and PK-A activity (42, 43). The increases in these cAMP-binding proteins and PK-A activity were induced shortly after exposure to RA, and it has been proposed that these could enhance the responsiveness of RA-treated cells to cyclic nucleotides. RA treatment of human HL-60 promyelocytic leukemia cells also induced differentiation and enhanced PK-A activity (44). In these cell lines there was a specific increase in cytosolic RI and in the type I isozyme of PK-A (44). 8-Bromo cAMP (1 mm) could also induce these cells to differentiate, but a 1000-fold higher concentration of this cAMP analogue was required compared with RA (44). In contrast with these results, RA neither induced differentiation of the S20 neuroblastoma cells nor changed the activity of PK-A. The only effects of RA were stimulation of anchorage-independent growth and decrease in the amount of the RI cAMP-binding protein. Interestingly, a recent report described a decrease in cytosolic kinase activity and in the RI subunit following RA treatment of PCC4(RA)-1 cells which are mutant embryonal carcinoma cells resistant to differentiation induction by RA (69).

Although Bt2cAMP slightly decreased PK-A activity and the level of the catalytic subunit in the S20 neuroblastoma cells, it increased the level of the RI cAMP-binding protein. Similar results have been observed previously with the S20 cells (18–21) as well as with another clone (N18) of C13000 neuroblastoma (63, 64), and with neuroblastoma-glioma cell hybrids (64, 65). In all these cells there was no increase in PK-A activity, suggesting that the regulation of the expression of the regulatory subunits may be independent of the expression of the catalytic subunit. The mechanism by which Bt2cAMP increases RI expression in the S20 cells probably involves an increase in translatable mRNA for RI (20) and an increase in the stability of the RI-cAMP complex (21). Conversely, RA might decrease RI expression by altering the transcription or the translation of the mRNA for RI. Since the growth of these cells was inhibited by Bt2cAMP and their differentiation was stimulated, it has been proposed that the free RI cAMP-binding protein may play a role in the regulation of these cellular processes (18–21, 64). In prokaryotes, the cAMP-receptor protein complex binds to DNA and modifies gene transcription (70). It is intriguing to speculate that a similar mechanism might operate in eukaryotic cells. In this context it is interesting to note that Bt2cAMP treatment of the S20 neuroblastoma cells increased nuclear cAMP-binding protein and cAMP-binding to chromatin (19). Another possible mechanism by which free cAMP-binding protein could regulate gene expression is by maintaining a higher level of cAMP by forming a cAMP-binding protein complex, thus protecting cAMP from phosphodiesterase (71). cAMP has been implicated in the regulation of the phosphorylation of certain nuclear nonhistone proteins and in the regulation of gene transcription (72); therefore, its protection from degradation might increase the efficacy of its nuclear function.

Since Bt2cAMP and RA exert opposite effects on anchorage-independent cell growth and on the level of RI there seems to be an inverse relationship between the level of RI and the expression of the transformed phenotype of the S20 neuroblastoma cells.


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