CYCLIC AMP IN G1-ARRESTED BHK21 CELLS INFECTED WITH ADENOVIRUS TYPE 12.

Karel Raska, Jr.
Department of Microbiology, Rutgers Medical School
College of Medicine and Dentistry of New Jersey
Piscataway, New Jersey 08854

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SUMMARY. Changes in intracellular levels of cyclic AMP and activity of adenyl cyclase and cyclic AMP phosphodiesterase were studied in G1-arrested BHK21 cells infected with adenovirus type 12 (Ad12). The intracellular concentration of cyclic AMP is reduced at 8 hours after infection (h.p.i.) and reaches a minimum at 14 h.p.i. The decrease in activity of adenyl cyclase is detected at 7.5 h.p.i.; at 9 h.p.i. the activity is about 50% of that in mock-infected cells. No significant changes were observed in the activity of cyclic AMP phosphodiesterase until 13 h.p.i., thereafter the activity of cyclic AMP phosphodiesterase decreased. It is suggested that the observed changes in cyclic AMP metabolism are related to expression of the viral genome, as the first changes are detected only after appearance of Ad12 mRNA in the infected cells.

INTRODUCTION. Infection of BHK21 cells, arrested in the G1 phase of the cell cycle by growth in medium containing a low concentration of serum, with an oncogenic adenovirus type 12 (Ad12), results in an abortive infection, with no detectable production of infectious progeny. In the infected cells early mRNA is transcribed (1), a round of cellular DNA synthesis is induced, and the cells synthesize T-antigen (2), but no viral DNA, late mRNA or viral capsid proteins can be detected after infection (3). The cells enter mitosis, but the chromosomes become fragmented and most of the cells die (4). The initiation of cellular DNA synthesis under these conditions could be considered to be an analog of the altered control of cell multiplication exhibited by adenovirus-transformed cells.

Addition of dibutyryl-cyclic AMP to the incubation medium inhibits the induction of cellular DNA synthesis and reduces markedly the frequency of T-antigen positive cells; on this evidence it has been proposed that infection with Ad12 might lead to reduction of the intracellular concentration of cyclic AMP (5).

Here we present experimental evidence that the intracellular concentration

of cyclic AMP and the activity of adenyl cyclase decrease in G1-arrested BHK21 cells after Ad12 infection and this decrease is later followed by a reduction of cyclic AMP phosphodiesterase activity.

MATERIALS AND METHODS. Media, BHK21 cells, methods for achieving G1-arrest in these cells, infection with adenovirus type 12, immunofluorescent staining for T-antigen and determination of DNA synthesis have been previously described (1,2).

Cyclic AMP Assay. The cell monolayers were washed with phosphate-buffered saline (PBS), scraped and collected by low speed centrifugation. The cell pellets were extracted with trichloroacetic acid (TCA) and the concentration of cyclic AMP was determined as described by Gilman (6).

Adenyl Cyclase Activity. The cells were washed with PBS and homogenized in 0.3 M sucrose with 5 mM dithiothreitol in a tight fitting Dounce homogenizer. The reaction mixture consisted of 75 µl of 50 mM Tris pH 8.0, 3 mM $MgCl_2$, 10 mM NaF, 10 mM theophylline, 12.5 mM phosphoenolpyruvate, 4 µg/ml of PEP-kinase, and 6.25 mM dithiothreitol, 20 µl of 2 mM ATP-^{3}H and 5 µl of the cell homogenate. The reaction mixture was supplemented with unlabeled cyclic AMP as described by Makman (7). Incubation at 37°C for 10 min. was stopped by adding 5% TCA; after ether extraction and $Ba(OH)_2$ and $ZnSO_4$ precipitations, ^{3}H-cyclic AMP was isolated by chromatography on BioRad AG 50W-X8 resin, as described by Krishna et al. (8).

Cyclic AMP Phosphodiesterase Assay. The cells in monolayers were washed with PBS and then with homogenizing solution (10.9% sucrose in 0.04 M Tris pH 8.0). The washed cells were then scraped and homogenized in a Dounce homogenizer. Activity of the enzyme was determined in an incubation mixture consisting of 100 µl of 40 mM Tris pH 8.0, 10 mM $MgCl_2$ and 4 mM 2-mercaptoethanol, 75 µl of the cell homogenate and 10 µl of an appropriate concentration of ^{3}H-cyclic AMP. The reaction was stimulated by 50 µg of snake venom. Liberated ^{3}H-adenosine was separated using BioRad AG1 X-2 resin as described by Monard et al. (9).

Protein Determination. Concentration of protein was determined in aliquots of washed cells or cell homogenates by the method of Lowry et al. (10).

RESULTS. The change in intracellular concentration of cyclic AMP and activities of adenyl cyclase and cyclic AMP phosphodiesterase after infection with Ad12 were studied in BHK21 cells brought to G1-arrest by 50 hours growth in medium containing 0.5% fetal calf serum. Because the timing of events after infection with Ad12 is dependent on the input multiplicity of infection (m.o.i.), it is useful to monitor the induction of cellular DNA synthesis in infected cells. The cells were infected at input m.o.i. of approximately 100 plaque-forming units (p.f.u.) per cell. T-antigen synthesis was detected in >94% of cells. Under such conditions the first increase in ^{3}H-thymidine incorporation is observed at 12 h.p.i., incorporation reaches maximum at 18 h.p.i. and thereafter decreases (Fig. 1).

Changes in cyclic AMP concentration in BHK21 cells at various times after infection with Ad12 are summarized in Fig. 2. It is apparent that at 8 h.p.i. the detected concentration of cyclic AMP was reduced to about 50% of that found at the time of infection; thereafter a further decrease was observed, reaching a minimum level at 14 h.p.i. This minimum level (about 1/6 of control) was maintained in infected cells through at least 22 h.p.i. In mock-infected cells, the intracellular concentration of cyclic AMP did not significantly change through 22 hours after mock-infection.

The intracellular concentration of cyclic AMP is controlled by two basic enzymatic activities, adenyl cyclase and cyclic AMP phosphodiesterase. To obtain some information about the possible mechanism by which the concentration of cyclic AMP decreases in Ad12-infected cells, activities of adenyl cyclase and cyclic AMP phosphodiesterase were studied at various times after infection with Ad12. Results summarized in Fig. 3 show the observed activities of adenyl cyclase. In infected cells, the first significant change of activity was detected at 7.5 h.p.i.; from 9 h.p.i. through 22 h.p.i. the observed activity of adenyl cyclase corresponded to slightly less than 50% of that

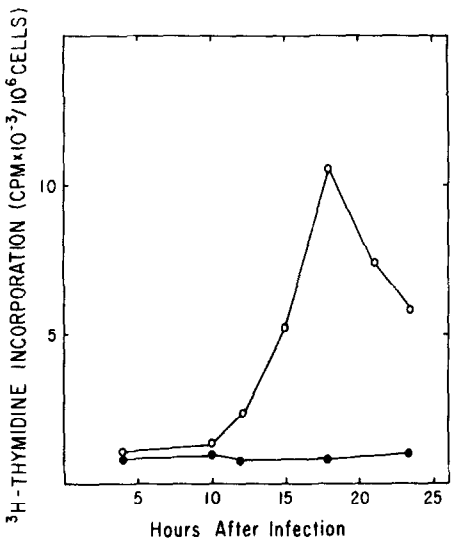

Fig. 1.

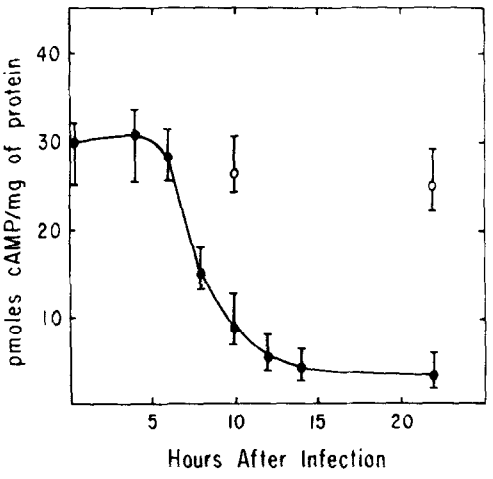

Fig. 2.

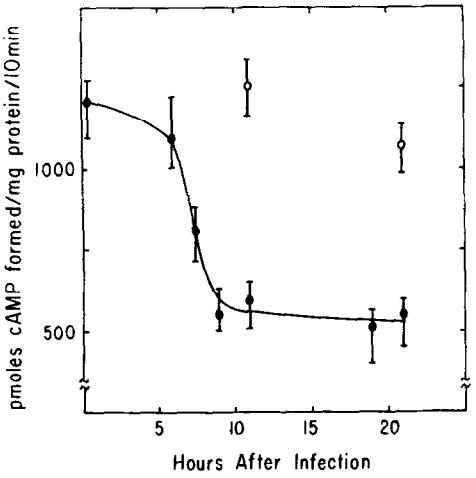

Fig. 3.

Figure 1. DNA synthesis in G1-arrested BHK21 cells infected with Ad12. The cells were pulse-labeled for 60 min. periods with thymidine-methyl-^{3}H at times indicated. The points are average of duplicate determinations. T-antigen was induced in >94% of infected cells. O G1-arrested cells infected with Ad12; ● mock-infected, G1-arrested cells.

Figure 2. Levels of cyclic AMP in G1-arrested BHK21 cells infected with Ad12. The points represent averages of 3-7 determinations. Ranges give minimum and maximum values observed. ● G1-arrested cells infected with Ad12 (input m.o.i. ∿ 100 p.f.u./cell); O mock-infected G1-arrested BHK21 cells.

Figure 3. Activity of adenyl cyclase in G1-arrested BHK21 cells infected with Ad12. The points represent averages of 3-4 determinations. Ranges give minimum and maximum values observed. ● G1-arrested cells infected with Ad12 (input m.o.i. ∿ 100 p.f.u./cell); O mock-infected, G1-arrested cells.

TABLE 1

ACTIVITY OF CYCLIC AMP PHOSPHODIESTERASE IN AD-12 INFECTED BHK21 CELLS.

Source of enzyme activity	Phosphodiesterase activity[a] (n mole per mg protein per 10 min.)[b]		
	60 μM cyclic AMP	5 μM cyclic AMP	0.5 μM cyclic AMP
G1-arrested cells	9.6	0.94	0.085
Infected cells (hours after infection)			
5	10.1	N.D.[c]	N.D.
7	9.9	1.07	0.086
10	9.9	N.D.	0.081
13	9.4	0.93	0.075
19	6.5	0.66	0.049
22	7.8	0.46	N.D.
24	4.1	0.39	0.045

(a) Assayed with 60 μM, 5 μM and 0.5 μM cyclic AMP as substrate.
(b) Values presented are averages of 2-4 determinations which varied not more than ± 10%.
(c) Not done.

observed in G1-arrested cells. Mock-infection did not significantly change the activity of the enzyme.

The activity of cyclic AMP phosphodiesterase in G1-arrested BHK21 cells at different times after infection with Ad12 was determined using three different concentrations of cyclic AMP substrate (Table 1). It is evident that no significant change in the activity of the enzyme was observed until 13 h.p.i. with all three tested concentrations of the substrate in the incubation mixture. At 19 h.p.i. and later, the decrease in activity of the enzyme was detected in all cases.

DISCUSSION. Ad12 infection of G1-arrested BHK21 cells leads to a release

from contact inhibition and to a progression through the mitotic cycle. It has been proposed that contact inhibition is mediated by an increase in intracellular concentration of cyclic AMP (11). Loss of contact inhibition is one of the important characteristics of transformed cells which are reported to have lower concentrations of cyclic AMP than their parental fibroblasts (11, 12). It is therefore possible that an early event in transformation might lead to lower than normal cyclic AMP levels.

The data presented here indicate that abortive infection with Ad12 results in reduction of cyclic AMP concentration in G1-arrested BHK21 cells. This result is in good agreement with the observation that addition of dibutyryl-cyclic AMP would bypass or prevent the virus-induced change in concentration of cyclic AMP and most cells would remain in a G1-arrested state (5). It remains to be determined what is the relation of the observed changes in cyclic AMP metabolism to cell killing under these conditions.

It was previously demonstrated that infection of cells with Rous sarcoma virus (RSV) leads to a marked drop in cyclic AMP concentration. An elegant experiment using temperature sensitive mutant of RSV indicates that this decrease in cyclic AMP level is related to transformation of cells by the virus and not to the virus replication, *per se* (13).

The decreased activity of adenyl cyclase in Ad12 infected cells fits well the earlier report that levels of the enzyme are lower in BHK21 cells transformed with polyoma virus (14).

The synthesis of cyclic AMP phosphodiesterase is regulated by intracellular concentrations of cyclic AMP which functions as an inducer of the enzyme (15). It is therefore logical to detect a decrease of cyclic AMP phosphodiesterase activity after reduction of intracellular level of cyclic AMP has occurred.

The results presented here suggest that the reduction of intracellular concentration of cyclic AMP may possibly be mediated by a decrease in activity of adenyl cyclase.

The timing of changes in cyclic AMP metabolism in that the first significant changes occur only after 7.5 h.p.i. is in good correlation with an observation that inhibitory effects of dibutyryl-cyclic AMP are undiminished when the drug is added as late as at least 6 h.p.i. (5). The changes in the level of cyclic AMP and activity of adenyl cyclase occur only after Ad12 mRNA appears in the infected cells: under conditions used in this report, no Ad12 mRNA was detected in BHK21 cells prior to 7 h.p.i. (1). This suggests that the observed changes in cyclic AMP metabolism are related to expression of the viral genome rather than to virus effects on the cell membrane during adsorption.

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