IN VIVO ACTIVATION OF CYCLIC ADENOSINE 3':5'-PHOSPHATE-DEPENDENT PROTEIN KINASE IN CHINESE HAMSTER OVARY CELLS TREATED WITH $N^6,O^{2'}$-DIBUTYRYL CYCLIC ADENOSINE 3':5'-PHOSPHATE

Albert P. Li*, Kohtaro Kawashima†, and Abraham W. Hsie
The University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences & Biology Division, Oak Ridge National Laboratory§, Oak Ridge, Tennessee 37830

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SUMMARY: Treatment of Chinese hamster ovary cells with dibutyryl cyclic AMP, which results in a net increase of the intracellular cyclic AMP level, converts the epithelial-like cells to a fibroblast-like shape. Protein kinase activity in cells treated with 1 mM dibutyryl cyclic AMP show a 3-fold increase in V_{max} but no appreciable changes in the apparent K_m for ATP. When cells are treated with dibutyryl cyclic AMP, there is a time-dependent conversion of cyclic AMP-stimulable protein kinase to cyclic AMP-independent catalytic subunits, as demonstrated by Sephadex G-100 gel filtration. These experiments demonstrate the activation of the cyclic AMP-dependent protein kinase in vivo. This activation may lead to phosphorylation of certain cellular constituent(s) and thus may be involved in the observed morphological transformation.

INTRODUCTION: It is well known that activation of protein kinases is the major mechanism by which cAMP exerts its physiological function as a second messenger in response to various hormonal actions in eukaryotic cells. Work from many laboratories has established that the enzyme in its so-called complex form consists of catalytic and regulatory subunits. Cyclic AMP activates protein kinases by binding to the regulatory subunit, resulting in the dissociation of the enzyme complex to yield the cAMP-independent catalytic subunit (e.g. 1, 2).

We have previously demonstrated that treatment of Chinese hamster ovary (CHO) cells with $N^6,O^{2'}$-dibutyryl cyclic adenosine 3':5'-phosphate (Bt_2cAMP) converts the compact, epithelial-like cells to a spindle-shaped, fibroblast-like form (3). The addition of Bt_2cAMP (1 mM) causes a 10- to 20-fold

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*Predoctoral Fellow and †Postdoctoral Investigator, respectively, of the University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences.
†Presently at the Department of Physiological Chemistry, University of Tokyo, Japan.
§Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.
Abbreviations: CHO cells, Chinese hamster ovary cells; Bt_2cAMP, $N^6,O^{2'}$-dibutyryl cyclic adenosine 3':5'-phosphate.

increase in the intracellular cAMP level (within 1 hour or longer), presumably as a result of the competitive inhibition by the intracellularly accumulated N^6-monobutyryl cAMP on a cAMP phosphodiesterase with low K_m (4). Thus it appears certain that the various physiologic and biochemical effects seen in CHO cells upon treatment with Bt_2cAMP (3, 5-7) are mediated by the elevated intracellular cAMP level.

In this communication, evidence is presented to demonstrate that treatment with Bt_2cAMP activates the protein kinase within CHO cells in culture (in vivo). Such activation might play a role in the Bt_2cAMP-mediated morphological transformation of CHO cells.

MATERIALS AND METHODS:

Cell Culture — CHO-K_1 cells were grown in plastic culture dishes (Falcon) in medium F12 supplemented with 10% heat-inactivated (56° for 30 min) dialyzed fetal calf serum. Standard tissue culture conditions were maintained at 5% CO_2 in the air at 37° in a 100% humidified incubator, as described previously (3, 4).

Preparation of Cell Extract — CHO-K_1 cells were grown in the presence or absence of 1 mM Bt_2cAMP (Boehringer-Mannheim) until they were approximately 80% confluent in 100-mm plastic tissue culture dishes. Cells were washed four times with warm saline (0.9% NaCl solution) and scraped off the plates with a rubber policeman in cold phosphate-glycerol buffer (0.05 M potassium phosphate, pH 7.0; 0.05 M NaCl; 10% glycerol; 0.014 M mercaptoethanol). The cells were then homogenized in a glass Dounce homogenizer with a Teflon pestle (tight pestle, 20 strokes) at 4°. The resulting homogenate was centrifuged at 27,000 g in a Sorvall RC2-B centrifuge for 10 min. This supernatant, called 27K sup, was used for the measurement of protein kinase activity.

Protein Kinase Assay — Protein kinase was assayed by measuring the incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP (Amersham/Searle, 12 Ci per mmole) into FIIA histone mixture (Sigma) according to the procedure of Corbin et al. (8). Protein kinase activity is expressed as picomoles of phosphate incorporated per minute per milligram of protein. All biochemicals used for enzyme assays were obtained from Sigma unless otherwise indicated.

Sephadex G-100 Gel Filtration — Four plates of approximately 80% confluent cells grown in F12 medium, either in the presence (treated) or absence (control) of 1 mM Bt_2cAMP, were homogenized in 1 ml of phosphate-glycerol buffer, resulting in a 27K sup that had a protein concentration of 1 to 2 mg per ml. A volume of 0.5 ml of the 27K sup was applied to the Sephadex G-100 column (Pharmacia), pre-equilibrated with elution buffer for separating the catalytic subunit from the enzyme complex of protein kinase (8). Void volume was monitored using blue dextran. After a buffer volume of 2.0 ml was eluted, 0.5-ml fractions were collected, and 50 µl of each fraction was used for the assay of protein kinase activity. Protein concentration was determined by the method of Lowry et al. (9).

RESULTS AND DISCUSSION: Under our standard assay conditions, the incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP into FIIA histone increased linearly with reaction time up to 15 min and with protein concentration from 20 to 150 µg

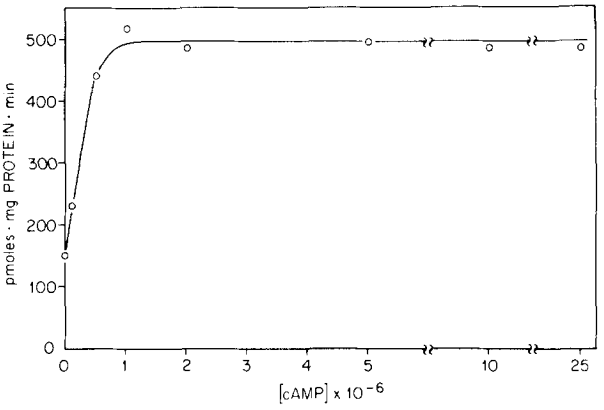

Fig. 1. Activation of protein kinase in CHO cells as a function of cAMP concentration. The protein kinase activities were measured with an enzyme source (containing 50 µg protein) prepared from cells grown in regular medium. Reaction time, 10 min.

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of 27K sup from untreated control CHO cells. Cyclic AMP stimulated the protein kinase activity, to a maximum of approximately 3-fold with 1.0 µM cAMP. Higher concentrations of cAMP tested (up to 25 µM) did not significantly further stimulate the activity (Fig. 1).

Kinetic studies showed that addition of 0.5 µM cAMP to the 27K sup of the untreated control cells increased the apparent V_{max} of the protein kinase activity from 200 to 500 pmoles/min/mg protein but did not appreciably alter the apparent K_m of this enzyme for ATP, which is approximately 50 µM (Fig. 2a). When CHO cells were treated with 1 mM Bt_2cAMP for 24 hours, the V_{max} of the protein kinase activity in the 27K sup reached approximately the same level as that seen when the enzyme from untreated control cells was nearly maximally stimulated *in vitro* by 0.5 µM cAMP (Fig. 2b). Addition of 0.5 µM cAMP to the reaction mixture only minimally stimulated the protein kinase activity of the treated cells (Fig. 2b). This indicates that most of the protein kinase activity in the treated cells probably existed in the activated form, so that treatment of CHO cells with Bt_2cAMP, which elevates intracellular cAMP (4), caused the activation of protein kinase activity *in vivo*. The possibility that such activation might lead to a time-dependent dissociation

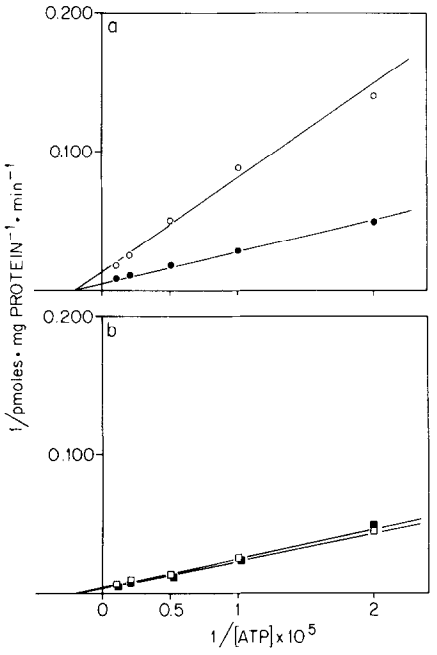

Fig. 2. Lineweaver-Burke plot of cAMP-activated protein kinase activity as a function of ATP concentration in the 27K sup of CHO cells grown in (a) regular growth medium (untreated control) or (b) medium supplemented with 1 mM Bt_2cAMP (treated) for 24 hours. The activity of protein kinase was assayed in the presence and absence of 0.5 µM cAMP. Protein kinase activity from control untreated cells was assayed in the presence (●–●) or absence (○–○) of cAMP, and that from treated cells was assayed in the presence (■–■) or absence (□–□) of cAMP.

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of the enzyme complex into regulatory and catalytic subunits within the cells was studied by Sephadex G-100 chromatography.

When the 27K sup of the untreated control cells was chromatographed, one species of protein kinase activity (mol wt greater than 100,000) eluted immediately after the void volume. Protein kinase activity in each fraction of this peak was highly stimulated by 0.5 µM cAMP, usually up to three times the basal activity (Fig. 3a). After a 30-min treatment with 1 mM Bt_2cAMP, the CHO-K_1 cells gave a 27K sup that separated into two species of protein kinase activity upon Sephadex G-100 gel filtration (Fig. 3b). The first species, which was stimulated by cAMP, presumably represents the enzyme complex; the second, which was not stimulated by cAMP, represents the catalytic

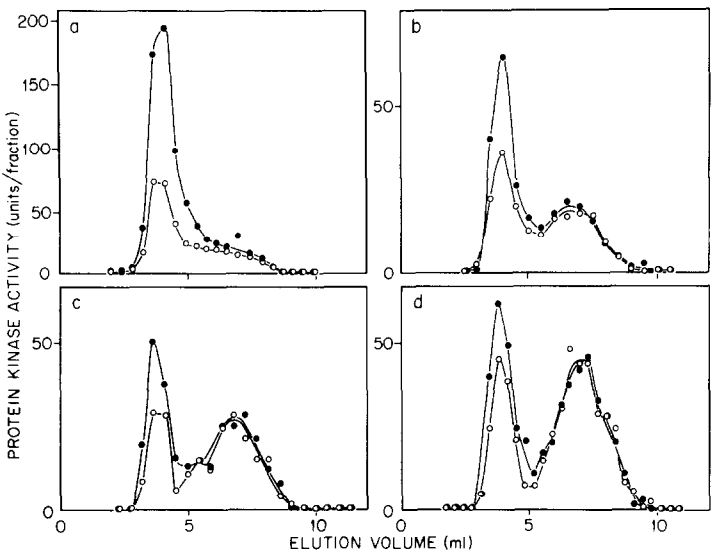

Fig. 3. The chromatographic profile of protein kinase activity on a Sephadex G-100 (0.9 X 20 cm) column. 0.5 ml of the 27K sup, which contains approximately 0.5 mg to 1.0 mg protein, was applied to the column. Protein kinase activity of each 50 µl fraction was assayed by our standard assay condition as described in Materials and Methods. The 27K sup were prepared from (a) control (untreated) cells, (b) cells incubated with 1 mM Bt_2cAMP for 30 min, (c) cells incubated with 1 mM Bt_2cAMP for 1 hour, and (d) cells incubated with 1 mM Bt_2cAMP for 24 hours. Protein kinase activities were assayed in the absence (○—○) and presence (●—●) of 0.5 µM cAMP. One unit of enzyme activity represents 1 pmole of $^{32}PO_4$ transferred from [γ-^{32}P]ATP to histone per min.

subunit activity. More catalytic subunits were seen from cells treated for longer periods of time: Treatment of cells for 1 hour gave more catalytic subunit activity (Fig. 3c) than treatment for 30 min (Fig. 3b). There was essentially no difference between the elution profiles for cells treated for 1 hour (Fig. 3c) or 6 hours (not shown) and those treated for 24 hours (Fig. 3d). Thus, in CHO cells treated with 1 mM Bt_2cAMP for 1 hour or longer, the protein kinase was almost fully activated, resulting in almost complete dissociation into the catalytic subunits. When 200 µM cAMP was added *in vitro* to the 27K sup from untreated cells, the protein kinase was activated, resulting in its dissociation into the same two species of protein kinase activity (Fig. 4) as seen in cells treated with 1 mM Bt_2cAMP (Fig. 3b, c and d).

These results demonstrate that treatment of CHO cells with Bt_2cAMP

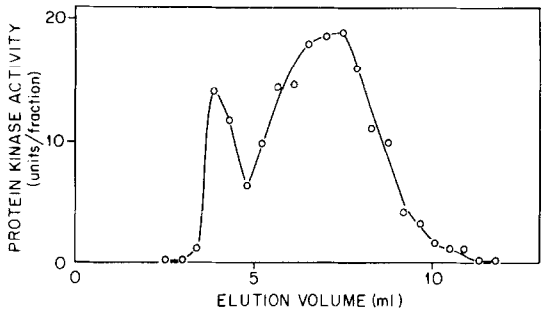

Fig. 4. The chromatographic profile of protein kinase activity of the untreated control cells on a Sephadex G-100 column in the presence of 200 μM cAMP prior to and during the separation procedure. Control cells were washed four times with warm (37°) saline (0.9% NaCl solution) and homogenized with 0.05 M potassium phosphate-glycerol buffer (pH 7.0) containing 200 μM cAMP, 0.5 M NaCl, 10% glycerol, and 0.014 M mercaptoethanol at 4°. A volume of 0.5 ml of 27K sup was applied to the column and eluted with the same cAMP-containing buffer used in homogenization. Protein kinase activity in each fraction was assayed as described.

causes the activation of cAMP-dependent protein kinase within the cells. As previously reported (10), the CHO cells also appear to contain considerable cAMP-independent protein kinase activity (Fig. 3d).

Previous time-lapse photomicrographic studies established that the first manifestation of morphological transformation into CHO cells by exogenous addition of Bt_2cAMP is the disappearance of knobbed structures on the cell membrane (6). The cells then elongate to assume a fibroblast-like morphology, presumably as a result of an increase in the number of microtubules and their organization into parallel arrays within 1 hour (3, 6). Such cells remain in the fibroblast-like form in the continuous presence of Bt_2cAMP. Independent time-course studies have shown that within 1 hour after addition of Bt_2cAMP to CHO cells, the intracellular cAMP level increases 10- to 20-fold and remains at that elevated level for at least another 24 hours (4). The almost identical time-course profiles of modification of surface membrane and cellular microtubules (5, 6), of elevation of intracellular cAMP level (4), and of activation of protein kinase with subsequent dissociation into cAMP-independent catalytic subunits (Fig. 3a to d) make it reasonable to speculate

that phosphorylation of some cellular constituents (such as membrane and microtubules) by protein kinase is involved in the morphological transformation induced by exogenous addition of Bt_2cAMP to CHO cells.

Both membrane and microtubule proteins are substrates for protein kinases from various mammalian tissues (10-14). In view of the demonstration that enucleated CHO cells can undergo morphological transformation by Bt_2cAMP (7), the nuclear components per se are unlikely to be involved in morphological transformation. Studies on the phosphorylation of natural cytoplasmic substrates for cAMP-dependent protein kinase and their possible involvement in the morphological transformation of CHO cells by Bt_2cAMP are in progress.

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