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# Induction of the Regulatory Subunit of Type I Adenosine Cyclic 3′:5′-Monophosphate-dependent Protein Kinase in Differentiated N-18 Mouse Neuroblastoma Cells

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## ABSTRACT

The expression of a adenosine cyclic 3′:5′-monophosphate (cAMP)-binding protein, regulatory subunit of the type I cAMP-dependent protein kinase ($R_I$), and its functional significance in the differentiation of N-18 mouse neuroblastoma cells were examined. 8-Azidoadenosine cyclic 3′:5′-[$^{32}$P]monophosphate, a photoaffinity-labeling analog of cAMP, and high-resolution sodium dodecyl sulfate-polyacrylamide gel electrophoresis were used to identify and quantitate cAMP-binding proteins in cell extracts. The induction of differentiation of N-18 mouse neuroblastoma cells, initiated either by adding dibutyryl adenosine cyclic 3′:5′-monophosphate to the growth medium or by culturing cells in medium supplemented with 1% fetal calf serum, led to a 3-fold increase in the amount of 8-azidoadenosine cyclic 3′:5′-[$^{32}$P]monophosphate incorporated into $R_I$, when assayed *in vitro*. This increased incorporation was attributable to an increase in the amount of $R_I$ rather than to an increase in the affinity of $R_I$ for 8-azidoadenosine cyclic 3′:5′-[$^{32}$P]monophosphate. The subunit molecular weight, isoelectric point, and immunoreactivity of $R_I$ were found to be identical to that of the regulatory subunit of the type I cAMP-dependent protein kinase purified from bovine skeletal muscle. The increase in $R_I$ was not accompanied by an increase in the cAMP-dependent protein kinase activity. DEAE-cellulose column chromatography confirmed the induction of $R_I$ as a free cAMP-binding protein in the differentiated neuroblastoma cells. The possibility of a growth-dependent regulation of $R_I$ was also examined. Addition of 2% dimethyl sulfoxide to cultures of N-18 mouse neuroblastoma cells inhibited cell growth without increasing the specific activity of $R_I$. Dimethyl sulfoxide had no effect on neurite outgrowth or acetylcholinesterase activity, two parameters characteristic of differentiated cells. The fact that the induction of $R_I$ coincided with differentiation of the neuroblastoma cells suggests that the expression of $R_I$ may be used as a biochemical index of differentiation in these cells. The presence of a free cAMP-binding protein, not associated with cAMP-dependent protein kinase in neuroblastoma cells, raises important considerations concerning the action of cAMP in the regulation of growth and differentiation.

## INTRODUCTION

Interest in the mouse neuroblastoma cells has centered around the expression of a catalog of morphological, biochemical, and electrophysiological functions which occur when cells are grown in defined conditions (22, 27). For example, the addition of $Bt_2cAMP$[3] to cultures of neuroblastoma cells results in the transformation of morphologically less complex cells, the neuroblastoma round cells, to cells with elongated neurites, a more complex phenotype (23). Morphological differentiation of neuroblastoma cells is generally associated with increases in the specific activity of enzymes involved in the synthesis and degradation of neurotransmitters. Electrophysiological recordings from a population of neuroblastoma cells exhibiting various stages of neurite formation suggested that there is a concerted sequence of development of discrete electrical membrane properties, reflecting different stages of neuroblastoma differentiation (27).

The ability of cAMP (or agents which increase intracellular levels of cAMP) to produce large changes in morphology, as well as in enzymatic and electrical activities, of the mouse neuroblastoma cells renders these cells an excellent system for studying the action of cAMP in modulating the expression of various differentiated functions in eukaryotic cells. Work from a large number of laboratories has suggested that cAMP-dependent protein kinase may be the primary, if not the only, mediator of cAMP action in eukaryotic cells. Thus, greater than 95% of the cAMP-binding protein present in a variety of tissues examined was identified as the regulatory subunit of cAMP-dependent protein kinase (32). This finding and the observation of equimolar concentrations of regulatory and catalytic subunits of cAMP-dependent protein kinase in various mammalian tissues (11) certainly suggest that the action of cAMP in eukaryotic cells is mediated through activation of cAMP-dependent protein kinase and phosphorylation of specific substrate proteins (19, 25) and that the cAMP-binding regulatory subunit is unlikely to be directly involved in modulating the activity of a wide variety of enzymes.

In mouse neuroblastoma cells, the induction of differentiation, initiated either by adding $Bt_2cAMP$ to the growth medium or by culturing cells under serum-deprived conditions, is accompanied by a large increase in cAMP-binding activity (17, 24, 31). The purpose of this report is (*a*) to characterize and identify the induced cAMP-binding protein in differentiated N-

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[3] The abbreviations used are: $Bt_2cAMP$, $N^6,O^{2'}$-dibutyryl adenosine cyclic 3′:5′-monophosphate; cAMP, adenosine cyclic 3′:5′-monophosphate; IBMX, 3-isobutyl-1-methylxanthine; 8-$N_3$-[$^{32}$P]cAMP, 8-azidoadenosine cyclic 3′:5′-[$^{32}$P]monophosphate; FCS, fetal calf serum; DMSO, dimethyl sulfoxide; PMSF, phenylmethylsulfonylfluoride; MES, 2-(*N*-morpholino)ethanesulfonate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; M.W., molecular weight(s); $R_I$, regulatory subunit of the type I adenosine cyclic 3′:5′-monophosphate-dependent protein kinase; $R_{II}$, regulatory subunit of the type II adenosine cyclic 3′:5′-monophosphate-dependent protein kinase; $R_I^*$, regulatory subunit of the type I adenosine cyclic 3′:5′-monophosphate-dependent protein kinase purified from bovine skeletal muscle.

18 mouse neuroblastoma cells, (*b*) to examine the possible functions of the increased cAMP-binding protein, either serving as a regulatory subunit of cAMP-dependent protein kinase or in some other capacity, and (*c*) to correlate the appearance of this cAMP-binding protein with the appearance of other differentiated functions, such as neurite extension or increased acetylcholinesterase activity.

## MATERIALS AND METHODS

### Materials

The following materials were purchased. AMP, cAMP, $Bt_2cAMP$, ATP, IBMX, neostigmine, and *Ophiophagus hannah* venom were from Sigma Chemical Co., St. Louis, Mo. [$^3$H]cAMP was from New England Nuclear, Boston, Mass. 8-$N_3$-[$^{32}$P]cAMP was obtained from ICN Pharmaceuticals, Inc., Irvine, Calif.; purity of the compound was checked by thin-layer chromatography using cellulose $F_{254}$-coated thin-layer chromatographic sheets (EM Laboratories, Inc., Elmsford, N. Y.) in a solvent system of *n*-butyl alcohol/acetic acid/water (5/2/3, v/v/v). [$\gamma$-$^{32}$P]ATP was synthesized according to the method of Post and Sen (21). 1-[$^{14}$C]Acetylcholine was obtained from Amersham/Searle Corp., Arlington Heights, Ill. Cation-exchange resin AG 50 W-X8 (200 to 400 mesh) and AG 1-X2 (200 to 400 mesh) were from Bio-Rad Laboratories, Richmond, Calif. Tissue culture supplies were from Grand Island Biological Co., Grand Island, N. Y.

### Experimental Procedures

**Cell Growth and Differentiation.** The N-18 mouse neuroblastoma cell line was obtained from Dr. E. S. Canellakis, Department of Pharmacology, Yale University School of Medicine, and was cultured under conditions as described previously (17).

Three experimental conditions were used to modulate growth and differentiation of N-18 neuroblastoma cells. These include (*a*) the addition of 1 mM $Bt_2cAMP$ to the growth medium at 24 hr after plating, (*b*) culturing cells in medium containing 1%, rather than the usual 10%, FCS, and (*c*) treatment of cells with 2% DMSO, added at 24 hr after plating.

The 2 criteria used to quantitate differentiation of N-18 mouse neuroblastoma cells were (*a*) the existence of one or more elongated processes (neurites) extending from the perikaryon which were greater than 50 $\mu$m in length and (*b*) increased specific activity of acetylcholinesterase. By these criteria, it was shown that cells treated with 1 mM $Bt_2cAMP$ or 1% FCS were differentiated, while cells treated with 2% DMSO were not.

**Preparation of Cell Extracts.** Unless otherwise stated, cells at an early stationary phase of growth were used. The 100,000 $\times$ *g* supernatant and pellet were prepared from cell homogenate according to methods described previously (17) and were used as the cytosol and membrane fractions, respectively. For routine analysis, all cytosol preparations were either passed through a Sephadex G-25 column or dialyzed against 10 mM Tris-HCl buffer (pH 7.4) containing 50 $\mu$g per ml PMSF and 1 mM EDTA, at 4° for 16 hr to remove low-molecular-weight endogenous substances. Protein concentration was determined by the method of Lowry *et al.* (18) using bovine serum albumin as the standard.

**Photoaffinity Labeling of cAMP Receptor Proteins.** Photoaffinity labeling experiments with 8-$N_3$-[$^{32}$P]cAMP were performed as described previously (17). The standard reaction mixture (final volume, 0.1 ml) contained 50 mM MES (pH 6.2), 10 mM $MgCl_2$, 0.5 mM IBMX, 0.1 nM to 1 $\mu$M 8-$N_3$-[$^{32}$P]cAMP (specific activity, 4 to 10 Ci/mmol), and various amounts of cytosol protein up to 200 $\mu$g. (Although 8-$N_3$-[$^{32}$P]cAMP is not a good substrate for phosphodiesterase, it is not entirely resistant to hydrolysis by this enzyme. The hydrolysis of 8-$N_3$-[$^{32}$P]cAMP by phosphodiesterase was of particular concern when submaximal concentrations of the ligand were used and when phosphodiesterase activity was elevated, *e.g.*, in extracts obtained from $Bt_2cAMP$- and 1% FCS-treated cells. The presence of a phosphodiesterase inhibitor, such as IBMX, is therefore necessary to maintain the concentration of 8-$N_3$-[$^{32}$P]cAMP in the assay mixture during the 60-min preincubation period and to obtain an accurate estimate of the $K_d$.) Samples were incubated at 4° for 60 min in the dark to allow equilibrium binding of 8-$N_3$-[$^{32}$P]cAMP, followed by photolysis of the samples with a mineralite UVS-11 hand lamp for 10 min. To each sample, 25 $\mu$l of a sodium dodecyl sulfate stop solution was added. The amount of radioactivity incorporated into proteins was analyzed by SDS-PAGE and autoradiography as described previously (17). Autoradiographs were scanned with a Schoeffel SD-3000 spectromicrodensitometer, and the peak heights of the optical tracings were used as a quantitative measure of the incorporation of $^{32}$P. Where indicated, the absolute amounts of radioactivity were also determined by slicing the dried gels and counting by liquid scintillation spectrometry.

In studying the photoactivated incorporation of 8-$N_3$-[$^{32}$P]cAMP into cAMP receptor proteins present in extracts of N-18 neuroblastoma cells, we observed that, in addition to the incorporation of 8-$N_3$-[$^{32}$P]cAMP into the specific cAMP-binding proteins, $R_I$ and $R_{II}$, occasionally other protein bands with molecular weights both higher and lower than that of $R_I$ and $R_{II}$ were also labeled. The labeling of these proteins could not be accounted for by the presence of trace amounts of contaminants in several batches of the 8-$N_3$-[$^{32}$P]cAMP preparations obtained from ICN Pharmaceuticals, Inc. These proteins were categorically termed nonspecific cAMP-binding proteins, since the inclusion of a 20-fold excess of cold cAMP in the assay mixture had little effect on the amount of 8-$N_3$-[$^{32}$P]cAMP incorporated. In attempting to reduce the extent of this nonspecific incorporation, we added 0.25 mM 2-mercaptoethanol to the assay mixture, hoping that it would serve as a scavenger to react with the photoactivated 8-$N_3$-[$^{32}$P]cAMP present in solution rather than that at the binding site. Results demonstrated a significant reduction in the amount of nonspecific labeling by 8-$N_3$-[$^{32}$P]cAMP in the presence of 2-mercaptoethanol. More importantly, however, was the concentration-dependent inhibition by 2-mercaptoethanol of the incorporation of 8-$N_3$-[$^{32}$P]cAMP into the specific cAMP-binding proteins $R_I$ and $R_{II}$ of N-18 neuroblastoma cells. This and the necessity of photolyzing the samples for prolonged periods of time to maximize the amount of 8-$N_3$-[$^{32}$P]cAMP incorporated (approximately 8 to 10 min) suggest the possibility of a pseudophotoaffinity-labeling mechanism for the incorporation of 8-$N_3$-[$^{32}$P]cAMP into cAMP-binding proteins (26). Alternatively, it is possible that the photolabile 8-$N_3$-[$^{32}$P]cAMP may react with 2-mercaptoethanol in a time- and concentration-dependent manner during the 60-min preincubation period in the dark to form the photostable 8-aminoadenosine cyclic 3':5'-monophosphate. The reduction of 8-azidoadenosine derivatives and arylazides by thiols has been demonstrated in previous studies (4, 28).

Thus, in order to quantitate the concentration of various cAMP receptor proteins present in extracts of N-18 neuroblastoma cells, it becomes essential to delete both dithiol and monothiol reducing agents from the assay mixture. The presence of 2-mercaptoethanol during photolysis was useful for some purposes, however, since it strongly inhibited the amount of nonspecific labeling. The presence of 2-mercaptoethanol in the binding assay mixture, when used, is indicated in the appropriate figure legends.

**Reversible Binding of [$^3$H]cAMP.** The binding of [$^3$H]cAMP to cytosolic proteins was performed according to the method of Gilman (8) as described previously (17).

**Isoelectric Focusing.** Isoelectric focusing was carried out on a LKB Multiphor unit using a pH 3.5 to 9.5 LKB Ampholine polyacrylamide gel plate. At the end of the focusing procedure, the pH gradient of the gel was determined by a surface electrode.

**Purification of $R_I$* and the Preparation of Rabbit Anti-$R_I$* Antibody.** $R_I$* was purified according to the method of Beavo *et al.* (2). The purified protein was analyzed by SDS-PAGE, and purity was estimated to be greater than 95%. No contamination from $R_{II}$ was detectable as

assayed by the incorporation of 8-$N_3$-[$^{32}$P]cAMP. For the generation of antiserum directed against the purified $R_I$*, approximately 200 µg of the purified protein in 1 ml of phosphate-buffered saline (0.15 M NaCl, 10 mM sodium phosphate, pH 7.4) was emulsified in equal volume of Freund's complete adjuvant and injected s.c. at multiple sites along the neck of a male New Zealand albino rabbit. A booster injection of the same amount of protein emulsified with Freund's incomplete adjuvant was given 2 weeks later. The rabbit was bled from the ear vein approximately 2 weeks after the booster injection. The antiserum thus obtained was purified by ammonium sulfate fractionation and DEAE-cellulose column chromatography as described previously (6).

**Immunoprecipitation of $R_I$ from Extracts of N-18 Mouse Neuroblastoma Cells using Anti-$R_I$* Antibody.** cAMP receptor proteins present in extracts of N-18 neuroblastoma cells (both control and differentiated) were first labeled with 8-$N_3$-[$^{32}$P]cAMP. Fifty µl of the purified rabbit anti-$R_I$* antibody were then added and incubated overnight at 4°. The antigen-antibody complex was then isolated by incubation with Protein A-Sepharose CL-4B at room temperature for 30 min. The Sepharose beads were pelleted by centrifugation and washed 3 times with 0.5 ml of 10 mM MES (pH 7.2) containing 50 mM NaCl. The bound proteins were eluted by the addition of 50 µl of 15% sodium dodecyl sulfate. The amount of radioactivity present in the eluate was then determined by liquid scintillation spectrometry.

**Histone Kinase Assay.** For determination of cAMP-dependent protein kinase activity, cytosols from N-18 mouse neuroblastoma cells were first dialyzed against 500 to 1000 volumes of 10 mM Tris-HCl (pH 7.4), containing 50 µg PMSF per ml, 1 mM EDTA, and 1 mM dithiothreitol. The cAMP-dependent protein kinase activity was measured according to the methods of Witt and Roskoski (33).

The standard assay mixture (final volume, 100 µl) contained 10 to 30 µg cytosol protein, 50 mM MES (pH 6.2), 10 mM $MgCl_2$, 50 µM [γ-$^{32}$P]ATP (specific activity, 5 to 20 × $10^5$ cpm/nmol), and 50 µg histone f2b, with or without 5 µM cAMP. Under these conditions, the phosphorylation of histone was proportional to time of incubation and amount of cytosol protein used, and neither [γ-$^{32}$P]ATP nor histone was rate limiting during the 10-min reaction time at 30°. One unit of histone kinase activity was defined as that amount of enzyme which catalyzed the transfer of 1 pmol of $^{32}$P from [γ-$^{32}$P]ATP to recovered histone in 1 min at 30°. A partially purified protein inhibitor from bovine brain, prepared according to the methods of Walsh *et al.* (30) (up to the step of DEAE-cellulose column chromatography), was used to discriminate the activity attributable to that of the cAMP-dependent protein kinase from the cAMP-independent protein kinase.

**DEAE-Cellulose Column Chromatography.** DEAE-cellulose column chromatography was performed as described previously (16). The column (0.9 × 4 cm) was preequilibrated in 10 mM Tris-HCl (pH 7.4), 50 µg PMSF per ml, and 1 mM EDTA. Enzymes were eluted from the column with a linear gradient of 0 to 0.3 M NaCl in the same buffer.

**Measurement of cAMP.** Measurement of intracellular cAMP was done according to methods described (17). The assay is based on competitive binding of [$^3$H]cAMP and cAMP to the type II cAMP-dependent protein kinase obtained from bovine heart muscle.

**Phosphodiesterase Assay.** Phosphodiesterase activity was determined according to the method of Thompson *et al.* (29). The 100-µl assay mixture contained 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 10 mM AMP, 30 µM cAMP, 50 nM [$^3$H]cAMP (140,000 cpm), 20 µg of snake venom from *Ophiophagus hannah*, and 5 to 20 µg of protein sample. The sample and venom were added to initiate the reaction. After incubation at 30° for 10 min, the reaction was stopped with the addition of 1 ml of a 1/2 slurry of Bio-Rad resin AG 1-X2 (200 to 400 mesh; in water at pH 5.0). After 10 min of equilibration, the supernatant was transferred into scintillation vials. Ten ml of Aquasol were then added, and radioactivity was determined by liquid scintillation spectrometry. Alternatively, the reaction product was added to a minicolumn of Bio-Rad resin AG 1-X2. The column eluate was collected, and the amount of radioactivity was determined. Similar results were obtained by the batch and column absorption methods, although the background activity was significantly lower in the column method.

**Acetylcholinesterase Assay.** Acetylcholinesterase activity was assayed by a radiometric method as described previously (17). All assays were carried out at 37° with 5 µM 1-[$^{14}$C]acetylcholine. The cation exchange resin (Bio-Rad AG50 W-X8) was used for the separation of [$^{14}$C]acetate from [$^{14}$C]acetylcholine and choline.

## RESULTS

**The Induction of the cAMP-binding Protein, $R_I$.** The identity and amount of cAMP-binding proteins present in membrane and cytosol fractions of N-18 mouse neuroblastoma cells were studied by the photoactivated incorporation of 8-$N_3$-[$^{32}$P]cAMP together with the techniques of SDS-PAGE and autoradiography. Comparison was made between extracts of control, 1 mM $Bt_2$cAMP-, 1% FCS-, and 2% DMSO-treated N-18 neuroblastoma cells (Fig. 1; Table 1). In both the membrane (Fig. 1*A*) and the cytosol (Fig. 1*B*) preparations, 3 protein bands incorporated 8-$N_3$-[$^{32}$P]cAMP, and the incorporation was blocked by a 20-fold excess of cold cAMP. The apparent M.W. of these proteins on SDS-PAGE were 47,000, 52,000, and 54,000. For reasons discussed previously (17), the M.W. 47,000 protein will be referred to as $R_I$; $R_{II}$ will be used as a collective term for the M.W. 52,000 and the M.W. 54,000 8-$N_3$-[$^{32}$P]cAMP-binding proteins.

Treatment of N-18 neuroblastoma cells with 1 mM $Bt_2$cAMP resulted in a 2.7- and a 3-fold increase in the incorporation of 8-$N_3$-[$^{32}$P]cAMP into $R_I$ present in the membrane and the cytosol fractions, respectively (Table 1). Similar increases in $R_I$ were observed when cells were grown in medium containing 1% FCS, a condition which increased intracellular cAMP concentration (17). For both the $Bt_2$cAMP- and the 1% FCS-treated cells, the amount of 8-$N_3$-[$^{32}$P]cAMP incorporated into $R_{II}$ was not significantly different from that of the control, although occasionally an increase (less than a doubling) in the amount of radioactivity incorporated into the M.W. 52,000 protein was observed. It should be noted that neither the specific activities of $R_I$ and $R_{II}$ in the particulate and soluble preparations nor the magnitudes of increase in $R_I$ were affected by the inclusion of 0.3 M KCl in the homogenization buffer (data not shown). This suggests that the $R_I$ and $R_{II}$ in the particulate preparation may

Table 1

*Concentrations of $R_I$ and $R_{II}$ present in membrane and cytosol fractions of control, 1 mM $Bt_2$cAMP-, 1% FCS-, and 2% DMSO-treated N-18 mouse neuroblastoma cells*

The incorporation of 8-$N_3$-[$^{32}$P]cAMP was done under standard condition using 1 µM 8-$N_3$-[$^{32}$P]cAMP, 0.5 mM IBMX, and 200 µg protein. Proteins were separated by SDS-PAGE. The amounts of radioactivity incorporated into $R_I$ and $R_{II}$ were quantitated by liquid scintillation counting of the dried gel slices.

| | 8-$N_3$-[$^{32}$P]cAMP incorporated (pmol/mg protein) | |
|---|---|---|
| | $R_I$ (M.W. 47,000) | $R_{II}$ (M.W. 52,000 and 54,000) |
| Membrane | | |
| Control | 0.66 ± 0.17[a] | 0.50 ± 0.15 |
| 1 mM $Bt_2$cAMP | 1.80 ± 0.42 | 0.68 ± 0.23 |
| 1% FCS | 1.70 ± 0.53 | 0.41 ± 0.11 |
| 2% DMSO | 0.58 ± 0.25 | 0.79 ± 0.29 |
| Cytosol | | |
| Control | 2.65 ± 0.38 | 1.06 ± 0.24 |
| 1 mM $Bt_2$cAMP | 7.72 ± 2.07 | 1.52 ± 0.32 |
| 1% FCS | 6.42 ± 2.54 | 1.44 ± 0.46 |
| 2% DMSO | 2.10 ± 0.18 | 1.90 ± 0.26 |

[a] Mean ± S.E. of 3 independent experiments.

represent proteins intrinsic to the membrane fraction, as opposed to proteins of cytoplasmic origin adsorbed onto membranes during the process of homogenization and fractionation. The artifactual translocation of cytoplasmic protein kinase to membrane fraction in buffers of low ionic strength has been described previously (13).

The induction of differentiation of the N-18 cells, initiated either by adding $Bt_2cAMP$ or by culturing cells under serum-limiting conditions, is accompanied by slowing of cell growth; the possibility of a growth-dependent regulation of $R_I$ must, therefore, be considered. DMSO (2%), while inhibiting cell growth, had no effect in promoting neurite outgrowth of the N-18 mouse neuroblastoma cells. Treatment of cells with 2% DMSO did not result in an increased level of $R_I$, when compared to the untreated counterpart; in fact, in 4 of 10 experiments carried out over a 6-month period, a 10 to 40% decrease in $R_I$ was observed. Interestingly, $R_{II}$ was increased in cells treated with 2% DMSO; the magnitude of this increase varied between 50 to 200% from experiment to experiment and was observed in both the membrane and cytosol fractions. This effect was not mimicked by the addition of DMSO directly to the 8-$N_3$-[$^{32}$P]cAMP-binding assay mixture. The significance of this observation is not clear.

In order to investigate whether the increased incorporation of 8-$N_3$-[$^{32}$P]cAMP into $R_I$ was attributable to an increase in the amount of $R_I$ or to an increase in the affinity of $R_I$ for 8-$N_3$-[$^{32}$P]cAMP, the concentration-dependent incorporation of 8-$N_3$-[$^{32}$P]cAMP into cytosol proteins of control, $Bt_2cAMP$-, 1% FCS-, and 2% DMSO-treated N-18 neuroblastoma cells was studied. Results are shown both in the forms of autoradiographs and semilog dose-response curves in Fig. 2. The apparent dissociation constants ($K_d$s) for the incorporation of 8-$N_3$-[$^{32}$P]cAMP into $R_I$ and $R_{II}$ of N-18 neuroblastoma cells were similar to values obtained for these 2 proteins from a variety of tissues and cell types studied previously (16, 32). In general, the $K_d$ values for the incorporation of 8-$N_3$-[$^{32}$P]cAMP into $R_I$ and $R_{II}$ of N-18 neuroblastoma cells were 10 to 15 and 80 to 100 nM, respectively. The increased incorporation of 8-$N_3$-[$^{32}$P]cAMP into $R_I$ of $Bt_2cAMP$- and 1% FCS-treated cells was evident at a saturating concentration of the ligand, indicating the presence of increased amounts of $R_I$ in these cell extracts.

**The Identification of $R_I$ as a Regulatory Subunit of the Type I Species.** $R_I$, the M.W. 47,000 8-$N_3$-[$^{32}$P]cAMP-binding protein of N-18 mouse neuroblastoma cells, has been tentatively identified as the regulatory subunit of the type I species (17). This assumption is based primarily on similarity of the apparent M.W. of these 2 proteins on SDS-PAGE (10, 17, 32). In this study, the behavior of the $R_I$ cAMP-binding protein of N-18 neuroblastoma cells in SDS-PAGE, isoelectric focusing, and immunoprecipitation reaction were compared to that of $R_I$*. Results in Figs. 3 and 4 demonstrate identical subunit molecular weights and isoelectric points between the $R_I$ cAMP-binding protein of N-18 cells and the purified regulatory subunit of the type I cAMP-dependent protein kinase. Furthermore, the increased 8-$N_3$-[$^{32}$P]cAMP-binding activity, observed in extracts of differentiated neuroblastoma cells, was precipitated by the rabbit anti-$R_I$* antibody (Table 2), while the preimmune serum was without effect (data not shown). Thus, based on results obtained on subunit M.W., isoelectric point, and immunoreactivity, it is concluded that the $R_I$ cAMP-binding protein of N-18 neuroblastoma cells is identical to $R_I$*.

**Catalytic Activity of cAMP-dependnet Protein Kinase.** Both the type I and the type II cAMP-dependent protein kinase are composed of 2 types of subunits, regulatory and catalytic (10, 11). There exists a variety of evidence suggesting a concerted expression of these 2 subunits. (*a*) Regulatory and catalytic subunits exist in equimolar concentrations in various mammalian tissues studied (11). (*b*) Both the cAMP-binding and the catalytic activities are greatly diminished in a mutant of the S-49 mouse lymphoma cells (12). (*c*) Both the cyclic nucleotide-binding domain and the catalytic domain are contained in a single polypeptide chain of the guanosine cyclic 3':5'-monophosphate-dependent protein kinase, and the cAMP- and guanosine cyclic 3':5'-monophosphate-dependent protein kinases are related (7, 15). The induction of $R_I$ by cAMP in the N-18 neuroblastoma cells and the identification of $R_I$ as a regulatory subunit of the type I species raise an interesting question concerning regulation of the levels of regulatory and catalytic subunits of cAMP-dependent protein kinase in these cells.

Catalytic activity of cAMP-dependent protein kinase was assayed using histone f2b as the substrate.The cytosolic histone kinase activity of the control, 1 mM $Bt_2cAMP$-, 1% FCS-, and 2% DMSO-treated N-18 cells is presented in Table 3. The cAMP-binding activity present in these cytosol preparations expressed in the amount of [$^3$H]cAMP bound is included. In the differentiated cells, induced either by 1 mM $Bt_2cAMP$ or 1% FCS treatment, the increase in cAMP-binding activity was not matched by an increase in the catalytic activity of cAMP-dependent protein kinase. In fact, in 6 of 8 experiments carried out over a 6-month period, there was a 20 to 50% decrease in the cytosolic histone kinase activity in both the $Bt_2cAMP$- and

Table 2

*Specific immunoprecipitation of 8-$N_3$-[$^{32}$P]cAMP-labeled protein of N-18 mouse neuroblastoma cells by antisera directed against $R_I$**

The incorporation of 8-$N_3$-[$^{32}$P]cAMP was carried out under standard conditions. Differences in the amounts of radioactivity precipitated by the immune and preimmune serum were taken as measurements of specific immunoprecipitation. Results are expressed in arbitrary units.

| | Amount of 8-$N_3$-[$^{32}$P]cAMP-labeled protein in immunoprecipitate | | |
|---|---|---|---|
| Treatment | Control | 1 mM $Bt_2cAMP$ | 1% FCS |
| Membrane | 1.00[a] | 2.33 | 2.22 |
| Cytosol | 1.00[b] | 2.45 | 2.27 |

[a] One unit = 0.9 pmol of 8-$N_3$-[$^{32}$P]cAMP-labeled protein in immunoprecipitate per mg of membrane protein used.

[b] One unit = 1.1 pmol of 8-$N_3$-[$^{32}$P]cAMP-labeled protein in immunoprecipitate per mg of cytosol protein used.

Table 3

*Specific activities of the regulatory and the catalytic subunits of cAMP-dependent protein kinase in cytosols of control, $Bt_2cAMP$-, 1% FCS-, and 2% DMSO-treated N-18 mouse neuroblastoma cells*

Measurement of the amount of specifically bound [$^3$H]cAMP was done under standard conditions. Histone kinase activity was assayed under optimal conditions in the presence of 5 μM cAMP.

| | Activity | |
|---|---|---|
| Cytosol | Regulatory subunit (pmol [$^3$H]cAMP bound/mg protein) | Catalytic subunit (pmol $^{32}$P transferred/min/mg protein) |
| Control | 3.35 ± 0.85[a] | 981 ± 168 |
| 1 mM $Bt_2cAMP$ | 8.10 ± 3.28 | 650 ± 79 |
| 1% FCS | 7.83 ± 4.16 | 720 ± 154 |
| 2% DMSO | 4.01 ± 1.05 | 690 ± 101 |

[a] Mean ± S.E. of 4 independent measurements.

1% FCS-treated N-18 neuroblastoma cells when compared to that of the control. The results suggest a dissociated expression of the regulatory and the catalytic subunits of cAMP-dependent protein kinase in the differentiated neuroblastoma cells and the likelihood that $R_I$ may exist as a distinct entity in these cells. The possibility of the presence of excess kinase inhibitor(s) (1, 30) in extracts of the differentiated cells, thus defying detection of an increased catalytic activity, seemed unlikely. Mixing cytosols from control and $Bt_2$cAMP-treated cells gave additive histone kinase activity, suggesting the absence of either a free kinase activator in extracts of control cells or an inhibitor in extracts of $Bt_2$cAMP-treated cells. It appears likely that the observed decrease in cytosolic kinase activity of the $Bt_2$cAMP- and 1% FCS-treated cells may be related to the growth-retarding effects of $Bt_2$cAMP and low-serum medium. This is based on the observation that the treatment of cells with 2% DMSO, while ineffective in promoting either morphological or biochemical differentiation, resulted in decreased cell growth and cAMP-dependent protein kinase activity.

DEAE-cellulose column chromatography has been used widely for the separation of the type I and the type II cAMP-dependent protein kinases in cell extracts; it has also been used for the resolution of free regulatory subunits of cAMP-dependent protein kinase from their respective holoenzymes. In this study, DEAE-cellulose column chromatography was used to examine the presence of free cAMP-binding protein in extracts of N-18 neuroblastoma cells and to resolve it from either the type I or the type II holoenzyme. Chart 1 illustrates the elution profiles from DEAE-cellulose columns of histone kinase and 8-$N_3$-[$^{32}$P]cAMP-binding activities of cytosol preparations from control, 1% FCS-, and 2% DMSO-treated neuroblastoma cells. In the control sample, 2 peaks of cAMP-dependent protein kinase activity, referred to as the type I and type II kinases, were eluted at 0.05 and 0.2 M NaCl, respectively, with the type I being the predominant form of the 2 (ratio of I/II, 3/1). In agreement with previous observations (32), a peak of M.W. 47,000 8-$N_3$-[$^{32}$P]cAMP-binding protein coeluted with the type I enzyme, while the M.W. 52,000 and 54,000 8-$N_3$-[$^{32}$P]cAMP-binding proteins coeluted with the type II enzyme. Perhaps of greater significance was the presence of a small but distinct peak of the M.W. 47,000 8-$N_3$-[$^{32}$P]cAMP-binding protein, unassociated with kinase activity, which was eluted by 0.16 M NaCl in the column eluate of the control cell extract. In the case of the 1% FCS-treated neuroblastoma cells, the M.W. 47,000 8-$N_3$-[$^{32}$P]cAMP-binding protein, $R_I$, was resolved into 3 components, 2 of which appeared to be associated with the type I kinase peak. The increase in $R_I$, observed in cytosol of the 1% FCS-treated cells, can be ascribed primarily to an increase in the component eluted at 0.15 to 0.18 M NaCl, which appeared not to be associated with histone kinase activity. Results qualitatively similar to that of the 1% FCS-treated sample were obtained with the cytosol of $Bt_2$cAMP-treated cells. In agreement with results obtained with the crude cytosol preparations, no increase in the $R_I$ peak was observed in the DEAE-cellulose column eluate of the DMSO-treated sample when compared to that of the control.

**Correlation of RI Induction and the Expression of Specific Differentiated Functions in N-18 Mouse Neuroblastoma Cells.** In order to probe the possible function(s) of $R_I$ in the differentiation of N-18 mouse neuroblastoma cells, one possible approach is to examine the various experimental manipulations which result in increased levels of $R_I$, *i.e.*, to correlate the expression of $R_I$ with other differentiated functions characteristic of mature neurons. A limited version of such an attempt is presented in Table 4. Four categories of changes were described: (*a*) cell growth; (*b*) morphological changes, characterized by neurite formation; (*c*) biochemical changes, as illustrated by increased acetylcholinesterase activity; and (*d*) changes in cAMP level and those proteins involved in cAMP action and metabolism.

The addition of 1 mM $Bt_2$cAMP or 2% DMSO to N-18 mouse

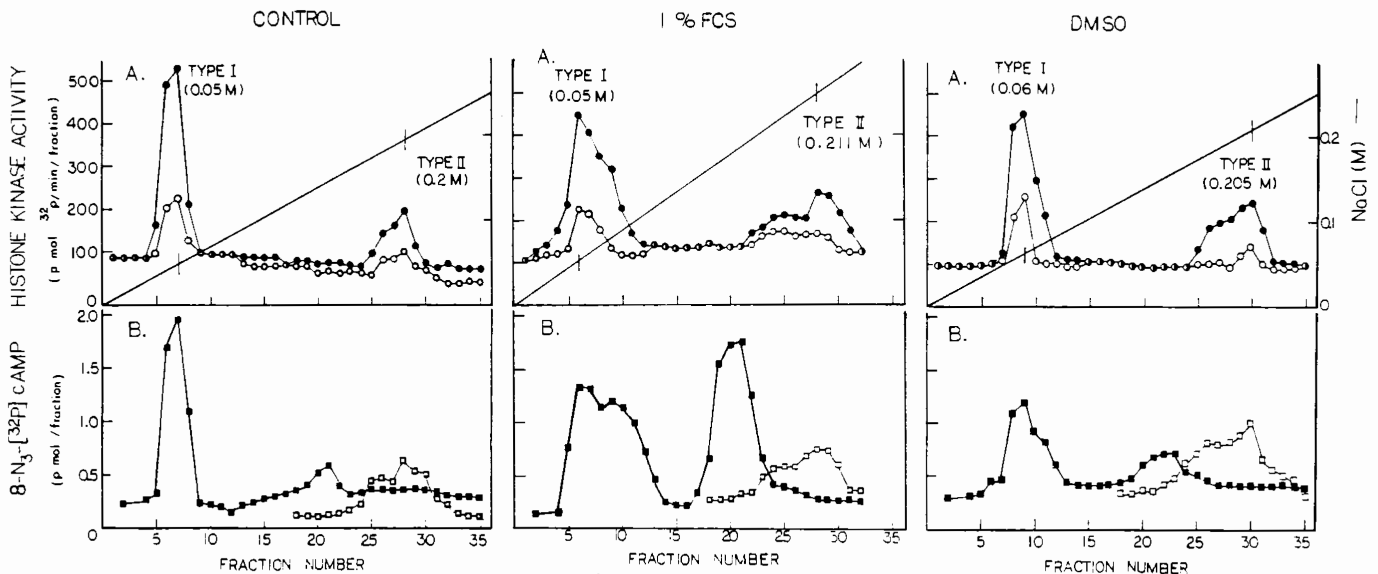


Chart 1. Elution profiles of cAMP-dependent protein kinase and 8-$N_3$-[$^{32}$P]cAMP-binding activity from DEAE-cellulose column using cytosols of control, 1% FCS-, and 2% DMSO-treated N-18 mouse neuroblastoma cells. Cytosol preparations, containing 3 mg protein, were loaded onto 3 similar DEAE-cellulose columns (0.9 x 4 cm) preequilibrated in 10 mM Tris-HCl (pH 7.4), 50 µg PMSF per ml, and 1 mM EDTA. Columns were washed with 6 ml of the same buffer, and proteins were then eluted by a linear gradient of 0 to 0.3 M NaCl (15 ml each) in the same buffer. Aliquots of individual fractions were assayed for histone kinase activity in the absence (○) or presence (●) of 5 µM cAMP (*A*) and the incorporation of 8-$N_3$-[$^{32}$P]cAMP into $R_I$ (■) and $R_{II}$ (□) (*B*). Results on histone kinase and 8-$N_3$-[$^{32}$P]cAMP-binding activities are presented in pmol of $^{32}$P transferred per min per fraction and pmol of 8-$N_3$-[$^{32}$P]cAMP incorporated per fraction, respectively.

Table 4

*Correlation of cell growth, differentiation, and changes in intracellular cAMP concentration and proteins involved in cAMP function and metabolism in N-18 mouse neuroblastoma cells*

All assays were carried out under standard conditions according to the described methods. The results are representative of 3 separate experiments.

| | Cell Growth | | Differentiation | | Intracellular cAMP | Proteins induced in cAMP function and metabolism | | | |
|---|---|---|---|---|---|---|---|---|---|
| Treatment | Doubling time (hr) | Saturation density (× $10^{-6}$/21 sq cm) | % of cells with neurites | Acetylcholinesterase (nmol/min/mg protein) | (pmol/mg protein) | 8-$N_3$-[$^{32}P$]cAMP incorporated (pmol/mg protein) $R_I$ | 8-$N_3$-[$^{32}P$]cAMP incorporated (pmol/mg protein) $R_{II}$ | Histone kinase (pmol $^{32}P$ transferred/min/mg protein) | Phosphodiesterase (pmol/min/mg protein) |
| Control | 22 | 5.8 | 5 | 7.1 | 5.32 | 2.65 | 1.06 | 981 | 24.0 |
| 1 mM $Bt_2cAMP$ | 31 | 2.8 | 90 | 62.5 | >180 | 7.72 | 1.52 | 650 | 74.6 |
| 1% FCS | 30 | 2.4 | 85 | 33.6 | 95.01 | 6.42 | 1.44 | 720 | 48.0 |
| 2% DMSO | 28 | 3.4 | 10 | 14.7 | 4.07 | 2.10 | 1.90 | 690 | 17.6 |

neuroblastoma cells or the culturing of cells in a medium containing 1% FCS all resulted in a slowing of cell growth, as determined by growth rate during the logarithmic phase and saturation density at the stationary phase of cell growth. DMSO, while inhibiting cell growth, was ineffective in inducing neurite outgrowth or acetylcholinesterase activity, suggesting that morphological differentiation is not a direct consequence of the inhibition of cell growth. On the other hand, the inhibition of cell growth by $Bt_2cAMP$ and 1% FCS treatment was associated with the expression of differentiated functions, namely, neurite extension and increased acetylcholinesterase activity. Concomitant increases in intracellular cAMP concentration, $R_I$ cAMP-binding protein, and cAMP phosphodiesterase activity were observed. Absence of these changes in the DMSO-treated N-18 neuroblastoma cells further supports the relatedness of these processes and cell differentiation.

## DISCUSSION

As with many other types of tissues, the neuroblastoma round cells were found to possess both the type I and type II cAMP-dependent protein kinases. Yet, unlike many of the tissues studied where greater than 90% of the cAMP-binding activity was found to associate with cAMP-dependent protein kinase activity, about 10 to 30% of $R_I$ in the N-18 neuroblastoma round cells was found to elute as a distinct entity from the DEAE-cellulose column, unassociated with protein kinase activity; it is this discrete peak of $R_I$ that increases during differentiation of the N-18 cells. Several lines of evidence indicate that the $R_I$ in neuroblastoma cells is similar to that of $R_I$*. These include (*a*) identical electrophoretic mobilities on SDS-PAGE and isoelectric focusing gels and (*b*) immunocross-reactivity of these 2 proteins, as determined by an immunoprecipitation assay.

The induction of $R_I$ appears to be correlated with the morphological appearance of neurites and the biochemical expression of increased acetylcholinesterase activity. Results demonstrated the concerted expression of all 3 functions in differentiated neuroblastoma cells, induced either by the addition of $Bt_2cAMP$ to the growth medium or by culturing cells in medium containing 1% FCS. These results suggest that $R_I$ may be used as a quantitative index of differentiation of the N-18 mouse neuroblastoma cells.

It appears unlikely that neurite extension and induction of $R_I$ in the N-18 cells are causally related to the slowing of growth in the differentiating cells. The addition of 2% DMSO to cultures of the N-18 mouse neuroblastoma cells inhibited cell growth but was without effect in either promoting neurite outgrowth or increasing acetylcholinesterase and $R_I$ cAMP-binding activities. Similar results were obtained when 1% DMSO was used. A previous study has also described the ineffectiveness of DMSO in inducing the morphological and biochemical maturation of a clonal line of mouse neuroblastoma cells (NB41A3) while $Bt_2cAMP$ was effective (5). In contrast, studies by Kimhi *et al.* (14) demonstrated the effects of DMSO (either 1 or 2%) in producing morphologically and electrically differentiated cultures of C-1300 mouse neuroblastoma cells; there was no clear correlation of these properties with the level of induction of either acetylcholinesterase or tyrosine hydroxylase. In a more recent study, it was noted that the addition of 1 to 1.5% DMSO to the NS20Y mouse neuroblastoma cells did not cause an increase in choline acetyltransferase activity (a differentiated phenotype in these cells), although "some morphological effects consistent with differentiation were observed" (9). These results suggest that DMSO is ineffective in promoting biochemical differentiation of mouse neuroblastoma cells and that it may have different effects in promoting morphological differentiation of different clones of mouse neuroblastoma cells.

The functional significance of the presence of a free cAMP-binding protein in the neuroblastoma cells and its marked increase during cell differentiation is not clear. Of the many possible and plausible models, 2 will be discussed. The first is that cAMP-binding protein(s) may not function solely to regulate and inhibit the catalytic activity of cAMP-dependent protein kinase in eukaryotic cells, *i.e.*, that cAMP-binding protein(s) may have intrinsic enzymatic and/or structural functions, whose activity is modulated by the binding of cAMP. A well-studied example of this sort is illustrated by the presence of a cAMP receptor protein in prokaryotes, whose function is to regulate the expression of various genes (20). In this regard, the recent report of increased cAMP binding to chromatin of differentiated neuroblastoma cells is particularly enlightening (24). Alternatively, it is possible that the cAMP-binding protein may serve as a scavenger of free cAMP in the mouse neuroblastoma cells, to prevent the prolonged activation of intracellular cAMP-dependent protein kinase. The induction of this free cAMP-binding protein under conditions which increase intracellular cAMP concentration and the concomitant increase in cAMP phosphodiesterase activity would be compatible with such a hypothesis. The hypothesis should be given some consideration in view of the dynamic nature of proteins involved in cAMP metabolism. A number of studies on the S-49 mouse lymphoma cells have demonstrated that phosphodiesterase activity could be adaptive, being specifically increased when

the intracellular cAMP level is elevated (3). These results provide important insights into the significance of phosphodiesterase activity in the control of cAMP levels. Far from being the simple kinetic brake as originally conceived, a number of discoveries in recent years have pointed to the complexity of the system. In the case of the N-18 mouse neuroblastoma cells, it is conceivable that cAMP is capable of regulating its own level not only through the regulation of phosphodiesterase activity but also through regulation of the level of a free cAMP-binding protein which serves as a sink for a pool of excess cAMP.

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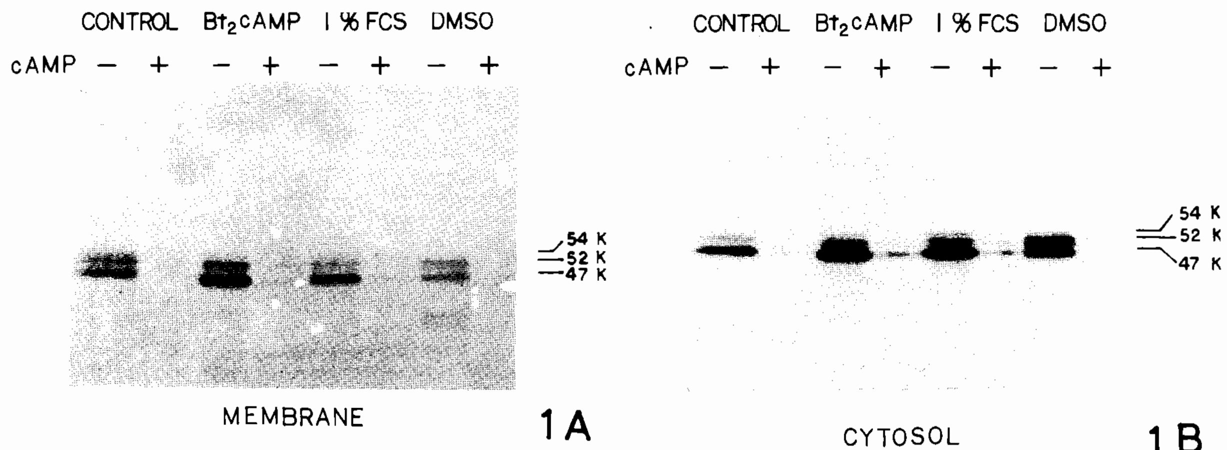



Fig. 1. Autoradiographs illustrating the incorporation of 8-$N_3$-[$^{32}$P]cAMP into membrane (*A*) and cytosol (*B*) proteins of control, $Bt_2$cAMP-, 1% FCS-, and 2% DMSO-treated N-18 mouse neuroblastoma cells. The incorporation of 8-$N_3$-[$^{32}$P]cAMP was done under standard conditions using 1 $\mu$M 8-$N_3$-[$^{32}$P]cAMP, in the presence of 0.25 mM 2-mercaptoethanol and 0.5 mM IBMX, with or without 20 $\mu$M cAMP. Each *channel* contained 200 $\mu$g protein. The radioactive bands with apparent M.W. of 47,000, 52,000, and 54,000 are indicated. The terms $R_I$ and $R_{II}$ are used to identify the M.W. 47,000 and the M.W. 52,000 and 54,000 proteins, respectively.

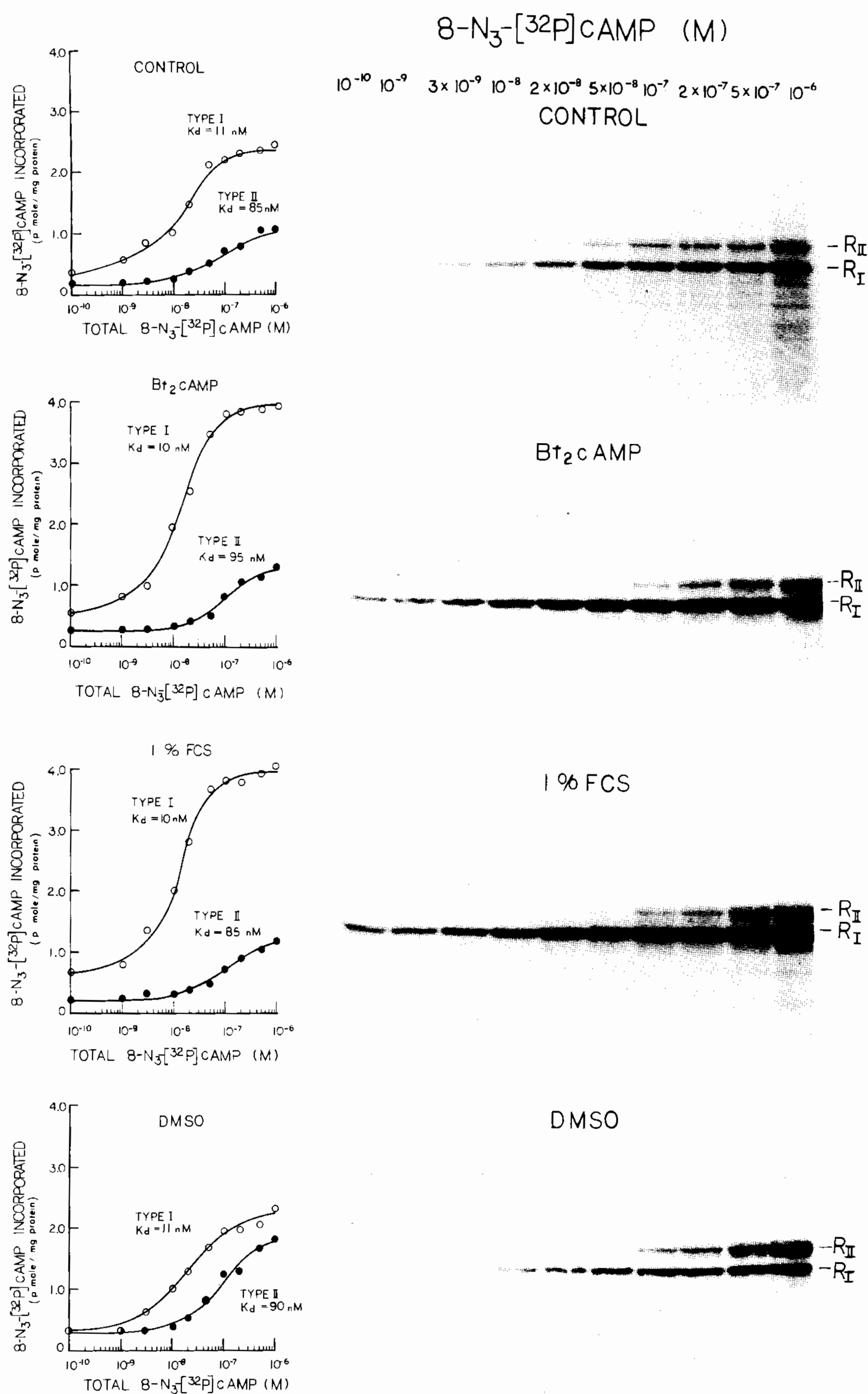
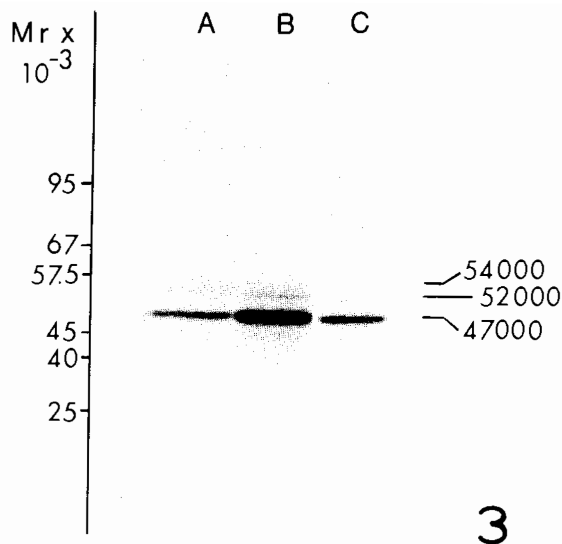



Fig. 2. Autoradiographs and semilog dose-response plots showing the concentration-dependent photoactivated incorporation of 8-$N_3$-[$^{32}$P]cAMP into cytosol proteins of control, $Bt_2$cAMP-, 1% FCS-, and 2% DMSO-treated N-18 mouse neuroblastoma cells. The incorporation of 8-$N_3$-[$^{32}$P]cAMP was done under standard conditions in the presence of 0.25 mM 2-mercaptoethanol and 0.5 mM IBMX. (The presence of 0.25 mM 2-mercaptoethanol in the binding assay mixture inhibited the incorporation of 8-$N_3$-[$^{32}$P]cAMP into $R_I$ and $R_{II}$ by approximately 45 and 25%, respectively.) Each *channel* contained 200 $\mu$g protein. $K_d$ is defined as the concentration of 8-$N_3$-[$^{32}$P]cAMP required to give half-maximal incorporation of the ligand into a specific protein.



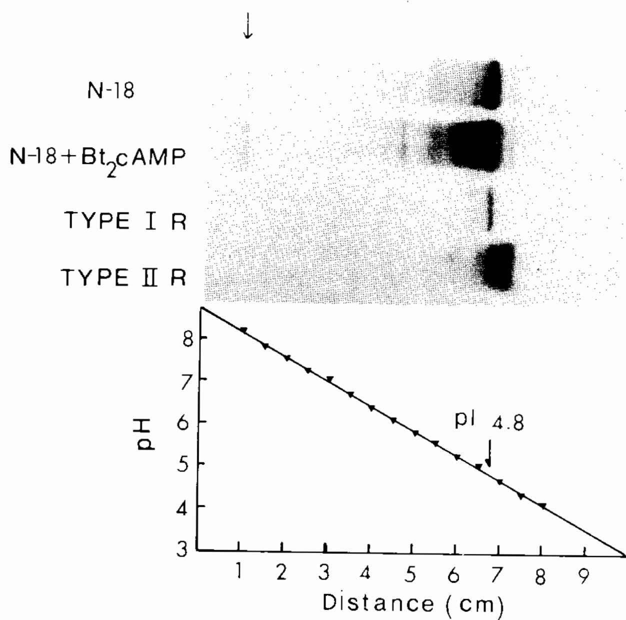




Fig. 3. Comparison of electrophoretic mobility of $R_I$ from control (*A*), $Bt_2$cAMP-treated N-18 mouse neuroblastoma cells (*B*), and $R_I$* (*C*) on a SDS 5 to 15% polyacrylamide slab gel. The incorporation of 8-$N_3$-[$^{32}$P]cAMP was done under standard conditions using 1 $\mu$M 8-$N_3$-[$^{32}$P]cAMP in the presence of 0.25 mM 2-mercaptoethanol and 0.5 mM IBMX. The specific activity of 8-$N_3$-[$^{32}$P]cAMP used for the incorporation into cytosol proteins was 5 times higher than that used for the incorporation into the purified regulatory subunit, $R_I$*. The amounts of protein used for *Lanes A*, *B*, and *C* were 200, 200, and <1 $\mu$g, respectively.

Fig. 4. Isoelectric focusing of the 8-$N_3$-[$^{32}$P]cAMP-labeled proteins of control and $Bt_2$cAMP-treated N-18 mouse neuroblastoma cells and $R_I$* and $R_{II}$*. The incorporation of 8-$N_3$-[$^{32}$P]cAMP was carried out under standard conditions with 50 $\mu$g cytosol protein and 1 $\mu$M 8-$N_3$-[$^{32}$P]cAMP, in the presence of 0.25 mM 2-mercaptoethanol and 0.5 mM IBMX. The 8-$N_3$-[$^{32}$P]cAMP-labeled $R_I$* and regulatory subunit of the type II cAMP-dependent protein kinase from bovine cardiac muscle were used as markers for the identification of $R_I$ of N-18 neuroblastoma cells. Isoelectric focusing was carried out on a pH 3.5 to 9.5 LKB polyacrylamide gel plate at 20 watts for 2 hr. The pH gradient of the gel at the end of the focusing procedure was determined by a surface pH electrode. The *arrow* identifies the position on the gel of the sample application strips.