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Effects of Cyclic Adenosine 3′,5′-Monophosphate and Insulin on Plasma Cell Tumor (MPC-11) in Culture[1] (39034)

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Exogenous cyclic adenosine 3′,5′-monophosphate (cAMP) inhibits the growth of plasma cell tumor (MPC-11) in culture and the inhibition is entirely prevented by the addition of insulin to the culture medium (1). Opposite effects of cyclic AMP and insulin have been described in a number of non-neoplastic cells (2–6), a finding which prompted the search for biochemical effects altered in opposite directions by these two agents.

Materials and methods. Cyclic AMP, N^6-O^2-dibutyryl adenosine 3′,5′-cyclic monophosphate (DBcAMP) and 5′-adenosine monophosphate (AMP) were purchased from Sigma Chemical Company, adenosine from Schwarz/Mann, crystalline zinc porcine insulin (containing 0.002% glucagon) was a gift from Lilly Research Laboratory. Denatured insulin was prepared as described by Lotten and Sneyd (7). ^{14}C-thymidine was purchased from Schwarz/Mann, ^{3}H-leucine and ^{14}C-inulin were obtained from New England Nuclear. The purity of all nucleotides and nucleosides was checked by thin-layer chromatography in several systems.

Incubation conditions. MPC-11 cells, which have been in culture for many months, were used in these experiments. The culture and growth characteristics have been described previously (1). Cells were washed with Dulbecco's modified Eagle's medium without serum, and resuspended in regular growth medium at a density of 2×10^6 cells/ml. Incubations were carried out in 25 ml siliconized Erlenmeyer flasks in 5% CO_2, 95% air at 37° in a PsycroTherm incubator shaker (80 rpm) for 60 min. Each incubation flask contained 5.0 ml of suspension and 0.5 ml Hanks' Balanced Salt Solution (HBSS) or other indicated additions. Incubations were carried out for 60 min prior to addition of tracers (0.2 μCi/ml ^{14}C-thymidine, 0.5 μCi/ml ^{3}H-leucine, or 0.1 μCi/ml ^{14}C-inulin and continued for 30 min thereafter.

After incubation the cells were centrifuged, washed three times with HBSS and resuspended in 5.0 ml HBSS. Results are expressed in terms of the total viable cells in this suspension. Viability in control and treated flasks was always greater than 95% (Trypan blue exclusion). No significant effect of insulin on cell density after incubation was seen. The washed cells were centrifuged and homogenized in 0.6 ml 10% TCA with a Duall glass homogenizer (Kontes). The acid-soluble fraction was removed and the acid-insoluble pellets were washed three times with 10% TCA. The pellets were dissolved in 0.6 ml of NCS solubilizer (Nuclear Chicago). Radioactivity of the acid-soluble fraction was determined in Toluene–Triton X114, the solubilized acid-insoluble fraction in Bray's scintillation mixture (8). Radioactive transport and incorporation of thymidine and leucine by plasma cell tumor was linear for at least 60 min.

Results. Table I shows that incubation of MPC-11 with 0.05–0.3 mM cAMP for 90 min markedly increased the thymidine radioactivity in the acid-soluble fraction, while no significant effects were observed when cAMP concentration was reduced to 0.01 m*M* (not shown). In spite of the increased thymidine pool, cAMP-treated cells showed no increased incorporation into the acid-insoluble fraction, except at the 0.1 m*M* level. In order to determine whether insulin could antagonize this action of cAMP, we studied nucleoside uptake in cells treated with cAMP and insulin together (Table I). The highly significant increase in acid-

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TABLE I. EFFECT OF CONCENTRATION OF cAMP AND INSULIN ON THYMIDINE UPTAKE.[a]

Treatment	Acid-soluble		Acid-insoluble	
	Control (%)	*P* versus Control	Control (%)	*P* versus Control
Control	100	—	100	—
0.3 m*M* cAMP	184. ± 3.5	<0.005	106 ± 4.6	NS
0.1 m*M* cAMP	171 ± 2.4	<0.005	145 ± 1.0	<0.025
0.05 m*M* cAMP	140 ± .15	<0.05	114 ± 4.9	NS
0.1 m*M* cAMP + 1 mU/ml insulin	110 ± 3.4	NS	110 ± 2.5	NS
+ 10 mU/ml insulin	116[b]	NS	100 ± 2.5	NS
+ 100 mU/ml insulin	106 ± 1.8	NS	102 ± 2.1	NS
+ 100 mU/ml Denatured insulin	157 ± 6.1	<0.01	137 ± 2.0	<0.005

[a] Cells treated with cAMP alone or with varying concentrations of insulin incubated at 37° for 90 min then ^{14}C-thymidine added to 3.5×10^{-6} *M* for 30 min more. Data represent acid-soluble and insoluble radioactivity in washed cell. Insulin alone at the concentrations tested had no significant effect. Each value represents mean ± SEM of three experiments. Control radioactivity in acid-soluble fraction was $1.50 \pm 0.14 \times 10^5$ dpm/10^8 cells. Control radioactivity in the acid-insoluble fraction was $1.26 \pm 0.04 \times 10^6$ dpm/10^8 cells.

[b] Single value.

soluble and insoluble radioactivity produced by incubation with 0.1 m*M* cAMP was entirely prevented by addition of 1–100 microunits/ml insulin had no such effect. Insulin alone at the same concentration did not exert any effect on thymidine metabolism by tumor cells.

Table II shows the effect of cAMP on incorporation of leucine into the acid-insoluble fraction of incubated cells. No effects were seen on the soluble fraction, it was not studied further nor were the negative data inserted into a table. cAMP, 0.1 m*M*, significantly enhanced the incorporation of leucine into the acid-insoluble fraction (Table II). Again, the highly significant increase by 0.1 m*M* cAMP on leucine incorporation was completely prevented by 10 and 100 mU/ml insulin, while 1 mU/ml reduced, but did not prevent the cAMP effect. Denatured insulin was inactive in this system. Table III shows that the addition of insulin without cAMP to the incubation mixture increased the incorporation of leucine, whereas denatured insulin had no such effect. This experiment shows that the prevention of cAMP-induced enhancement of incorporation cannot be attributed to inhibition on the part of insulin alone.

0.05–0.3 m*M* DBcAMP and AMP were tested in experiments designed to measure the nucleotide effect on ^{14}C-thymidine and ^{3}H-leucine entry into MPC-11. Neither AMP nor DBcAMP enhanced the labeling of either acid-soluble or insoluble fractions. However, Table IV shows that adenosine stimulated the uptake of ^{14}C-thymidine and the incorporation of ^{3}H-leucine, but insulin did not significantly lower the labeling of the acid-soluble and insoluble fraction of thymidine radioactivity. The increase in entry of leucine was deemed too small to study further.

TABLE II. EFFECT OF cAMP AND INSULIN ON LEUCINE INCORPORATION.[a]

Treatment	Control (%)	*P*
Control	100	—
0.1 m*M* cAMP	155 ± 2.5	0.001
0.05 m*M* cAMP	142 ± 5.1	0.01
0.1 m*M* cAMP + 100 mU/ml Insulin	94 ± 3.2	N.S.
+ 10 mU/ml Insulin	100 ± 1.2	N.S.
+ 1 mU/ml Insulin	113 ± 2.3	0.05
+ 100 mU/ml Denatured Insulin	138 ± 4.5	0.01

[a] Incubation with cAMP alone or with varying concentrations of insulin for a total of 90 min; during the final 30 min ^{3}H-leucine was present at 0.5 μCi/ml. Leucine at 8×10^{-4} *M* was present in the culture medium throughout the incubation. The data represent acid-insoluble radioactivity in washed cells from three experiments. Coltrol radioactivity was $6.14 \pm 0.09 \times 10^5$ dpm/10^8 cells.

^{14}C-inulin was incubated with the tumor cells in experiments analogous to those described above. Insignificant radioactivity was present in the washed cells in either the control, insulin or cAMP treated samples. In none of the experiments described was there a significant decrease in trypan blue exclusion after incubation with cAMP. These results exclude nonspecific cell damage by cyclic nucleotide as a basis for the observed transport changes.

Discussion. Insulin reversal of cAMP-mediated growth inhibition of MPC-11 was interpreted as evidence for the existence of a control system in a tumor cell similar to those found in a number of other studies (2–6).

TABLE III. EFFECT OF INSULIN ON INCORPORATION OF LEUCINE.[a]

	Control (%)	*P* (from control)
Control	100	
100 mU/ml insulin	121 ± 4.7	0.05
10 mU/ml insulin	128 ± 2.6	0.005
1 mU/ml insulin	120 ± 3.7	0.025
100 mU/ml denatured insulin	99 ± 3.1	NS

[a] Conditions were identical to those in Table II, except that cAMP was omitted. Each value represents mean ± SEM of three experiments.

Thymidine incorporation studies were planned to investigate cAMP growth control. We were surprised to learn that although cAMP inhibited growth, thymidine incorporation was significantly increased by cAMP at 0.1 m*M*, but not at 0.05 or 0.3 m*M* concentration. The effect of cAMP on thymidine incorporation may invovle more than one factor, since all but the lowest concentrations of cAMP enhanced entry of thymidine into the acid-soluble fraction, so that the specific activity of precursor material must have been materially increased. Kaukel *et al.* (9) found that cAMP increased thymidine incorporation in HeLa cell cultures although net DNA synthesis was decreased. Roller *et al.* (10) also found an increase in uptake of thymidine into the nucleotide pool of monkey kidney cells incubated with cAMP. Kaukel (9) found opposite effects of cAMP and DBcAMP on thymidine incorporation into HeLa cells, although in the present system the dibutyryl derivative gave no significant effect. Burger and Knyscynski (11) demonstrated that 1 m*M* cAMP produced increased incorporation of ^{3}H-thymidine into nucleic acid of normal spleen and marrow cells as well as leukemic thymic cells. No effect with DBcAMP and AMP was observed, just as we found in the present day study. Both Burger and Kaukel (11, 9) noted that 1 mM cAMP produced increased

TABLE IV. EFFECT OF ADENOSINE AND INSULIN ON THYMIDINE AND LEUCINE UPTAKE BY MPC-11.[a]

	Acid-Soluble		Acid-Insoluble			
	^{14}C-thymidine		^{14}C-thymidine		^{3}H-leucine	
Treatment	% Control	*P*	% Control	*P*	% Control	*P*
0.1 m*M* adenosine	126 ± 2.8	.005	113 ± 1.5	.025	—	
0.1 m*M* adenosine + 100 mU/ml insulin	118 ± 4.7	.025	109 ± 2.2	.05	—	
0.05 m*M* adenosine	124 ± 2.3	.025	124 ± 5.4	.025	108 ± 6.7	.05
0.05 m*M* adenosine + 100 mU/ml insulin	114 ± 5.4	.025	120 ± 1.8	.01	N.D.	—

[a] Incubation with adenosine for total of 90 min: leucine at 8×10^{-4} *M* was present in culture medium throughout the incubation. During the final 30 min, ^{3}H-leucine 0.5 μCi/ml or ^{14}C-thymidine 3.5×10^{-6} *M* was present. Addition of 100 mU/ml insulin without adenosine had no effect. Control radioactivity in acid-soluble and insoluble fractions of thymidine was $1.28 \pm 0.23 \times 10^{5}$ dmp/10^{8} cells and $5.68 \pm 0.51 \times 10^{5}$ dpm/10^{8} cells respectively and control for leucine incorporation was $6.61 \pm 0.31 \times 10^{5}$ dpm/10^{8}. The 0.1 m*M* adenosine experiment represents the mean of three flasks and the 0.05 m*M* adenosine conc. experiment represents the mean of six flasks. The *P* value between adenosine treatment and adenosine + 100 mU insulin treatment is not significant.

incorporation of ^{14}C-leucine into HeLa, normal and leukemic thymic cells. In the present system a very clear increase in incorporation of leucine is noted. The experiments with adenosine require further study. Kaukel (9) also showed some activity of adenosine on stimulation of thymidine incorporation into HeLa cultures. The effect of cAMP might result from degradation to AMP and adenosine. AMP is completely ineffective, while adenosine shows qualitatively the same effects as that of cAMP. However, insulin had different effects on cAMP and adenosine stimulation of thymidine uptake. In addition, cAMP and adenosine do not share a common transport carrier, since adenosine itself does not inhibit the transport of cAMP (manuscript in preparation). In view of these findings, it can be concluded that the mechanism of cAMP and adenosine action must be fundamentally different. The concentration of insulin required to affect the tumor cells is higher than that found in plasma. Perhaps insulin resembles peptides in plasma which control or moderate cAMP transport effects. The unique feature of these studies lies in the description of cAMP effects in tumor cells by insulin.

1. Naseem, S. M., and Hollander, V. P., Cancer Res. **33,** 2909 (1973).
2. Exton, J. H., Jefferson, L. S., Jr., Butcher, R. W., and Park, C. R., Amer. J. Med. **40,** 709 (1966).
3. Glinsmann, W. H., and Mortimore, G. E., Amer. J. Physiol. **215,** 553 (1968).
4. Goodman, H. M., Proc. Soc. Exp. Biol. Med. **130,** 97 (1969).
5. Manganiello, V. C., Murad, F., and Vaughan, M., J. Biol. Chem. **246,** 2195 (1971).
6. Rozengurt, E., and DeAsua, L. J., Proc. Nat Acad. Sci. U.S.A. **70,** 3609 (1973).
7. Lotten, E. G., and Sneyd, J. G. T., Biochem. J. **120,** 187 (1970).
8. Stevens, J., Stevens, Y. W., and Hollander, V. P., Cancer Res. **33,** 370 (1973).
9. Kaukel, E., Fuhrmann, U., and Hilz, H., Biochem. Biophys. Res. Commun. **48,** 1516 (1972).
10. Roller, B., Hirai, K., and Defendi, V., J. Cell. Physiol. **83,** 163 (1974).
11. Burger, M., and Knyscynski, A., Hoppe, Seyler's Z. Physiol. Chem. **352,** 1019 (1971).

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