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Cyclic AMP-dependent protein kinase mediates a cyclic AMP-stimulated decrease in ornithine and *S*-adenosylmethionine decarboxylase activities

(S49 cells/polyamines/enzyme induction)

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ABSTRACT **Incubation of S49 lymphoma cells with N^6,$O^{2'}$-dibutyryl cyclic AMP (Bt_2cAMP) decreases the activities of ornithine decarboxylase (L-ornithine carboxy-lyase; EC 4.1.1.17) and *S*-adenosylmethionine decarboxylase (*S*-adenosyl-L-methionine carboxy-lyase; EC 4.1.1.50), the two principal enzymes in the pathway of polyamine synthesis. This decrease is dose-dependent, commences after a 3-hr delay, virtually abolishes the assayable activities of the two enzymes, and is not associated with a soluble inhibitor of the enzyme activities. Studies in mutant S49 clones that have altered protein kinase indicate that cAMP-dependent protein kinase mediates the decreases in enzyme activities. The dose-response pattern for the cAMP-stimulated decrease in enzyme activities parallels the pattern for the cAMP-stimulated, cell cycle-specific (G_1) growth arrest of S49 cells. The activity of ornithine decarboxylase decreases faster than Bt_2cAMP arrests wild-type S49 cells and, similarly, release of cells from the cAMP-stimulated arrest in G_1 increases the activity of ornithine decarboxylase faster than cells exit from G_1. These findings contrast with reports that cAMP induces ornithine decarboxylase in other cell types and further suggest that passage of cells through the cell cycle is required for maintaining the activities of ornithine and S-adenosylmethionine decarboxylases.**

Polyamines have been implicated as regulators of protein synthesis in a variety of biologic tissues (1–3). In certain regenerating tissues and cultured cells, increased levels of ornithine decarboxylase (L-ornithine carboxy-lyase; EC 4.1.1.17), the rate-limiting enzyme in polyamine biosynthesis in eukaryotes, closely correlate with increased rates of cellular growth and proliferation (2, 3). In fibroblasts (4), mammary tissue (5), chinese hamster V79 cells (6), hepatoma (7), glioma, and neuroblastoma cells (8), and L cells (9), adenosine 3′:5′cyclic monophosphate (cAMP) appears to induce ornithine decarboxylase; but in other model systems (10, 11) changes in cAMP levels can be dissociated from increased ornithine decarboxylase activity. Russell and colleagues (12) have suggested that activation of cAMP-dependent protein kinase mediates the induction by cAMP. A recent report (13) indicates that S-adenosylmethionine decarboxylase (*S*-adenosyl-L-methionine carboxy-lyase; EC 4.1.1.50) can be induced in glioma and neuroblastoma cells by agents that elevate cAMP.

In S49 mouse lymphoma cells (14), elevation of cAMP levels by dibutryl cAMP (Bt_2cAMP) induces phosphodiesterase (15), arrests cell growth in the G_1 phase of the cell cycle (16), and subsequently kills the cells (14). Mutant S49 clones have been isolated that are resistant to such cytolysis and that have decreased cellular responses to cAMP (17, 18). These cAMP-resistant clones have been classified into three general types, based on cellular responses to Bt_2cAMP and cytosolic activity of cAMP-dependent protein kinase (14, 17): (*i*) "kinase-negative"—cells with no cAMP-dependent protein kinase activity and no response to Bt_2cAMP; (*ii*) "K_a mutants"—cells whose kinase has a decrease in apparent affinity for cAMP to 10% or less of the value in wild-type cells and whose responses to Bt_2cAMP have an increased requirement for the nucleotide; and (*iii*) "V_{max} mutants"—cells whose maximal kinase activity and maximal response to Bt_2cAMP are both decreased. The close correlation between the alterations in cAMP-dependent protein kinase activity and the altered responses of these mutant cells to cAMP strongly suggests that protein kinase mediates the responses of intact S49 cells to cAMP (17). We therefore used S49 cells to test the hypothesis (12, 13) that cAMP and protein kinase induce ornithine decarboxylase, but found instead that in these cells, in contrast with all other cells reported previously, cAMP strikingly decreases the activities of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase and that this effect is mediated by cAMP-dependent protein kinase.

MATERIALS AND METHODS

S49.1 mouse lymphoma cells (19) were propagated in suspension culture in Dulbecco's modified Eagle's medium that contained 10% heat-inactivated horse serum as described (14, 17). Cell concentrations and viability were determined by a hemocytometer and by trypan blue exclusion, respectively. The isolation and characterization of the mutant clones have been described (17, 20).

Cellular lysates were obtained by suspending cell pellets in ice-cold hypotonic buffer (5 mM Tris·HCl, pH 7.5/2 mM dithiothreitol) at a concentration of 4×10^7 cells per ml and lysing the cells with two cycles of rapid freeze-thawing. The homogenates were centrifuged at $100,000 \times g$ for 60 min, and the resulting supernatant fractions were used for enzyme assays.

Ornithine decarboxylase activity was determined by measuring the release of $^{14}CO_2$ from (−)- or (±)-[*1*-^{14}C]ornithine in an assay system adapted from Jannë and Williams-Ashman (21). The standard incubation mixture contained, in a final volume of 0.25 ml: 0.1 ml of cell extract; 25 μmol of glycylglycine buffer, pH 7.2; 0.05 μmol of pyridoxal 5-phosphate; 1.2 μmol of dithiothreitol; 0.04 μmol of nonradioactive (−)-ornithine; and 0.12–0.25 μCi of (−)-[*1*-^{14}C]ornithine (specific activity, 57 Ci/mol, Amersham-Searle) or 0.5 μCi of (±)-[*1*-^{14}C]ornithine (specific activity, 54 Ci/mol, New England Nuclear). The released CO_2 was trapped in 0.1 ml of 1 M hyamine hydroxide contained in a polypropylene well, which was attached to a rubber stopper that capped the incubation

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Abbreviations: cAMP, cyclic AMP; Bt_2cAMP, dibutyryl cyclic AMP.

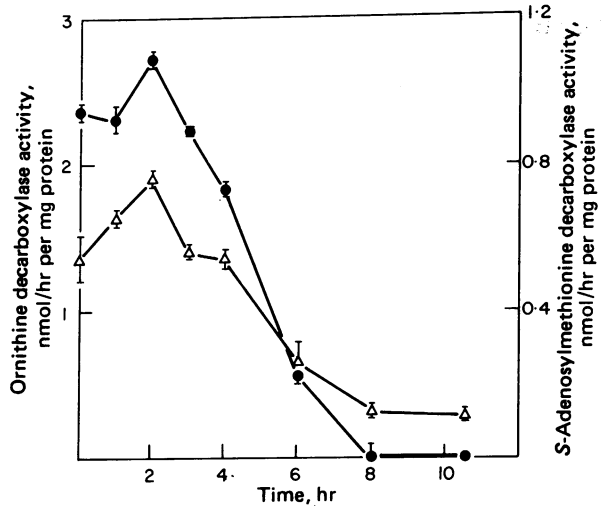

FIG. 1. Time course of response of ornithine decarboxylase (●) and S-adenosylmethionine decarboxylase (△) to Bt_2cAMP treatment in wild-type S49 cells. Logarithmically growing cells were incubated with 0.5 mM Bt_2cAMP at 37° and aliquots were removed at the indicated times. Assays were in triplicate and are plotted as mean ± SD.

tubes. After a 60-min incubation at 37°, the reactions were halted by injecting 1 ml of 40% trichloroacetic acid through the rubber stopper. The tubes were incubated for at least 20 min at 37° to ensure that all the dissolved CO_2 was released from the acidified medium. The well was removed and radioactivity was determined in a scintillation vial that contained 2 ml of ethanol and 10 ml of Omnifluor (New England Nuclear) toluene. All values were corrected for radioactivity in control samples containing buffer in lieu of cell extract. Release of $^{14}CO_2$ from the substrate in the assay was linear with time for at least 90 min and with protein [assayed by the method of Lowry *et al.* (22)] from 30 to 500 μg. The apparent K_m for ornithine was 35 μM. Radioactive ornithine was lyophilized and reconstituted in 10 mM HCl in order to decrease the nonenzymic control activity.

Putrescine-activated S-adenosylmethionine decarboxylase activity was determined by measuring the release of $^{14}CO_2$ from (−)-S-adenosyl-L-[*carboxyl*-^{14}C]methionine in the presence of saturating levels (0.5 mM) of putrescine in an assay system adapted from a described procedure (23). The reaction mixture contained, in a final volume of 0.2 ml: 0.1 ml of supernatant protein; 20 μmol of glycylglycine buffer, pH 7.2; 1.2 μmol of dithiothreitol, 0.1 μmol of putrescine, 0.007 μmol of nonradioactive S-adenosylmethionine, and 0.1–0.2 μCi of (−)-S-adenosyl-L-[*carboxyl*-^{14}C]methionine (specific activity 54–59 Ci/mol, New England Nuclear). Incubations were performed and radioactivity was measured as described above for the ornithine decarboxylase assay. The release of $^{14}CO_2$ from the substrate was linear for at least 80 min and with protein from 30 to 500 μg. The apparent K_m for S-adenosylmethionine was 15 μM.

[^{3}H]Leucine incorporation was determined as described in the legend to Fig. 3. Cell cycle distribution of cells was determined by flow microfluorimetry, as described (16).

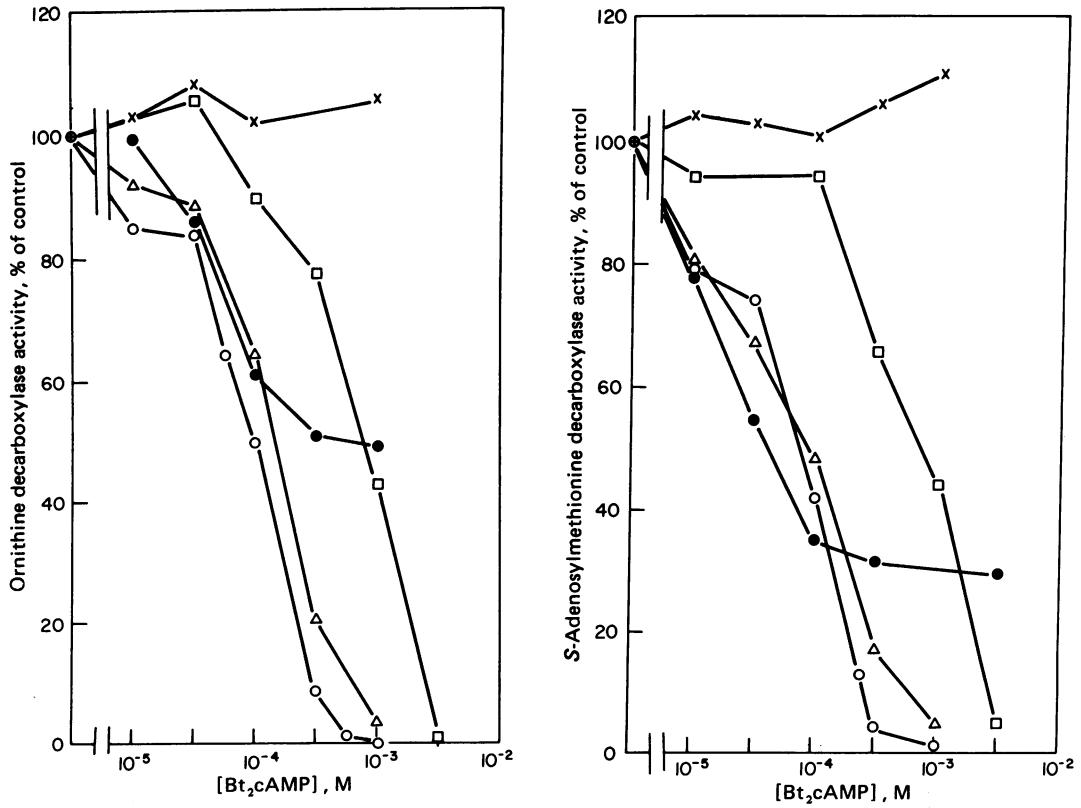

FIG. 2. Inhibition of ornithine decarboxylase activity (*Left*) and S-adenosylmethionine decarboxylase activity (*Right*) in wild-type and mutant S49 cells after Bt_2cAMP treatment. Logarithmically growing wild-type (○), "kinase-negative mutant" (×), "K_a mutant" (□), "V_{max} mutant" (●), or "cAMP-deathless mutant" (△) cells were incubated with the indicated concentrations of Bt_2cAMP for 16–22 hr. The data are expressed as the percent decrease in enzyme activity compared to control samples having no Bt_2cAMP. Each point is the mean value of data from two to seven separate experiments run in duplicate. The control activities for ornithine and S-adenosylmethionine decarboxylase, respectively, were (mean ± SD, nmol/hr per 4×10^6 cells): wild-type (0.97 ± 0.37 and 0.11 ± 0.06); "kinase-negative mutant" (2.28 ± 0.37 and 0.18 ±0.14); "K_a mutant" (0.82 ± 0.17 and 0.16 ± 0.05); "V_{max} mutant" (0.69 ± 0.06 and 0.20 ± 0.01); and "cAMP-deathless mutant" (1.38 ± 0.36 and 0.26 ± 0.04).

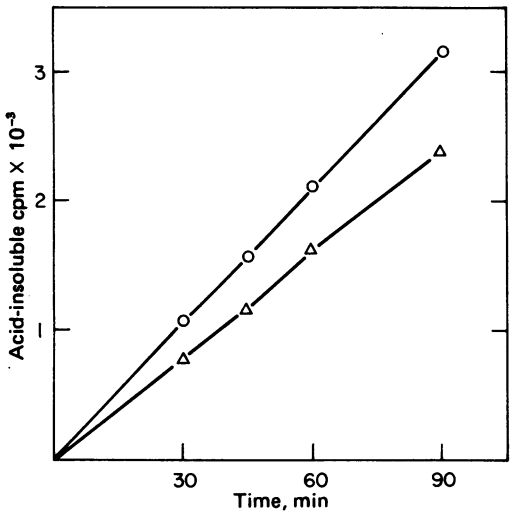

FIG. 3. Effect of Bt_2cAMP on [^{3}H]leucine incorporation into trichloroacetic acid-insoluble material in wild-type S49 cells. Wild-type S49 cells (1.8×10^6 cells per ml) were incubated in a volume of 20 ml in the presence (△) and absence (○) of Bt_2cAMP (0.4 mM) for 8 hr. [^{3}H]Leucine (1 μCi/ml) was added and 1.5-ml aliquots were removed in duplicate at the indicated times. Samples were centrifuged for 3 min at $500 \times g$, the medium was aspirated, and the pellets were resuspended in 5 ml of phosphate-buffered saline. The samples were centrifuged again at $500 \times g$ for 3 min, the buffer was aspirated, and the pellets were resuspended in 0.3 ml of 125 mM potassium phosphate buffer (pH 7.4). Two milliliters of 10% trichloroacetic acid was added. After the samples were left in the cold for exactly 5 min, each was filtered over a Whatman GFC filter. The filters were washed with 10 ml of 5% trichloroacetic acid and then radioactivity was determined in a liquid scintillation counter.

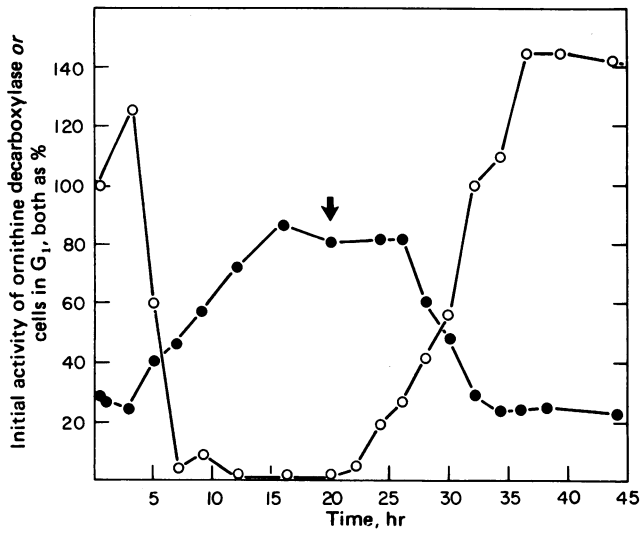

FIG. 4. Time course of development and reversal of G_1 arrest and decrease in ornithine decarboxylase activity by Bt_2cAMP in wild-type S49 cells. Logarithmically growing wild-type S49 cells were incubated with 0.5 mM Bt_2cAMP at 0 time and aliquots were removed at the indicated times for assay of ornithine decarboxylase (○) and for fraction of cells in G_1 (●) by flow microfluorimeter analysis. Data are plotted as percent of initial ornithine decarboxylase activity and percent of cells in G_1. At the time indicated by the arrow, cells were washed to remove the Bt_2cAMP, resuspended in fresh medium, and incubated at 37°.

RESULTS AND DISCUSSION

To examine the effect of cAMP on the activities of ornithine and *S*-adenosylmethionine decarboxylases, we incubated wild-type S49 cells with 0.5 mM Bt_2cAMP (Fig. 1). Although it was anticipated (4–9, 12, 13) that the activities of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase would increase, we found instead that Bt_2cAMP decreased the activities of both enzymes (Fig. 1). After an initial 3-hr period during which a transient, small (less than 30%) increase in the two activities occurred, both enzyme activities fell to levels less than 10% of initial values. In some experiments, this low level could not be distinguished from the control samples, which had no cell extracts. The decrease in activities was completed within 10 hr, prior to the time of maximal accumulation of the arrested cells in the G_1 phase of the cell cycle (see Fig. 4). Maximal growth arrest requires a full cell generation time, 16–18 hr (16).

In order to investigate the role of cAMP-dependent protein kinase in the inhibition of enzyme activities, we used a series of mutant S49 clones that are resistant to killing by Bt_2cAMP and that have altered activities of cAMP-dependent protein kinase (17). Incubation of wild-type cells and cells from the three types of mutant clones with varying concentrations of Bt_2cAMP for 16–20 hr resulted in decreases in the activities of ornithine and *S*-adenosylmethionine decarboxylases (Fig. 2). These decreases closely resembled the dose-response patterns observed for the other responses to cAMP in these clones: half-maximal effect was at approximately 0.1 mM Bt_2cAMP in wild-type and "V_{max} mutant" cells and 1 mM in "K_a mutant" cells; there was no decrease in "kinase-negative mutant" cells and a decreased maximal response in the "V_{max} mutant." These results strongly suggest that the decrease in activities of the polyamine-synthesizing enzymes is mediated by cAMP-dependent protein kinase.

The decrease in the activities of the two decarboxylase enzymes after treatment with Bt_2cAMP distinguishes S49 cells from several other types of cells (4–9) in which Bt_2cAMP increases the level of ornithine decarboxylase activity. In S49 cells cAMP induces at least one protein (phosphodiesterase) at a time when ornithine and *S*-adenosylmethione decarboxylase activities are falling. Induction of phosphodiesterase becomes apparent at 2 hr and is maximal by 8 hr after treatment with Bt_2cAMP (15); this, as well as independent measurements of effects of Bt_2cAMP on [^{3}H]leucine incorporation into acid-precipitable material of wild-type cells (ref. 24, Fig. 3) in which only a 25–30% decrease in the rate of incorporation is noted after 8 hr, indicate that the decreases of decarboxylase activity that occur during this period of time do not result from a general loss of the ability of the cells to synthesize new protein.

Using another mutant S49 clone, termed "cAMP-deathless, we found that the decrease of activities of the polyamine-synthesizing enzymes is independent of cellular events that mediate

Table 1. Additivity of activities (pmol/hr) of ornithine and *S*-adenosylmethionine decarboxylases in extracts of wild-type S49 cells

Extract	Ornithine decarboxylase	*S*-Adenosylmethionine decarboxylase
Control	330	85
Bt_2cAMP-treated	5	2
Control + Bt_2cAMP-treated	357 (335)	82 (87)

Supernatant fractions (50 μl) from control cells and cells incubated for 16 hr with 1 mM Bt_2cAMP were prepared and ornithine and *S*-adenosylmethionine decarboxylase activities were assayed. Numbers in parentheses are predicted values of control + treated extracts, assuming additivity of activity.

cAMP-induced cell lysis. The "camp-deathless" clone (20) has cAMP-dependent protein kinase activity and cAMP-stimulated induction of phosphodiesterase and growth arrest in G_1 that resemble the responses of wild-type cells, but in contrast with wild-type cells, cells of this clone do not die after prolonged (up to 9 days) treatment with Bt_2cAMP. The response of the "cAMP-deathless" cells to varying concentrations of Bt_2cAMP was similar to the response of wild-type cells (Fig. 2).

The mechanism whereby Bt_2cAMP decreases the enzymatic activities in S49 cells is unknown. In other systems addition of putrescine blocks induction of ornithine decarboxylase (25–28). In some, (26, 28) but not all (27) cases, this inhibition is associated with the appearance of an intracellular inhibitor of the enzyme that has been termed an antizyme (28). Such a soluble inhibitor of either ornithine or *S*-adenosylmethionine decarboxylase activity was not detectable in mixing experiments with extracts of S49 cells (Table 1). That inhibition by Bt_2cAMP was not caused by some nonspecific effect of the nucleotide is indicated by results with the mutant clone that lacks protein kinase (Fig. 2) and by the lack of effect of Bt_2cAMP (50 μM) itself on the enzyme assays (data not shown).

Studies of all the S49 clones indicate that the dose-response relationship for the decrease in enzyme activities directly parallels the curves for cAMP-dependent protein kinase activity in cell extracts as well as the curves for arrest of cell growth in G_1 and induction of phosphodiesterase. Our data do not distinguish between direct regulation of the polyamine-synthesizing enzymes by phosphorylation and decreases of the activities of these enzymes by other cellular events, such as G_1 growth arrest, that are regulated by phosphorylation.

One experimental approach to test this was to compare the time courses for the development of reversal of cells arrested in G_1 by Bt_2cAMP with the time course for the changes in activity of ornithine decarboxylase. We found (Fig. 4) that changes in the activity of ornithine and *S*-adenosylmethionine decarboxylases (data not shown) preceded both the arrest of cells in G_1 during exposure of cells to Bt_2cAMP and the release of cells from the G_1 block when Bt_2cAMP was removed. Although this may only reflect the greater sensitivity of the methods for detecting changes in the activity of ornithine decarboxylase than changes in distribution of cells in G_1, this experiment raises the possibility that the fall in the activities of polyamine-synthesizing enzymes may be a primary factor in determining entry and exit of S49 cells from a block in G_1. Of perhaps greater likelihood is that the G_1 block produced by cAMP precedes the point of regulation of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase activities and that a randomly distributed population of cells takes longer to fully traverse S, G_2, and M than to pass the point of regulation of the activities of the polyamine-synthesizing enzymes. Experiments to measure the activity of these enzymes in synchronized populations of S49 cells at various points in the cell cycle should help distinguish whether changes in the polyamine-synthesizing enzymes determine, are determined by, or are unrelated to progression of cells through the cell cycle.

Thus, the data emphasize that ornithine decarboxylase activity may decrease in response to elevated cellular levels of cAMP and that, as previously suggested (16), cAMP-dependent protein kinase mediates all the observed effects of cAMP in S49 cells. In addition, our results suggest that continued passage of cells through the cell cycle is necessary for maintaining the activities of ornithine and *S*-adenosylmethionine decarboxylases and that growth arrest of cells by cAMP is related to the decrease in activities of the polyamine-synthesizing enzymes.

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