A COMPARATIVE STUDY ON THE METABOLISM OF N^6,$O^{2'}$-DIBUTYRYL ADENOSINE 3',5'-MONOPHOSPHATE IN VARIOUS RAT HEPATOMA CELL LINES IN CULTURE

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1. Introduction

Studies with four rat hepatoma cell lines revealed that although N^6,$O^{2'}$-dibutyryl cyclic AMP (DBcAMP) inhibited the growth and caused a substantial increase in certain enzyme activities of Reuber H35 and MH_1C_1 cell lines, DBcAMP was only marginally active in HTC and RLC cells [1,2].

This report deals with a study of the metabolism of DBcAMP in the hepatoma cell lines MH_1C_1 Reuber H35, HTC and RLC in order to find out if a different metabolism of DBcAMP in HTC and RLC cells compared with MH_1C_1 and Reuber H35 cells, is responsible for the insensitivity of both cell lines towards DBcAMP. From the results it is concluded that the concentrations of the active compounds, i.e., N^6-monobutyryl cyclic AMP (N^6-MBcAMP) and cyclic AMP, in HTC and RLC cells are quite similar to the concentrations measured in MH_1C_1 and Reuber H35 cells, although some minor differences in the metabolism of DBcAMP were observed between the various cell lines.

Moreover, it is shown that a more complicated breakdown of DBcAMP occurred in the four hepatoma cell lines than has been published recently for HTC and RLC cells [3].

2. Materials and methods

2.1. *Cell growth*

All the rat hepatoma cell lines, MH_1C_1, Reuber H35, HTC and RLC, were seeded at a density of 10^6 cells/flask and were grown in monolayer culture as described previously [2].

Experiments were performed during the late logarithmic phase of growth. The final change of medium was twelve hours before the start of the experiments.

2.2. *Uptake and metabolism of ^{3}H-labelled DBcAMP in monolayer cultures*

Cells were incubated with 0.5 mM [^{3}H]DBcAMP (spec. act. 5866 dpm/nmol) and harvested as described [2]. The amount of cell associated radioactivity at 60 and 120 min of incubation was corrected for the amount of radioactivity absorbed to the cell surface by subtraction of the amount of radioactivity measured at zero time. Analysis of the radioactivity was performed by descending paperchromatography on Whatman 1 MM paper, using a solvent system of ethanol/0.5 M ammoniumacetate (5 : 2, v/v) [4]. Chromatograms were cut into 1 cm strips and counted in a Mark II liquid scintillation counter. Radioactivity in the medium was analyzed in the same way. Standards were run to locate the positions of the various components. Protein was measured according to Lowry et al. [5]. The monobutyryl cyclic AMP spots on the paperchromatograms were cut out, eluted with water and treated with 0.1 N NaOH for 10 min and rechromatographed to determine the percentage of $O^{2'}$-MBcAMP and N^6-MBcAMP [6].

2.3. *Measurement of intracellular concentrations of cyclic AMP*

After incubation of the monolayers with 0.5 mM DBcAMP for two hours the medium was poured off,

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Table 1

Distribution of radioactivity during incubation of various hepatoma cell lines with 0.5 mM [^{3}H]DBcAMP

Cell line	Incubation time (min)	ATP + ADP	AMP	Unidentified	Cyclic AMP N^6-MBAMP $O^{2'}$-MBAMP $N^6,O^{2'}$-DBAMP	A	N^6-MBcAMP $O^{2'}$-MBcAMP	N^6-MBA $O^{2'}$-MBA	$N^6,O^{2'}$-DBA
		(nmoles per milligram of protein)							
		R_F = 0.03	R_F = 0.13	R_F = 0.21	R_F = 0.33	R_F = 0.50	R_F = 0.65	R_F = 0.77	R_F = 0.90
MH_1C_1	60	0.20	–	–	0.18	–	0.14	0.06	0.12
	120	0.63	0.10	0.08	0.35	0.04	0.22	0.06	0.21
Reuber H35	60	0.07	–	0.02	0.07	0.02	0.04	0.05	0.12
	120	0.15	0.07	0.04	0.17	0.05	0.09	0.08	0.21
HTC	60	0.04	0.02	0.02	0.05	–	0.06	0.05	0.16
	120	0.08	0.03	0.04	0.13		0.13	0.08	0.29
RLC	60	0.02	0.02	0.04	0.05	–	0.13	0.05	0.12
	120	0.11	0.04	0.04	0.18	–	0.22	0.08	0.17

Cells were grown for 60 h. Then the medium was changed and 12 h later 0.5 mM [^{3}H]DBcAMP was added. At various incubation times the cells were harvested and the radioactivity analyzed as described under Materials and methods. The results shown were obtained in one representative experiment. The same pattern was observed in two additional experiments, although the actual composition varied depending on the cell preparation used. MBAMP = monobutyryl adenosine monophosphate, A = adenosine, DBA = dibutyryl adenosine, MBA = monobutyryl adenosine, – = absent or non detectable.

the cells were quickly washed five times with ice-cold saline and 0.5 ml of 0.5 N perchloric acid was added.

The cells were scraped, centrifuged and the sediment was dissolved in 1 N NaOH for protein estimation. The supernatant was neutralized with KOH, centrifuged and the resulting precipitate was discarded. The remaining supernatant was concentrated. Part of it was applied to Whatman 3 MM and chromatographed with the solvent system of ethanol/0.5 M ammonium-acetate (5 : 2, v/v). The cyclic AMP area was eluted with water and concentrated. The amount of cyclic AMP was measured in these samples by the binding assay described by Gilman [7]. Samples treated with purified beef heart phosphodiesterase served as controls.

2.4. *Chemicals*

[8-^{3}H]DBcAMP was from the Radiochemical Centre, Amersham, England. All nucleotides, cyclic nucleotides and the enzymes acid phosphatase and beefheart phosphodiesterase were obtained from Boehringer, Mannheim, Germany.

3. Results

Table 1 shows the amounts of the radioactive compounds (calculated as nmol per mg protein based on the specific activity of [^{3}H]DBcAMP) after 60 and 120 min of incubation with 0.5 mM [^{3}H]DBcAMP. No intracellular increase in DBcAMP could be detected during the incubation period. However, during the incubation period the amount of intracellular MBcAMP increased.

Analysis of the composition of MBcAMP after one hour of incubation revealed that in the four cell lines the increase was due predominantly to N^6-MBcAMP. Since this compound was not detected in the culture medium (neither in the presence nor in the absence of the hepatoma cells), this indicates an intracellular production of N^6-MBcAMP. Concomitant with the appearance of N^6-MBcAMP there is also formation of other compounds, which are not detectable in the culture medium. The amount of radioactivity at the spot with R_F = 0.33 reached a level of about 0.17 nmol/mg protein after two hours of incubation with exception of the MH_1C_1 cell line. Since this fraction contains cAMP, analogous experiments were performed in which the total cyclic AMP content was determined in this fraction of R_F = 0.33 of control cells and of cells treated with DBcAMP for two hours. From the results in table 2 it appears that the amount of cyclic AMP is slightly increased in DBcAMP treated cells indicating that not more than 5% of the radioactivity found at the R_F = 0.33 area could be cyclic AMP.

It appears from table 1 that there are three peaks of radioactive compounds (R_F = 0.33, 0.77 and 0.90), which are not produced by the known degradation of DBcAMP via deacylases, which results in MBcAMP and cyclic AMP and ultimately (via the action of cyclic AMP phosphodiesterase) to the adenosine phosphates.

These peaks were found in the cellular fraction and neither one was detected in the medium.

According to the investigations of O'Neill et al. [8] the appearance of these particular peaks indicates that DBcAMP in the four hepatoma cell lines is also partly degraded by a direct attack of the cyclic AMP phosphodiesterase on butyryl compounds.

In order to substantiate these findings the degradation of 0.5 mM [^{3}H]DBcAMP by extracts of MH_1C_1, Reuber H35, HTC and RLC cells was studied. After various incubation times samples were taken and the radioactivity was analyzed after deproteinisation by paperchromatography. Analysis of the radioactivity which cochromatographed with MBcAMP showed that during the incubation in all four cell line-extracts N^6-MBcAMP accumulated. Since no formation of $O^{2'}$-MBcAMP was found this indicated a relatively

Table 2
The effect of incubation with 0.5 mM DBcAMP for two hours on the cyclic AMP content of cultured hepatoma cells

Cell line	Cyclic AMP (pmol/mg protein)	
	Control	DBcAMP treated
MH_1C_1	3.1	6.4
Reuber H35	2.5	7.9
HTC	2.2	8.9
RLC	2.2	8.2

Incubation conditions and determinations of cyclic AMP were as described under Materials and methods. The values presented are the averages of three determinations in one particular experiment which agreed within 5%. The actual values differ from one experiment to another.

high $O^{2'}$-butyrylesterase activity and a relatively low or totally absent N^6-butyrylamidohydrolase activity [9]. Incubation with acid-phosphatase of the radioactivity which cochromatographed with cyclic AMP (R_F = 0.33) followed by rechromatography showed that only a small percentage of the total amount was resistant to degradation by the enzyme and thus presumably cAMP [8]. Treatment of the R_F = 0.33 fraction with 0.1 N NaOH for 60 min followed by rechromatography, resulted in a partial shift of radioactivity to the position of AMP which indicated the presence in the original fraction of $O^{2'}$-MBAMP, which is completely degraded to AMP by alkali treatment. The other part of the R_F = 0.33 fraction minus cyclic AMP is presumably a mixture of DBAMP and N^6-MBAMP, because the latter is almost completely resistant to alkali treatment.

The presence of compounds like N^6-MBAMP and $O^{2'}$-MBAMP is supported by the fact that degradation of DBcAMP by the cell line-extracts resulted in the appearance of radioactive spots with R_F values of 0.77 and 0.90 indicating a direct attack of cyclic AMP phosphodiesterase in the cell extracts on MBcAMP and DBcAMP [8]. No important differences in the in vitro degradation of [^{3}H]DBcAMP by the four cell extracts resulting in MBA and DBA were observed.

4. Discussion

In contrast to a recent report by Granner et al. [3] the metabolism of DBcAMP in hepatoma cells appeared to be more complicated. It seems to be similar to the degradation as observed by O'Neill et al. [8] in CHO-cells. In all four cell lines compounds which are presumed to be active in cyclic AMP regulated processes (i.e. N^6-MBcAMP and cyclic AMP) were observed. Therefore, further experiments will be performed to explain the low response of DBcAMP on growth and certain enzyme activities in the fast growing HTC and RLC cell lines.

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