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Activation of Cyclic AMP-Dependent Protein Kinase and Stimulation of Protein Phosphorylation in Response to Adenosine in C-1300 Murine Neuroblastoma

Richard D. Green and Thomas A. Noland, Jr.

Department of Pharmacology, University of Illinois at the Medical Center, Chicago, Illinois 60680

DEAE-cellulose chromatography of the 20,000g supernatant fraction of homogenates of C-1300 murine neuroblastoma (clone N2a) yields one major and two minor peaks of cyclic AMP-dependent protein kinase activity. Assessment of the endogenous activation state of the enzyme(s) reveals that the enzyme is fully activated by the treatment of whole cells with adenosine (10 μM) in the presence of the phosphodiesterase inhibitor Ro 20 1724 (0.7 mM). This treatment produces a large elevation in the cyclic AMP content of the cells. The treatment of whole cells with adenosine alone (1–100 μM) or Ro 20 1724 alone (0.1–0.7 mM) produces minimal elevations in cyclic AMP but nevertheless causes significant activations of cyclic AMP-dependent protein kinase. The autophosphorylation of whole homogenates of treated and untreated cells was studied using [γ-^{32}P] ATP, sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Treatments which activate cyclic AMP-dependent protein kinase selectively stimulate the incorporation of ^{32}P into several proteins. This stimulation is most prominent in the 15,000-dalton protein band. The addition of cyclic AMP to phosphorylation reactions containing homogenate of untreated cells stimulates the phosphorylation of the same protein bands. These results indicate that adenosine may have regulatory functions through its effect on the cyclic AMP:cyclic AMP-dependent protein kinase system.

Key words: protein phosphorylation, cAMP-dependent protein kinases, adenosine on cyclic AMP, C1300 neuroblastoma

Various tissues and cells respond to exogenous adenosine with an elevation in intracellular cyclic AMP [1–5]. While this effect of adenosine is minimal in several clones of C1300 murine neuroblastoma, adenosine produces large elevations in the cyclic AMP

Abbreviations: DEAE) diethylaminoethyl, AMP) adenosine monophosphate, ATP) adenosine triphosphate, EDTA) ethylenediaminetetracetic acid

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content of the same cells when a phosphodiesterase inhibitor such as Ro 20 1724 (4-[3-butoxy-4-methoxybenzyl]-2-imidazolidinone) is also present [6–8]. The failure of blockers of adenosine uptake to inhibit this response [5, 8], along with the demonstration that adenosine stimulates the adenylate cyclase activities of some broken cell preparations [9–11], gives support to the hypothesis that adenosine acts at an extracellular receptor site to stimulate adenylate cyclase and thus elevate cyclic AMP.

Previous work from this laboratory has given indirect evidence that adenosine is continually released by neuroblastoma cells into the growth medium and that this adenosine acts extracellularly to modulate the sensitivity of the cells to the cyclic AMP-elevating effect of adenosine [12]. Reports of the abilities of various substances to cause significant elevations in the endogenous activation state of cyclic AMP-dependent protein kinase in the absence of sizable elevations in cyclic AMP [13, 14] leave open the possibility that adenosine may play a regulatory role in the neuroblastoma cells via modulation of protein phosphorylation reactions catalyzed by cyclic AMP-dependent protein kinase. Experiments reported in this communication show that adenosine can cause significant activations of cyclic AMP-dependent protein kinase and stimulate the phosphorylation of select proteins even though it produces minimal changes in the intracellular content of cyclic AMP. It is anticipated that further experimentation will better define a regulatory function of adenosine in these cells as well as certain other cells and tissues.

METHODS AND MATERIALS

C1300 murine neuroblastoma cells (cloneN2a) were grown and maintained as previously described [8]. Cyclic AMP determinations, protein kinase activity ratios, and autophosphorylation experiments were all performed in cultures rinsed and incubated (15–30 min) in serum-free Dulbecco's Modified Eagle's Medium (DMEM). Cyclic AMP was assayed by the method of Gilman [15] as previously detailed [8].

Protein kinase activity ratios (histone phosphorylation in the absence of added cyclic AMP divided by histone phosphorylation in the presence of a maximally effective concentration of cyclic AMP) were determined using methods developed by Corbin and co-workers [13, 16]. Briefly, cultures (control or treated) were rinsed with cold saline, homogenized in buffer A (10 mM Tris-acetate buffer (pH 6.5); 10 mM EDTA; 0.1 mM isobutylmethylxanthine; ± 150 mM KCl), and centrifuged at 20,000g for 20 min (0°C). The phosphotransferase activity in the supernatant was then immediately assayed by incubating 50 μl of the supernatant with 150 μl of a protein kinase cocktail (67 μM Tris-acetate buffer (pH 6.5); 5.3 mM $MgCl_2$; 0.13 mM [γ-^{32}P] ATP [20–40 cpm/pmole]; 2.4 mg/ml histone [Sigma type IIA]; ± 1.3 μM cyclic AMP) for 10 min at 30°C. The reaction was terminated by the addition of 10% trichloroacetic acid (TCA), the protein was collected on glass fiber filter disks [17], and the radioactivity was counted in a liquid scintillation counter. Samples that contained the supernatant fraction but no histone were used to correct for the phosphorylation of endogenous proteins [18]. A 2.5-fold increase in the concentration of $MgCl_2$ did not affect histone phosphorylation (± cyclic AMP), indicating that the relatively high concentration of EDTA in the incubation mixture did not chelate sufficient Mg^{++} to inhibit the phosphorylation reaction.

The autophosphorylation of whole homogenates was studied by methods similar to those used to determine protein kinase activity ratios. In these experiments the final concentration of [γ-^{32}P] ATP (200–400 cpm/pmole) was 0.2 mM, and the reactions were terminated by the addition of a sodium dodecyl sulfate stopping solution [19] and

a 2-min incubation at 100°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [19] using 13% resolving gels. Gels were stained (Coomassie Brilliant Blue R), destained [20], and dried, and autoradiograms were prepared and scanned with a Zeineh Soft-Laser densitometer [21]. Molecular weights were determined using standard proteins of known molecular weights. Proteins were determined by the method of Lowry et al [22].

Tissue culture supplies were obtained from Gibco. [γ-^{32}P] ATP was purchased from ICN. Electrophoresis supplies were from Bio Rad. Autoradiograms were prepared from Kodak NoScreen X-ray film. Ro 20 1724 was kindly supplied by Hoffmann-LaRoche, Inc. All other reagents were obtained from Sigma Chemical Co. or other commercial sources.

RESULTS

DEAE-Cellulose Column Profile of Soluble Protein Kinase Activity

Twenty-seven medium-dense (near confluence) cultures (100-mm tissue culture plates) were rinsed with cold saline, scraped, homogenized in 5 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA (buffer B), and centrifuged at 20,000g for 30 min. The supernatant was applied to a 1- X 5-cm DEAE-cellulose column, washed with 175 ml of buffer B, and eluted with an 80-ml linear gradient of 0–0.4 M NaCl in the same buffer. Aliquots (50 μl) of each fraction were assayed for their ability to catalyze the phosphorylation of histone at pH 6.5 and casein at pH 8.5 (Fig. 1A). The column resolved three peaks of protein kinase activity that catalyzed the phosphorylation of histone in a cyclic AMP-dependent manner. There was one additional peak of protein kinase activity that phosphorylated casein in a cyclic AMP-independent manner. Cyclic AMP binding activity in 50 μl of each fraction was determined (Fig. 1B) using the Millipore filtration technique [15]. The major peak of cyclic AMP binding activity corresponded to the major peak of cyclic AMP-dependent protein kinase activity. Phosphotransferase activities of all three peaks of cyclic AMP-dependent activity were optimal at pH 6.5–8.0 and $MgCl_2$ concentrations > 0.4 mM.

Assessment of Endogenous Activation State of Soluble Cyclic AMP-Dependent Protein Kinase Activity

Various homogenization buffers were tested for their utility in determining the endogenous activation state of soluble cyclic AMP-dependent protein kinase. The buffer chosen for use (buffer A) was similar to that employed by Keely et al [13] to study the endogenous activation state of cyclic AMP-dependent protein kinase in preparations containing primarily type I kinase activity (nomenclature of Corbin et al [16]). Work of Keely et al [23] has shown that free catalytic subunits of soluble cyclic AMP-dependent protein kinase in rat heart nonspecifically bound to particulate material when the heart was homogenized in hypotonic buffer and that this binding was not present when the salt concentration of the buffer was raised to isotonicity.

Table I summarizes an experiment in which control and treated (Ro 20 1724, 0.7 mM + adenosine, 0.1 mM) cultures were homogenized in buffer A with or without 150 mM KCl. In this experiment kinase activity was measured in the supernatant and in the 20,000g pellet which was rinsed and resupended in the homogenization buffer before assay. Although the activity ratios in both the soluble and particulate fractions were little affected by the presence of KCl in the homogenization buffer (Table IA), it is very clear that a large fall in the total soluble protein kinase activity occurred when "treated" cultures were homogenized in the absence of KCl (Table IB). A loss in particulate protein

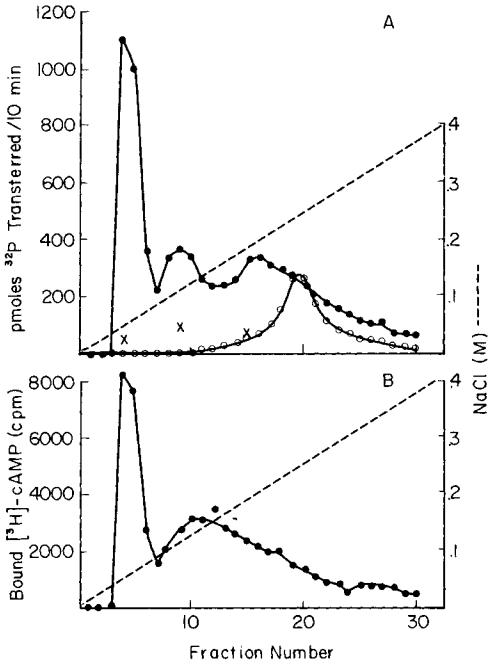


Fig. 1. A) DEAE-cellulose column profiles of protein kinase activity. Each assay (200 μl total volume) contained 50 μl column eluate; 4 mM $MgCl_2$; 0.1 mM [γ^{32}P] ATP; 400 μg phosphoryl acceptor; ± 1 μM cyclic AMP and 50 mM buffer [Tris-HCl (pH 6.5) for histone and glycine-NaOH (pH 8.5) for casein]. ●) Histone plus cyclic AMP; X) Histone minus cyclic AMP; ○) Casein minus cyclic AMP. B) Cyclic AMP binding. A 105-μl binding reaction contained 50 μl column eluate, 50 mM Tris-acetate buffer (pH 6.5), 60 μg albumin, and 5 pmoles [^{3}H]-cyclic AMP. After 90 min incubation (4°C) the bound [^{3}H]-cyclic AMP was collected using the Millipore filtration technique [15].

TABLE I. Effect of KCl (150 mM) in the Homogenization Buffer on the Distribution of cAMP-Dependent Protein Kinase Between Particulate and Soluble Fractions and the Activity Ratios (−cAMP/+cAMP) Therein.

Fraction	Control		Activated[a]	
	−KCl	+KCl	−KCl	+KCl
A. Activity ratio[b]				
Soluble	0.09 ± 0.02	0.15 ± 0.01	0.98 ± 0.03	1.03 ± 0.04
Particulate	0.42 ± 0.06	0.42 ± 0.08	0.92 ± 0.02	0.91 ± 0.03
B. Total activity[b,c]				
Soluble	5,033 ± 204	3,910 ± 394	1,467 ± 85	3,945 ± 281
Particulate	2,559 ± 133	1,008 ± 48	1,623 ± 131	1,074 ± 185

[a]Treatment for 15 min with Ro 20 1724 (0.7 mM) + adenosine (10^{-4}M).

[b]All values are means ± SEM for four determinations.

[c]Units: pmoles ^{32}P transferred to histone per milligram protein per 10 min in the presence of 1 μM cAMP.

kinase activity also occurred in treated cultures homogenized without KCl, indicating that the loss in soluble kinase activity was not simply due to nonspecific binding of the catalytic subunit to the particulate fraction. All further experiments were performed with homogenization buffer containing 150 mM KCl even though the total protein kinase activity appeared to be slightly depressed by its presence.

Table II summarizes several experiments in which the effects of various treatments on soluble protein kinase activity ratios were determined. The effects of similar treatments on intracellular cyclic AMP levels were determined in the two experiments summarized in Table III. It is evident that adenosine alone (1–100 μM) produced minimal elevations in cyclic AMP (Table III) but significant activations of cyclic AMP-dependent protein kinase (Table II). In a separate experiment samples of untreated cells were incubated with phosphodiesterase before assay to determine if there was a large blank value under conditions in which minimal amounts of cyclic AMP were assayed. These samples assayed 2.51 ± 0.17 (N = 5) pmole/mg protein, indicating that the relative increase in cyclic AMP in response to adenosine alone (Table III, Expt. 1) was not grossly underestimated due to a large blank value.

Autophosphorylation of Whole Homogenates

Figure 2 shows an experiment designed to determine if a treatment which causes a modest elevation in cyclic AMP and the subsequent activation of cyclic AMP-dependent protein kinase causes a differnce in the autophosphorylation of the endogenous protein substrates in whole homogenates. In this experiment control and treated (Ro 20 1724,

TABLE II. Effect of Various Treatments on Protein Kinase Activity Ratios (–cAMP/cAMP)

Treatment		Protein kinase activity ratio [mean + SEM (N)]	
Agent	Time (min)	Control	Treated
Adenosine, 10^{-7} M	15	0.30 ± 0.02 (4)	0.30 ± 0.02 (4)
Adenosine, 10^{-6} M	15	0.16 ± 0.02 (3)	0.34 ± 0.02 (4)[b]
Adenosine, 10^{-5} M	15	0.26 ± 0.05 (4)	0.50 ± 0.05 (4)[c]
Adenosine, 10^{-4} M	15	0.20 ± 0.04 (4)	0.46 ± 0.05 (4)[d]
Ro 20 1724, 0.7 mM	15	0.24 ± 0.05 (4)	0.74 ± 0.05 (4)[c]
Ro 20 1724, 0.1 mM[a] +adenosine, 10^{-5} M	5	0.45 ± 0.02 (4)	0.89 ± 0.06 (4)[d]
Ro 20 1724, 0.03 mM[a] +adenosine, 10^{-5} M	5	0.24 ± 0.04 (4)	0.95 ± 0.05 (4)[c]
Ro 20 1724, 0.03 mM[a] +adenosine, 10^{-7} M	5	0.45 ± 0.06 (4)	0.82 ± 0.04 (4)[d]

[a]Control was treated with stated concentration of Ro 20 1724 for 5 min.

[b]Significantly different from control, $p < 0.025$.

[c]Significantly different from control, $p < 0.001$.

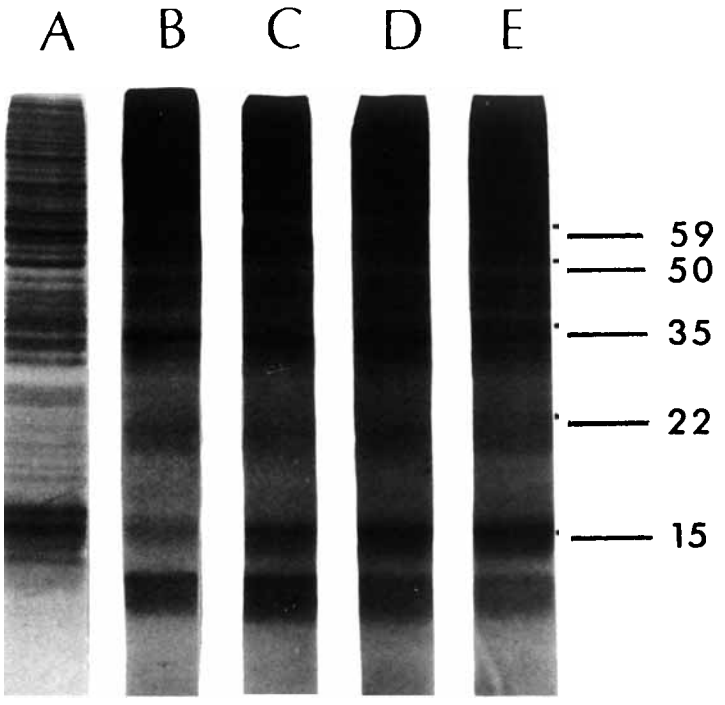
[d]Significantly different from control, $p < 0.005$.

TABLE III. Effect of Various Treatments on Cyclic AMP Content

Treatment		cAMP[a]
Agent	Time (min)	(pmoles/mg protein)
Experiment 1[b]		
Control		17.2 ± 1.4 (4)
Adenosine, 10^{-6} M	15	26.9 ± 4.7 (4)
Adenosine, 10^{-5} M	15	23.8 ± 1.4 (4)
Adenosine, 10^{-4} M	15	30.5 ± 1.6 (3)
Adenosine, 10^{-4} M + Ro 20 1724, 0.7 mM	15	2,245, 1,960
Experiment 2		
Control		30.1 ± 4.6 (4)
Ro 20 1724, 0.03 mM	5	29.5 ± 6.6 (4)
Ro 20 1724, 0.03 mM + adenosine, 10^{-7} M	5	101.7 ± 3.4 (4)
Ro 20 1724, 0.03 mM + adenosine, 10^{-5} M	5	480.6 ± 22.8 (4)
Ro 20 1724, 0.7 mM + adenosine, 10^{-5} M	5	1,164, 1,227

[a]Mean ± SEM (N) or individual values.

[b]Analysis of variance excluding adenosine + Ro 20 1724 gives $F = 3.82$, $P < 0.05$.



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0.03 mM + adenosine, 2 μM, 30 or 60 sec) cells were homogenized (buffer A), and the autophosphorylation pattern was determined after a 5-min phosphorylation reaction. Phosphorylation reactions containing the homogenate of control cells were run with and without the addition of 2 μM cyclic AMP. The figure shows a representative lane of the stained gel along with the autoradiogram of the dried gel. An increased phosphorylation of a protein band of approximately 15,000 daltons was clearly seen in the homogenate of treated cells and in the homogenate of control cells to which cyclic AMP had been added. Corresponding increases in the phosphorylations of protein bands with molecular weights of 59,000, 50,000, 35,000 and 22,000 occurred but were not as obvious.

Figure 3 shows densitometer tracings of an autoradiogram demonstrating the effect of treatment of cells with 2 μM adenosine for 5 min on the autophosphorylation pattern

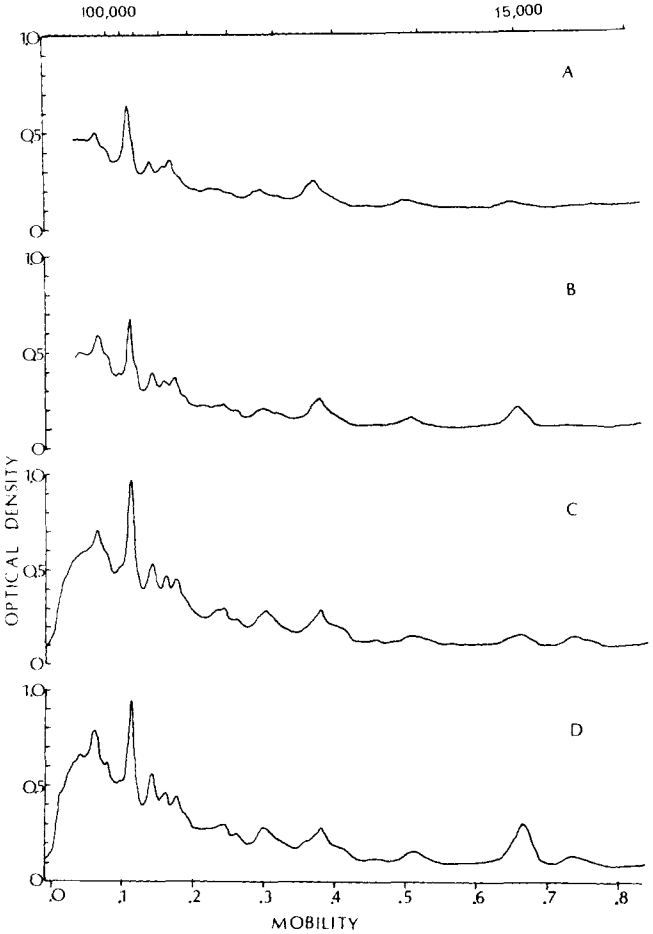

Fig. 3. Densitometer tracings of an autoradiogram showing the effect of adenosine treatment (2 μM, 5 min) on subsequent phosphorylation patterns. A, C) From homogenates uf untreated cells; B, D) from homogenates of treated cells. NaF (10mM) was present during the phosphorylation reactions in C and D. [γ-^{32}P] ATP 300 cpm/pmole; 80 μg protein/sample. Autoradiogram was exposed for five days. A logarithmic molecular weight scale is shown at the top.

Fig. 2. Representative stained gel (lane A) and autoradiogram (lanes B-E), showing different autophosphorylation patterns in homogenates of untreated (lane B, minus cyclic AMP; lane E, plus 2 μM cyclic AMP) and treated (lane C, 2 μM adenosine + 0.3 mM Ro 20 1724, 30 sec; lane D, same treatment for 60 sec) cultures. [^{32}P]-ATP, 300 cmp/pmole; 70μg protein/sample. Autoradiogram was exposed for five days. Molecular weight values X 10^{-3} are shown on the right.

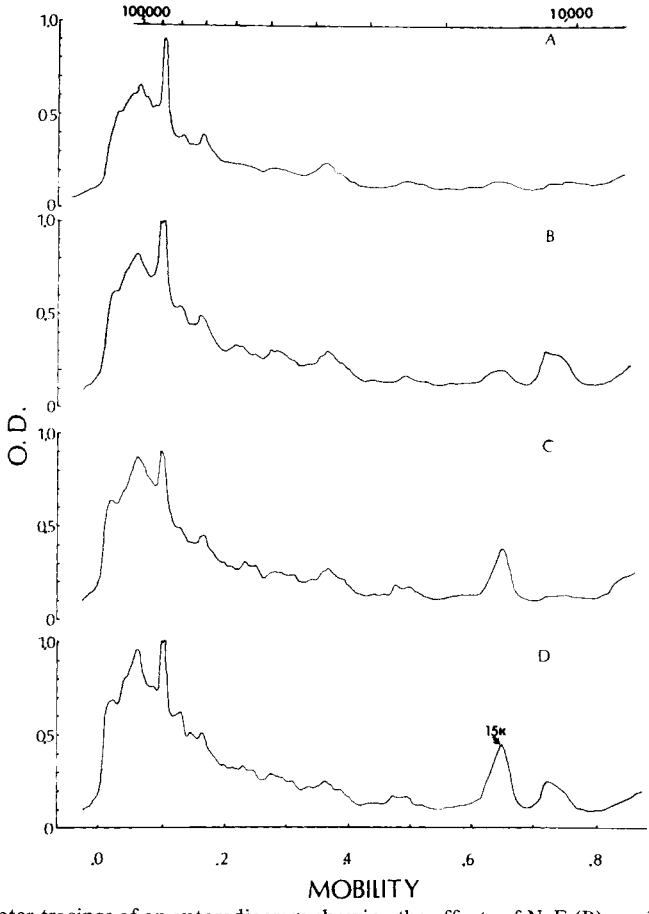

Fig. 4. Densitometer tracings of an autoradiogram showing the effects of NaF (B), cyclic AMP (C) and NaF + cyclic AMP (D) on the autophosphorylation pattern of a whole homogenate of untreated cells. A) A control tracing. [γ-^{32}P] ATP, 306 cpm/pmole; 80 μg protein/sample. Autoradiogram was exposed for five days. A logarithmic molecular weight scale is shown at the top.

in the corresponding homogenate. In this experiment the autophosphorylation reactions were carried out in the absence (A and B) and presence (C and D) of 10 mM NaF. The incorporation of ^{32}P into several protein bands was increased in the homogenate of adenosine-pretreated cells. Again the increased phosphorylation of a 15,000-dalton protein was most pronounced. The addition of NaF to the phosphorylation reactions increased the incorporation of the radioactivity into all protein bands and made the phosphorylations catalyzed by cyclic AMP-dependent protein kinase more apparent. Densitometer tracings of an autoradiogram of a similar experiment demonstrating the effects of cyclic AMP and NaF on the protein phosphorylation pattern of a whole homogenate of untreated cells are shown in Figure 4. Once more the results obtained by the addition of cyclic AMP to whole homogenates of untreated cells were identical to those obtained in homogenates of adenosine-treated cells.

DISCUSSION

Work from many laboratories has shown the existence of at least two forms of cyclic AMP-dependent protein kinase (for reviews see Hosey and Tao [24] and Nimmo and Cohen [25]). Cyclic AMP-dependent protein kinase that elutes from DEAE-cellulose at < 0.1 M NaCl (Type I kinase) differs from cyclic AMP-dependent protein kinase that elutes at concentrations of NaCl > 0.1 M (Type II kinase) in regard to conditions that stabilize the equilibrium between the holoenzyme and the dissociated subunits [15]. According to this classification, the major cyclic AMP-dependent protein kinase in N2a cells is a type I kinase. Although the same pattern of cyclic AMP-dependent kinases was found in all experiments, it is not known whether the two minor peaks are truly different or arise during the experimental procedure. A separate type of protein kinase that is independent of regulation by cyclic AMP and preferentially phosphorylates casein is also present in the soluble fraction. Cyclic AMP-independent kinases have been isolated from a variety of sources [24]. The major peak of cyclic AMP-dependent protein kinase activity was lost if the 20,000g supernatant of whole homogenate was subjected to $(NH_4)_2SO_4$ precipitation (50%) and dialysis (unpublished observation). Simantov and Sachs [26] have reported DEAE-cellulose profiles of soluble cyclic AMP-dependent protein kinase activity in another clone of C-1300 neuroblastoma. They did not detect the large peak of type I kinase that we observed in the present studies. It is not possible to know whether these workers lost this activity during their experimental procedure (which did not contain an $(NH_4)_2SO_4$ precipitation step) or if, in fact, the clone they studied did not contain a type I kinase

The baseline activity ratio of soluble cyclic AMP-dependent protein kinase was approximately 0.2 and was consistent with values reported for a variety of tissues [25]. Light cultures tended to have higher cyclic AMP levels and higher activity ratios than dense cultures (data not shown.) Previous work from this laboratory showed that cyclic AMP-dependent protein kinase activity ratios were linearly correlated with cyclic AMP levels in embryonic and newborn chick myocardium between ratios of 0.2 and 1.0 and baseline and maximal cyclic AMP levels [18]. Skala and Knight [27] reported a hyperbolic relationship between "histone kinase" activity ratios and cyclic AMP concentrations in rat brown fat. Such a relationship would be expected if the binding of cyclic AMP to the regulatory subunit of cyclic AMP-dependent protein kinase follows the bimolecular mass action equation under intracellular conditions. While neither relationship appears to exist in the N2a cells (Tables II and III), there is no reason to believe that the activation of cyclic AMP-dependent protein kinase by adenosine is not due to cyclic AMP generated in response to the stimulation of adenylate cyclase by adenosine. The occurrence of significant activations of cyclic AMP-dependent protein kinase, in unison with more moderate elevations in cyclic AMP, is not without precedent [13, 14].

Autophosphorylation experiments with whole homogenates showed that while adenosine was incapable of producing large elevations in cyclic AMP, it stimulated the transfer of ^{32}P from [γ-^{32}P] ATP to select cellular proteins. While the phosphorylation of several protein bands was increased, this stimulation was most marked in the protein band corresponding to a molecular weight of approximately 15,000 daltons. A similar pattern of increased protein phosphorylation was seen when cyclic AMP was added to homogenates of control cells. This finding, coupled to the demonstration that adenosine

does cause small elevations in cyclic AMP and rather large activations of cyclic AMP-dependent kinase, indicates that the stimulation in the autophosphorylation of certain bands in homogenates of adenosine-treated cells proceeds through the cyclic AMP-dependent protein kinase system. These effects were more apparent if 10 mM NaF was present in the kinase reaction mixture. This was no doubt due to the ability of NaF to inhibit ATPases and phosphoprotein phosphatases. The present experiments with whole homogenates give no clue as to the cellular origins of the proteins of interest. Furthermore as cyclic AMP-dependent protein kinase activity is present in particulate material (Table I) and in plasma membrane-enriched preparations of N2a cells (unpublished observation), the origin (soluble or particulate) of the cyclic AMP-dependent protein kinases involved in the adenosine-stimulated phosphorylations is unknown. Interestingly, cyclic AMP stimulates the phosphorylation of a 15,000-dalton protein in plasma membrane-enriched preparations (unpublished observation).

Although the ability of adenosine to elevate cyclic AMP in a number of preparations has now been demonstrated, the relationship between this effect of adenosine and any of the many "physiologic effects" of adenosine remains unknown. The finding that a relatively weak cyclic AMP-elevating effect of adenosine (in the absence of phosphodiesterase inhibiton) can stimulate the phosphorylation of endogenous substrates of cyclic AMP-dependent protein kinase leaves open the possibility that adenosine may perform a regulatory role in N2a cells as well as in other systems.

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