Proc. Nat. Acad. Sci. USA
Vol. 70, No. 1, pp. 76–79, January 1973

Induction of Cytolysis of Cultured Lymphoma Cells by Adenosine 3′:5′-Cyclic Monophosphate and the Isolation of Resistant Variants

(cytoplasmic receptor molecules/protein kinase)

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Communicated by Bruce N. Ames, October 26, 1972

ABSTRACT **Cultured mouse lymphosarcoma cells are killed on exposure to 0.1 mM N^6,$O^{2'}$-dibutyryl-adenosine 3′:5′-cyclic monophosphate. A population of cells resistant to the killing effect of dibutyryl cyclic AMP at concentrations as high as 1 mM was selected. The growth characteristics of the resistant cells were similar to those of the sensitive parental line. However, the resistant cells contain less cytoplasmic cyclic AMP-binding proteins and decreased cyclic AMP-stimulated protein kinase activity. It is proposed that transition from sensitivity to resistance to dibutyryl cyclic AMP in lymphoma cells is connected with a modification of the cyclic AMP-binding protein, which appears to be the regulatory subunit of the cyclic AMP-activated protein kinase.**

Exposure of cultured lymphoid cells to adrenal steroid hormones leads to inhibition of cell growth and, ultimately, to cytolysis (1). Various experiments suggest that specific protein synthesis induced by steroid hormones is required for this process (2, 3). Steroid-resistant variants have been isolated that contain decreased quantities of the specific cytoplasmic receptor molecules thought to mediate steroid action (4, 5).

Adenosine 3′:5′-cyclic monophosphate (cAMP), although not a direct intermediate in glucocorticoid action (6), frequently mimics the physiological effects of the steroids. In this publication, we demonstrate that the cyclic nucleotide can also promote the death of lymphoid cells and that cAMP-resistant cells can be obtained that contain lower concentrations than normal cells of cAMP-binding proteins. Furthermore, we show that the binding proteins that are diminished in the resistant variants behave like the regulatory subunits of the cyclic AMP-dependent protein kinase. Therefore, cAMP-induced cytolysis very probably involves the combination of the cyclic nucleotide with this protein subunit.

MATERIALS AND METHODS

The mouse lymphoma line S49.1 TB4, obtained from Ruth Epstein, Salk Institute, San Diego, was propagated in Dulbecco's medium (General Biochemicals) containing 10% heat-inactivated horse serum. Cell concentrations were assayed by a Coulter Counter model F with a cell-size threshold value that excluded the majority of cells with pycnotic nuclei (1). [^{3}H]cAMP, [^{3}H]N^6,$O^{2'}$-dibutyryl cAMP (But_2cAMP), and [γ-^{32}P]ATP were obtained from New England Nuclear Corp. Unlabeled But_2cAMP was purchased from Calbiochem, and unfractionated calf-thymus histones from Sigma Co.

Cyclic AMP binding to cellular proteins was measured by the competition assay of Gilman (7). The reaction mixture contained, in a final volume of 0.1 ml: 50 mM potassium phosphate buffer (pH 7.0) or the same concentration of sodium acetate (pH 6.5), 3 mM theophylline, and 0.5 μM [^{3}H]cAMP (24 Ci/mmol). The reactions were started by addition of the protein samples.

Protein kinase activity was determined by measurement of the incorporation of ^{32}P from γ-labeled ATP into histone. The reaction mixtures contained, in a final volume of 0.2 ml: [γ-^{32}P]ATP (8×10^4 cpm/nmol), 1 mM theophylline, 10 mM dithiothreitol, and enzyme protein. Incubations were at 30° for 5 min with or without 1 μM cAMP. Protein-bound ^{32}P was determined by the method of Kabat (8).

Protein concentrations were determined by the method of Lowry *et al.* (9).

RESULTS

Killing of cultured lymphoma cells by But_2cAMP and the emergence of a resistant population

Growth of the mouse lymphoma line S49.1 TB4 that is sensitive to glucocorticoids (1) was inhibited by But_2cAMP at concentrations as low as 10 μM, and at 0.1–1 mM But_2cAMP (with 0.2 mM theophylline) there was virtually complete cytolysis 48 hr after treatment (Fig. 1). Theophylline alone, at this concentration, had no effect on cell growth. Neither cAMP itself, adenine, adenosine, nor 5′-AMP at a concentration of 0.1 mM inhibited cellular growth.

We derived a But_2cAMP-resistant population of S49.1 TB4 cells by growing the sensitive cells in the presence of 0.2 mM theophylline and increasing But_2cAMP concentrations stepwise from 1 to 100 μM over a period of about 30 days. This partially resistant population was then exposed to 1 mM But_2cAMP for a week; the survivors of this treatment were used as the cAMP-resistant cells. The growth rates of both sensitive and resistant populations are similar in the absence of But_2cAMP (Fig. 1). No cytolysis occurred in the resistant cells at 1 mM But_2cAMP with 0.2 mM theophylline (Fig. 1), and the cells remained resistant to these concentrations when cultured in medium free of But_2cAMP and theophylline for up to 6 months.

Abbreviation; N^6,$O^{2'}$-dibutyryladenosine 3′:5′-cyclic monophosphate, But_2cAMP.

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Mechanism of But$_2$cAMP resistance

Because free cAMP itself does not inhibit the growth of even But$_2$cAMP-sensitive lymphoma cells, we investigated the possibility that resistance to But$_2$cAMP might be due to inability of the substituted nucleotide to enter the resistant cells. We therefore sought to increase the intracellular cAMP concentration indirectly by adding prostaglandin to the medium of both sensitive and resistant cells. In both cases there was a marked increase in the internal cAMP concentration (manuscript in preparation); however, only the growth of the But$_2$cAMP-sensitive cells was inhibited. These results suggest that the resistant cells are truly insensitive to intracellular cAMP and are not simply unable to take up exogenous But$_2$cAMP. Consistent with this conclusion was the finding that both sensitive and reistant cells showed an identical uptake of [^{3}H]But$_2$cAMP (0.4 pmol per 4 × 10^6 cells in a 3-hr incubation) at an external nucleotide concentration of 1.4 μM.

We next inquired whether cAMP resistance is due to impaired binding to a specific cytoplasmic receptor. We incubated [^{3}H]cAMP with cytosols from sensitive or resistant lymphoma cells and subjected the mixtures to gel filtration on Sephadex G-25. Fig. 2 shows that there is much greater macromolecular binding by cytoplasmic extracts of sensitive than of resistant cells, as shown by the amount of radioactivity excluded by the column. In seven different experiments, the binding to sensitive cell extracts was 4–8 times higher than to cytosols from resistant cells, but in each case some residual binding by resistant cell extracts was found.

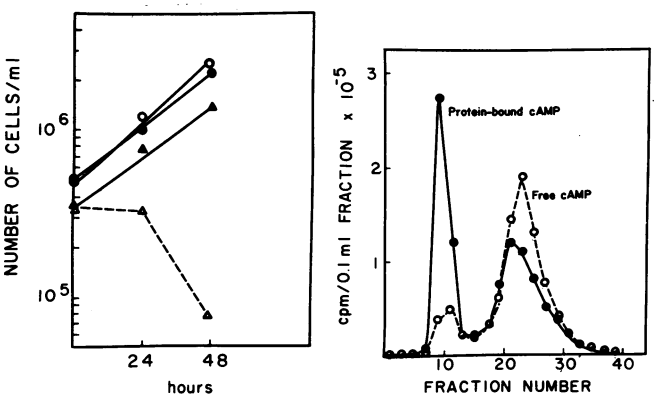

FIG. 1. (*left*). Effect of But$_2$cAMP on the growth of sensitive and resistant lymphoma cells. Cells sensitive to But$_2$cAMP were grown in the absence (▲——▲) or presence (△——△) of 1 mM But$_2$cAMP and 0.2 mM theophylline. Cells resistant to But$_2$cAMP were grown in the absence (●——●) or presence (○——○) of the same reagents.

FIG. 2. (*right*). Gel filtration of [^{3}H]cAMP-bound cytosols from But$_2$cAMP-sensitive and -resistant cells. Centrifuged packed cells (1–2 ml) were homogenized with an equal volume of 0.25 M sucrose in 0.05 M Tris·HCl buffer (pH 7.4) containing 3 mM $MgCl_2$ and 4 mM 2-mercaptoethanol. Particulate material was removed by a 90-min centrifugation in a Spinco preparative ultracentrifuge at 40,000 rpm (105,000 × *g*). The same amounts (25 mg of protein) of the resulting cytosols from both But$_2$cAMP-sensitive (●) and resistant (○) cells were incubated for 60 min at 0° with 0.5 μM [^{3}H]cAMP in the presence of 5 mM theophylline. The macromolecular-bound [^{3}H]cAMP was then separated from free [^{3}H]cAMP by filtration through a 1.2 × 20 cm Sephadex G-25 column equilibrated with 0.05 M Tris·HCl buffer (pH 7.5). 1-ml Fractions were collected, and samples of 0.1 ml were counted for radioactivity in a scintillation counter with a mixture of Triton X-100–water–Omnifluor–toluene (25:4:04:70).

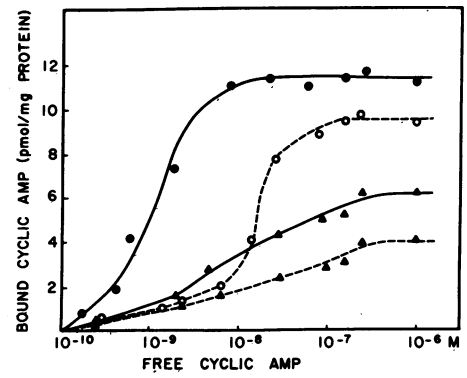

FIG. 3. Specific cAMP binding by cytoplasmic extracts from But$_2$cAMP-sensitive and resistant lymphoma cells at various cAMP concentrations. Reaction mixtures in a total volume of 0.1 ml contained 50 mM sodium acetate buffer (pH 4.5 or 6.0), 2 mM theophylline, 120 or 140 μg of cytosol protein from sensitive or resistant cells, and various concentrations of [^{3}H]cAMP (24 Ci/mmol) with or without 0.5 mM nonradioactive cAMP. The difference between the amount of [^{3}H]cAMP bound to cytoplasmic extracts in the absence and presence of excess nonradioactive cAMP is considered a measure of the "specifically-bound" cAMP. The concentration of free [^{3}H]cAMP is obtained by subtraction of the total bound [^{3}H]cAMP from total [^{3}H]cAMP present in the incubation. ●——●, sensitive cytosol (pH 4.5); ○——○, sensitive cytosol (pH 6.0); ▲——▲, resistant cytosol (pH 4.5); △——△, resistant cytosol (pH 6.0).

"Specific" binding curves were next compared at two pH values with cytosols from both types of cells. Specific binding was calculated by subtraction of a blank value (obtained at a given concentration of radioactive cAMP together with a 500- to 1000-fold excess of nonradioactive cAMP) from the value obtained with the radioactive nucleotide alone (10). Fig. 3 shows that at pH 4.5 the specific binding components in both cells become saturated at lower cAMP concentrations than at pH 6.0. At both pH values, however, there was substantially less specific cAMP binding by resistant cell cytosol at all the cAMP concentrations tested. These results, together with the experiments shown in Fig. 2, lead us to conclude that resistant cells contain fewer cAMP receptors than do sensitive cells.

Characteristics of "sensitive" and "resistant" cAMP-binding proteins

In addition to these quantitative differences, the shape of the curves in Fig. 2 suggest the possibility of qualitative differences between "sensitive" and "resistant" binding proteins. Although obviously a final decision cannot be based only on measurements made in a crude extract, this point was investigated further by examination of the influence of pH on the binding at two different concentrations of the cyclic nucleotide by "sensitive" and "resistant" cytosols. Fig. 4 shows that binding by resistant cytosol has a pH optimum of about 4.5 for both cAMP concentrations. At the lower concentrations of cAMP, sensitive cytosol shows optimal binding at the same pH. However, the pH-binding curve at the higher nucleotide concentration seems qualitatively different in the sensitive as compared to the resistant cells. The data in Fig. 4 indicate that the sensitive cytosol might contain two types of cAMP-binding sites, one with a higher affinity for the nucleo-

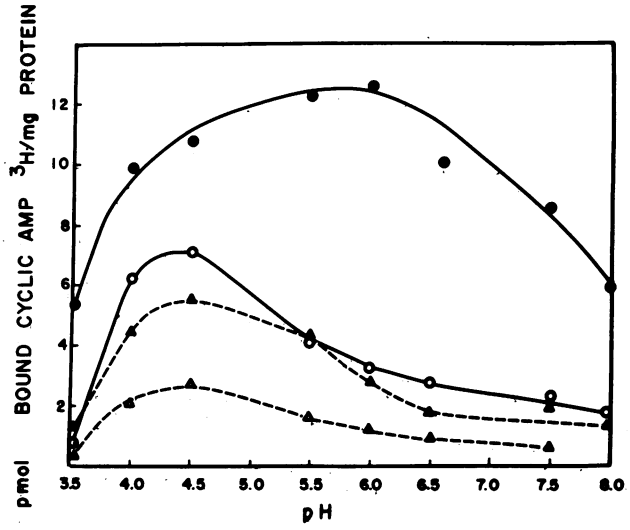

FIG. 4. Effect of pH on cAMP binding activity. In a total volume of 0.1 ml the reaction mixtures contained: 50 mM buffer (pH 3.5–6.5 sodium acetate and pH 7.5–8.0 Tris·HCl), 2 mM theophylline, 0.5 μM or 5 nM [^{3}H]cAMP (24 Ci/mmol), and 82 or 170 μg of cytosol protein from sensitive or resistant cells. The protein-bound cAMP was assayed on Millipore filters (9) with 20 mM buffers of the same pH. ●——●, sensitive cytosol (0.5 μM cAMP); ○——○, resistant cytosol (0.5 μM cAMP); ▲——▲, sensitive cytosol (5 nM cAMP); △——△, resistant cytosol (5 nM cAMP).

tide than the other. The resistant cytosol appears not to contain the lower affinity sites, and also seems to have a diminished concentration of the higher affinity sites compared to the sensitive cytosol.

We also bound ^{3}H-labeled cAMP to both sensitive and resistant extracts and subjected them to get filtration on Sephadex G-200 (data not shown). The binding activity in both extracts emerged as a single peak. The binding activity from sensitive cytosol was included in the gel and had, by comparison with known proteins, apparent molecular weights of 105,000 and 107,000 in two experiments. However, the binding activity from resistant cell cytosol (in three experiments) was excluded from the gel, suggesting that it may have a higher molecular weight. The signifiance of this apparent difference between the size of the sensitive and resistant binding activities, if any, cannot be assessed until further purification of the extracts.

Identity of the cAMP-binding protein in lymphoma cells

In many different mammalian tissues the cAMP receptor is a regulatory subunit that controls the activity of a protein phosphokinase (11, 12). Experiments were therefore performed to determine whether this is the case in our cultured lymphoma cells. Extracts of sensitive and resistant cells were chromatographed on DEAE-Sephadex, and the cAMP binding and histone kinase activities were identified (Fig. 5*A* and *B*). In both cases the cAMP-binding macromolecules migrate with the cAMP-activated kinase activity. A decreased binding activity in resistant cells was seen by this technique and was accompanied by a diminished cAMP-activated kinase. Similar conclusions were reached on the basis of sucrose density gradient centrifugation of the two cytosols (not shown). Since the cAMP-binding activity in both sensitive and resistant cell extracts cochromatographed on DEAE-Sephadex and cosedimented on sucrose gradient with their respective cAMP-activated histone kinase, it appears that in both types of cells the cAMP receptor is the regulatory subunit.

DISCUSSION

The results presented show that exogenous But$_2$cAMP inhibits the growth of cultured mouse lymphoma S49.1 TB4 cells and ultimately leads to their death. Experiments with prostaglandin E_1 suggest that endogenously generated cAMP has a similar effect. Variants of the parent cell line resistant to high concentrations of added But$_2$cAMP (and to prostaglandin) were easily derived from the sensitive parent line. Compared to the sensitive cells, these resistant variants contain lowered concentrations of cAMP-binding macromolecules, which appear to be the regulatory subunits of the cAMP-activated protein kinase. Our data suggest that cAMP resistance is accompanied by a decrease in the activity of cAMP receptors and, perhaps, by a change in their physical properties. However, none of our experiments indicates whether the transition from cAMP-sensitivity to resistance represents a genetic change or some type of heritable phontypic variation.

The mechanism by which cAMP induces the killing of sensitive lymphoma cells is unknown. The cylic nucleotide inhibits the growth of transformed, cultured mouse-embryo fibroblasts (13) and slows the uptake of added uridine, leucine, and 2-deoxyglucose by these cells (R. Kram, in preparation). Glucocorticoids that decrease the penetration of macromolecular precursors into thymic lymphocytes (2, 3) also

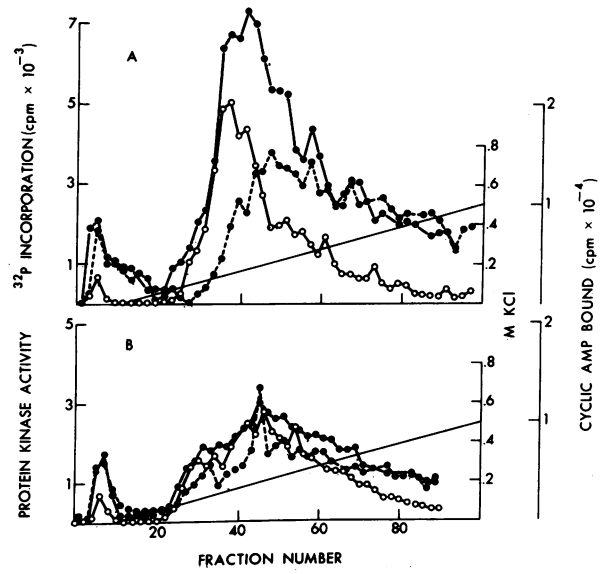

FIG. 5. DEAE-Sephadex A-50 chromotography of cytosols from But$_2$cAMP-sensitive or resistant cells. 60 mg of cytosol protein (prepared as described in legend to Fig. 2) was applied to a DEAE-Sephadex A-50 column (2.5 × 4 cm) equilibrated with 0.05 M Tris·HCl buffer (pH 7.4) containing 4 mM 2-mercaptoethanol and then eluted with a 100-ml linear (0.05–0.5 M) KCl gradient in the same buffer. 1.0-ml Fractions were collected and 50 1-ml samples were assayed for [^{3}H]cAMP binding (○——○), protein kinase (●——●), and cAMP-activated protein kinase (●——●) activities (see *Methods*). (*A*) Chromatography of But$_2$cAMP-sensitive cell cytosol; (*B*) chromatography of But$_2$cAMP-resistant cell cytosol.

induce lympholysis (1). We imagine, therefore, that the cytolytic effect of cAMP on lymphoma cells may also be related to decreased membrane transport.

This study was supported by Contract no. 72-3236 within the Special Virus-Cancer Program of the National Cancer Institute, and Grant GM 17239 from the National Institute of General Medical Sciences of the National Institutes of Health. The work reported in this paper was undertaken during the tenure of an American Cancer Society-Eleanor Roosevelt-International Cancer Fellowship awarded by the International Union Against Cancer.

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