Vol. 110, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 27, 1983 Pages 688-693

REPLICON INITIATION FREQUENCY AND INTRACELLULAR LEVELS OF ATP, ADP, AMP AND OF DIADENOSINE 5',5'''-P^1,P^4-TETRAPHOSPHATE IN EHRLICH ASCITES CELLS CULTURED AEROBICALLY AND ANAEROBICALLY

Hans Probst, Klaus Hamprecht and Volker Gekeler

Physiologisch-chemisches Institut der Universität Tübingen

Hoppe-Seyler-Straße 1, D-7400 Tübingen, Germany

Received December 10, 1982

SUMMARY: When Ehrlich ascites cells were cultured for 2 h under oxygen-free atmosphere, a shut-down of inititation of new replication units was observed by chain length analysis of the nascent daughter strands and by DNA fibre autoradiography. The intracellular level of ATP, ADP and AMP remained virtually normal in the anaerobized cells, while that of diadenosine 5',5'''-P^1,P^4-tetraphosphate was found reduced by about two orders of magnitude. It is proposed that the ceasing of DNA synthesis after O_2 removal is an actively controlled regulatory response of the cells in which diadenosine 5',5'''-P^1,P^4-tetraphosphate is probably involved.

In this communication we report on first results of our search for the intracellular factors involved in the suppression of DNA synthesis in Ehrlich ascites cells after withdrawal of O_2 (1, 2). According to the results of previous work this suppression most probably happens by a shut-down of the initiation of new replication units (3). In order to find out the causes of this shut-down we examined the energetic situation of the cells by measuring the intracellular levels of ATP, ADP and AMP. Then, we extended our studies to the determination of diadenosine 5',5'''-P^1,P^4-tetraphosphate (Ap_4A). This compound seemed to be of particular interest in the present context: Its intracellular level was found to be correlated with the proliferative activity of cultured cells (4). It triggered the initiation of DNA synthesis in cells made permeable in the growth-arrested phase of the cell cycle (5). It bound to a subunit of DNA polymeraseα (6, 7) and it primed DNA

0006-291X/83/020688-06$01.50/0

synthesis by this enzyme in vitro with a double stranded octadecamer as a template (8).

Primarily, we confined ourselves to study the cells at 2 h after the beginning of the O_2-deprivation. The suppression of the replicon initiation, occuring under these conditions, was monitored by two independent methods: Alkaline sucrose gradient centrifugation, as mentioned above, and DNA fibre autoradiography.

The results indicated that the shut-down of the replicon initiation occured while the ATP, ADP and AMP levels were virtually normal. The level of intracellular Ap_4A, however, was drastically diminished.

MATERIALS AND METHODS: The ascites cells and their growth in vivo and in vitro have been described (9). For experimentation we explanted 4 - 5 x 10^5 cells/ml from the logarithmic growth phase of the in vivo tumor (4 days after inoculation (9)) into culture medium of 37°C. The establishment of anaerobic conditions by means of an argon/5% CO_2 atmosphere is described in (3). All experiments of this work were performed 2 h after switching to argon/CO_2. Aerobic control cultures grew for the same time in normal air. This duration was chosen because prior experiments (3) had suggested a pronounced but not yet completed shut-down of replicon initiation at this time (2 h).

ATP/ADP/AMP in the cells were determined by bioluminometry depending on published methods (see ref.(10) for a survey). We obtained the most reliable results using the following procedure: Duplicate 0.5 ml cell culture aliquots were pipetted into 4.5 ml of boiling 50 mM Tris-acetate, 4 mM EDTA pH 7.75. Subsequently, 5 nMol ATP (Boehringer-Mannheim) in 50 µl H_2O were added to one of the two samples as a standard. The samples were kept boiling for 3 min and then rapidly chilled to -20°C. After thawing between 0 and +2°C 100 µl aliquots of each sample were mixed with (i) 100 µl of 25 mM HEPES, 10 mM KCl, 7 mM $MgCl_2$, 1 mM EDTA pH 7.75 (adjusted with KOH), (ii) 3.5 mM Na-phosphoenolpyruvate (Boehringer-Mannheim) and 2 U/ml pyruvate kinase (EC 2.7.1.40 from rabbit muscle, Boehringer-Mannheim) in the same buffer and, (iii) the same mixture as (ii) containing additionally 40 U/ml of myokinase (EC 2.7.4.3. from pig muscle, Boehringer-Mannheim). After incubating these three mixtures for 30 min at 30°C, 20 µl of them were added to 180 µl of a mixture of 40 µl ATP Monitoring Reagent 1243-200 (LKB-WALLAC) and 140 µl of 50 mM Tris-acetate buffer pH 7.75. Subsequently, the luminscence signal was measured at 25°C in polystyrene cuvettes in an LKB-Wallac Luminometer 1250 equipped with a chart recorder. The signals obtained were constant for several minutes and proportional to the ATP content in the final sample ranging between 10^{-16} and 10^{-10} Moles per sample. The measured signals were converted to amounts of ATP, ADP and AMP by means of the signal differences between the samples derived from the standardized and non-standardized cell extraxts.

Ap_4A was also determined by bioluminometry. We used the procedure of F.Grummt and coworkers (11) which relies on the same principle

as the method recently published by Ogilvie (12) but includes an additional digestion of ATP by alkaline phosphatase: About 5 x 10^7 cells were collected by centrifugation at 37°C (5 min 1000 x g). In the case of anaerobized cells the oxygen exclusion was maintained during centrifugation. The cell pellet was rapidly chilled to 0°C and then suspended in 10 ml of 5% cold formic acid. After centrifugation (15 min, 10000 x g, 0 - 3°C) the cell pellet was extracted again with another 4 ml of 5% HCOOH. The combined extracts were lyophilized, redissolved in 2 ml H_2O and mixed with 10 µl of 1 M Tris-base, 20 µl of 0.1 M $MgCl_2$ and 20 µl of 0.01 M $ZnCl_2$. The resulting pH was between 7.5 and 8.0. This mixture was incubated for 6 h at 37°C with 1.5 U of alkaline phosphatase. The enzyme (EC 3.1.3.1. from calf intestine, Boehringer-Mannheim) was freed from phosphodiesterase traces by chromatography on Sephadex G75. The digest was diluted with 20 ml of 0.12 M $(NH_4)HCO_3$ (pH 7.85) and applied to a 0.8 x 13 cm column of DEAE cellulose (Whatman DE 52) equilibrated with the same buffer. After washing the column with further 50 ml of the 0.12 M buffer, Ap_4A was eluted with 30 ml of 0.25 M $(NH_4)HCO_3$ (pH 8.0). The eluate was lyophilized and redissolved in 1 ml H_2O. 20 µl aliquots were mixed with 135 µl of 50 mM Tris-acetate (pH 7.75) and 40 µl of ATP Monitoring Reagent. The background signal was recorded at 25°C for about 30 sec. Then, 0.5 U of snake venom phosphodiesterase (EC 3.1.4.1., Boehringer-Mannheim) in 5 µl of H_2O were added and the signal was recorded for further 5 - 8 min. The Ap_4A amount was derived from the luminiscence maximum reached 1 - 2 min after phosphodiesterase addition. The peak height was proportional to the Ap_4A content of the final sample in the range between 10^{-15} and 10^{-10} Moles per sample. For calculating the amount of Ap_4A in the cells two kinds of standards were added to the initial HCOOH extracts: (i) 5 nMoles of Ap_4A (Li-salt, Boehringer-Mannheim) were added to one of duplicate cell extracts in analogy to the above ATP measurements; (ii) in order to determine the yield of the Ap_4A separation for the luminometry assay, 1 - 2 pMoles of [^{3}H]-Ap_4A of high specific activity were added to each extract (also to the extracts containing the non-radioactive standard). The [^{3}H]-Ap_4A was a gift of F.Grummt and was re-purified in our laboratory by chromatography on DEAE cellulose (Whatman DE 52, elution by NH_4HCO_3 gradient). The yield of the Ap_4A separation was calculated from dpm-measurements obtained by liquid scintillation counting of aliquots of the HCOOH extracts and of the final lyophilized and redissolved samples using internal standardization.
The proportion of postpulse- to prepulse-initiation figures, obtained by DNA fibre autoradiography, was taken as a measure of the relative replicon initiation frequency. The step-down labeling protocol, already described in (13), was used with the following modifications: The specific radioactivity of the Me-[^{3}H]-thymidine batch (obtained from Amersham-Buchler) was 82 C/mMol and the duration of the "hot" and the "warm pulse" was diminished to 20 min. Patterns which displayed a clearly distinguishable hot-pulse and warm-pulse part were evaluated. Replicons initiated during the hot pulse were classified as "post-pulse". The analysis of the chain length distribution of the pulse labeled daughter strand DNA by alkaline sucrose gradient centrifugation was exactly performed as described in (3).

RESULTS AND DISCUSSION: Fig.1 displays that, 2 h after switching to argon/CO_2, the population of DNA chains which can be labeled by a 5 min pulse with [^{3}H]-dThd became already distinctly depleted

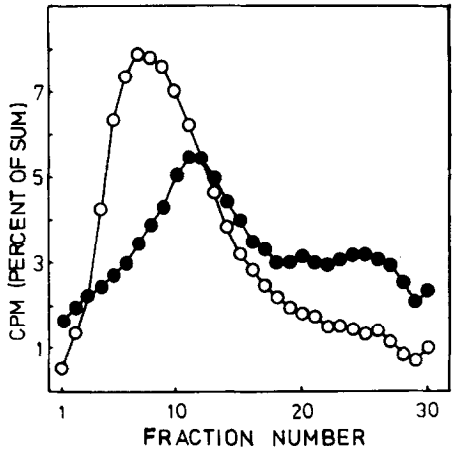

Fig. 1: Alkaline sucrose gradient centrifugation. A cell culture was divided and incubated aerobically or anaerobically resp. for 2 h and then labeled for 5 min with [^{3}H]-thymidine (20 µC/ml) under the respective conditions. Cell lysis on top of SW 27 gradients, centrifugation and calibration was performed as described. Sedimentation is from left to right. One fraction corresponds to 5.4 S. •–• = anaerobic cells (Σcpm = 25 300); o–o = aerobic cells (Σcpm = 34 400).

of molecules sedimenting below 50 - 60 S. By DNA fibre autoradiography it is confirmed impressively that this must be interpreted as a consequence of a severe reduction of the relative frequency of newly initiated replicons (table 1). The proportion of replicons initiated during the (hot) 20 min pulse to replicons initiated earlier and active during the pulse was found decreased by a factor of about 10 in relation to the aerobic control culture. (A detailed presentation of the influence of anaeroby on the parameters measurable by DNA fibre autoradiography will be published elsewhere.)

Table 1: DNA fibre autoradiography: Relation of post-pulse initiation patterns to pre-pulse initiation patterns. A cell culture was divided and incubated 2 h aerobically or anaerobically resp. and then labeled for DNA fibre autoradiography under the respective conditions and evaluated as described in Methods.

	post-pulse	pre-pulse	relation
aerobic culture (control)	196	144	1.36
anaerobic culture	22	134	0.16

Table 2: ATP/ADP/AMP and diadenosine 5',5'''-P',P⁴-tetraphosphate (Ap_4A) in cells cultured 2 h aerobically or anaerobically resp. *) AEC is the so-called adenylate energy charge as defined by Atkinson and Walton (14). According to (14), the AEC value represents the phosphorylating capacity of the cell with respect to the total adenylate system. It is calculated according to the formula

$$AEC = \frac{(ATP) + 0.5(ADP)}{(ATP)+(ADP)+(AMP)} .$$

AEC values between 0.80 and 0.85 mean that the energetic state of the cells is normal.

	n	ATP (nanomoles per million of cells)	ADP	AMP	AEC*	Ap_4A picomoles/10^6 cells
aerobic cultures	3	5.50 ±1.44	0.87 ±0.22	0.70 ±0.25	0.84 ±0.01	–
	1	4.65	0.61	0.66	0.84	7.13
	1	–	–	–	–	7.80
anaerobic cultures	3	5.09 ±0.96	0.51 ±0.17	0.73 ±0.19	0.85 ±0.04	–
	1	5.92	0.70	0.88	0.84	0.12

The ATP/ADP/AMP values compiled in table 2 indicate that this shut-down of replicon initation after 0 withdrawal occured at a virtually normal energetic state of the cells. Obviously, the anaerobic adenylate phosphorylation capabilities of the cells can completely substitute, in the present case, for the missing oxidative phosphorylation.

In further aerobic and anaerobic experiments we determined the intracellular level of Ap_4A besides ATP/ADP/AMP. We found that the energetic state of both kinds of cultures was normal and could not be distinguished from the other aerobic or anaerobic cultures. In the 2 h aerobically cultured cells we measured 7 - 8 picomoles of Ap_4A per 10^6 cells. This is in the same range as the value determined by Ogilvie (12), who found 4 pMol Ap_4A per 10^6 cells at an ATP content of 5.7 nMol/10^6 cells. However, after 2 h of anaerobic culturing, the Ap_4A level decreased to less than 2% of the above value. More detailed studies, which are in progress at this time and will be published elsewhere, indicate that, during prolonged aerobic as well as anaerobic cultur-

ing of the cells, the Ap_4A level increases significantly when compared to the value observed in the 2 h-cultures. The difference between aerobiosis and anaerobiosis, however, seems to remain essentially unchanged.

We conclude from the observations of this work that the suppression of replicon initiation after O_2 removal is rather a regulatory response, actively controlled by the cells, than simply a consequence of an impaired supply of energy. It seems possible that Ap_4A is part of the control system involved.

ACKNOWLEDGMENT

We are very grateful to F. Grummt for teaching us his method for measuring intracellular Ap_4A and for the gift of [^{3}H]-Ap_4A.

REFERENCES

1. Krause, H.P., Probst, H. and Schneider, Fr. (1971) Z.Naturforsch. 26b, 780-787.
2. Löffler, M., Postius, St. and Schneider, Fr. (1978) Virchows Arch.B.Cell Path. 26, 359-368.
3. Probst, H. and Gekeler, V. (1980) Biochem.Biophys.Res.Commun. 94, 55-60
4. Rapaport, E. and Zamecnik, P.C. (1976) Proc.Natl.Acad.Sci. USA 73, 3984-3988.
5. Grummt, F. (1978) Proc.Natl.Acad.Sci.USA 75, 371-375.
6. Grummt, F., Waltl, G., Jantzen, H.M., Hamprecht, K., Huebscher, U. and Kuenzle, C.C. (1979) Proc.Natl.Acad.Sci.USA 76, 6081- 6085.
7. Rapaport, E., Zamecnik, P.C. and Baril, E.F. (1981) Proc. Natl.Acad.Sci.USA 78, 838-842.
8. Zamecnik, P.C., Rapaport, E. and Baril, E.F. (1982) Proc. Natl.Acad.Sci.USA 79, 1791-1794.
9. Probst, H. and Maisenbacher, J. (1973) Exptl.Cell Res. 78, 335-344.
10. De Luca, M. and McElroy, W.D. (eds.) (1981) Bioluminiscence and Chemiluminiscence: Basic Chemistry and Analytical Applications, Academic Press, New York.
11. Grummt, F., personal communication
12. Ogilvie, A. (1981) Anal.Biochem. 115, 302-307.
13. Probst, H. Blütters, R. and Fielitz, J. (1980) Exp.Cell Res. 130, 1-13.
14. Atkinson, D.E. and Walton, G.M. (1967) J.Biol.Chem. 242, 3239-3247.