CYCLIC GMP METABOLISM IN *TETRAHYMENA PYRIFORMIS* SYNCHRONIZED BY A SINGLE HYPOXIC SHOCK

N. C. C. GRAY, J. R. DICKINSON and B. E. P. SWOBODA
Department of Molecular Sciences, University of Warwick, Coventry CV4 7AL, England

Received 11 July 1977

1. Introduction

Using selection synchronized cultures of *Tetrahymena pyriformis*, we have reported on the modulation of cyclic AMP and cyclic GMP in the natural cell cycle [1,2]. We have recently employed the technique of a single hypoxic shock to induce cell synchrony [3]. Comparison of cyclic nucleotide metabolism in this perturbed cell cycle system with the natural cell cycle enables identification of important control points in the cell cycle and an understanding of the roles of the cyclic nucleotide 'signals' involved.

Induction of division synchrony results in elevation of intracellular cyclic GMP. The increase in cellular cyclic GMP level was associated with an increase in guanyl cyclase and decreased cyclic GMP phosphodiesterase. Release from cell cycle blockade causes a fall in cyclic GMP and guanyl cyclase and an increase in cyclic GMP phosphodiesterase. The results suggest that either high intracellular GMP is a signal for inhibition of cell division, or that this pattern of modulation of cellular cyclic GMP reflects readjustment of the cell to the hypoxic conditions involved in the synchronization procedure. Cyclic AMP phosphodiesterase was assayed simultaneously with cyclic GMP phosphodiesterase. The two enzymes have different maximum activities at slightly different times immediately before the first synchronous division.

2. Materials and methods

2.1. *Synchronization of cells*

This technique has been described in detail elsewhere [3]. Essentially this involves growth of the cells at 28 ± 0.5°C in an orbital shaker at a rate of 180 rev/min to a cell density of approx. 2.0×10^5 cells/ml. Shaking of the cultures is then stopped for a period of 240 min (cessation of shaking causing hypoxia). After 240 min of hypoxia, the cultures are reshaken. After reshaking there is a lag period of about 105 min during which time no increase in cell density occurs. There is then a burst of synchronous division. The cell counting technique has been described previously [1,3].

2.2. *Extraction and assay of cyclic GMP*

The extraction and assay of cyclic GMP were performed as described previously [2] without modification.

2.3. *Assay of cyclic AMP phosphodiesterase*

Cyclic AMP phosphodiesterase was assayed as described previously [1].

2.4. *Assay of cyclic GMP phosphodiesterase*

Cyclic GMP phosphodiesterase was assayed as described previously [2], at a substrate concentration of 0.36 mM.

2.5. *Assay of guanyl cyclase*

Cells were harvested at 2800 × *g* for 5 min at growth temperature, washed with 25 mM Tris–HCl buffer, pH 7.4 (containing 0.25 M sucrose, 2 mM EDTA and 0.2 mM dithiothreitol), then resuspended to 1 ml in this buffer, cooled to 0°C and sonicated in a MSE 150 W sonicator at maximum power and amplitude for 15 s. This cell homogenate was then used immediately in the assay of guanyl cyclase which

comprised: 40 mM Tris–HCl, pH 7.4, 5 mM cyclic GMP, 10 mM $MnSO_4$, 10 mM theophylline, 1.9 mM [8-^{3}H]GTP (10 μCi/μmol), 25 mM phosphocreatine, 50 units/ml creatine phosphokinase and 0.04 ml sonicate in final vol. 0.1 ml. The reaction was terminated after 10 min at 30°C by rapid chilling to 0°C and addition of 0.6 ml ice-cold 5 mM EDTA, pH 7.5.

Protein was removed by centrifugation and the supernatant submitted to sequential chromatography (modified from Krishnan and Krishna [4]) on Dowex 1X8-400 (eluted with 2.0 M HCl) and neutral alumina (eluted with 40 mM Tris–HCl, pH 7.4). The product of this purification has been identified as cyclic GMP by hydrolysis with beef heart cyclic nucleotide phosphodiesterase (Boehringer Corp. (London) Ltd). Liquid scintillation counting was used to estimate the conversion of [8-^{3}H]GTP to cyclic GMP.

Incubation blanks (terminated prior to the addition of enzyme) contained less than 0.005% of total radioactivity. The recovery of cyclic GMP, which was 76.4 ± 3.2% in 8 experiments, was not affected by *Tetrahymena* homogenate containing up to 0.6 mg protein/assay.

3. Results and discussion

Figure 1 shows that induction of division synchrony results in the production of a plateau of intracellular cyclic GMP. After commencement of reshaking cyclic GMP falls to a minimum before cell division. The first synchronous division follows about 30 min after this minimum level of cyclic GMP. A 'spike' of intracellular cyclic GMP is found to coincide with the first division. Cellular cyclic GMP subsequently rises to another maximum 58 min after the onset of cell division. Intracellular cyclic GMP then falls to a new minimum immediately before the start of the second division. After this point cyclic GMP rises progressively.

After the start of hypoxia guanyl cyclase rises rapidly, and cyclic GMP phosphodiesterase quickly falls. The increased guanyl cyclase and decreased cyclic GMP phosphodiesterase activities are maintained at approximately the same levels throughout the 240 min hypoxic period. Upon reshaking guanyl cyclase activity falls to a minimum at 282 min and

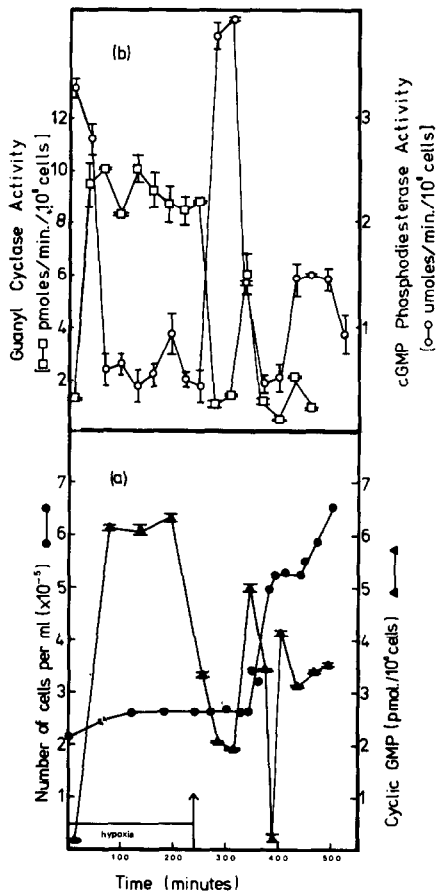

Fig.1. The time course of a typical culture of *Tetrahymena pyriformis* strain W synchronized by a 240 min hypoxic shock. The arrow indicates the point at which reshaking started. (a) Cell density and cyclic GMP measured per 10^6 cells. (b) Guanyl cyclase and cyclic GMP phosphodiesterase activities. These results represent the mean of at least two experiments.

cyclic GMP phosphodiesterase rises rapidly to a maximum at 312 min. These variations of guanyl cyclase and cyclic GMP phosphodiesterase can account for the observed fluctuations of intracellular cyclic GMP up to the first synchronous division. The subsequent pattern of modulation of guanyl cyclase is also consistent with the levels of intracellular cyclic GMP. We expected to see increased cyclic GMP phosphodiesterase immediately following the maximum level of intracellular cyclic GMP that coincides with the first synchronous division. Even

though cyclic GMP phosphodiesterase activity does rise shortly after the first synchronous division, it does not appear to rise sufficiently quickly to explain the rapid drop in cyclic GMP that is seen between 343 min and 384 min. This observation raises the possibility that cyclic GMP is excreted from the cell at this point in the cell cycle.The notion of excretion of cyclic GMP from the cell is not without precedent; indeed, this cyclic nucleotide was first found in rat urine [5]. Between 422 min and 492 min, cyclic GMP phosphodiesterase activity reaches a plateau and after 492 min cyclic GMP phosphodiesterase falls. This subsequent pattern of cyclic GMP phosphodiesterase activity is consistent with the observed levels of intracellular cyclic GMP.

The elevation of cyclic GMP within the cells associated with cell cycle blockade is an unexpected finding. We observed a broadly similar accumulation of cyclic AMP in hypoxic-shocked cells [3]. Previously it has been thought that cyclic AMP acts as a growth inhibitor and that cyclic GMP antagonizes the actions of cyclic AMP and promotes cellular growth [6,7]. Clearly our results are not in accordance with these ideas for it would appear that both cyclic AMP and cyclic GMP are elevated in the non-dividing hypoxic-shocked cells. Miller et al. have studied BA LB 3T3 cells and have reported that cyclic GMP rose as cell growth slowed and that addition of serum to resting cells caused a prompt fall in both cyclic GMP and cyclic AMP [8].

The 'spike' of intracellular cyclic GMP that coincides with the first synchronous division is similar to that we observed for cyclic AMP in both hypoxia-synchronized and selection synchronized cultures of *Tetrahymena pyriformis* [1,3]. Furthermore, we have observed that in selection synchronized *Tetrahymena pyriformis* cyclic GMP is highest during cell division and then declines progressively to its lowest level at a point immediately before cell division [2]. In this experiment cyclic GMP falls to minima which immediately precede the cell divisions. It is probable that cyclic GMP falls rapidly in hypoxia-synchronized *Tetrahymena pyriformis* after division, giving the characteristic 'spike' pattern observed because the cells have no GI phase in this cell cycle system [3]. If GI were present, the decline of cyclic GMP would presumably be more gradual as it is in the natural cell cycle [2].

The similarity in the patterns of cyclic AMP and cyclic GMP in hypoxia-synchronized *Tetrahymena pyriformis* led us to investigate the role of cyclic AMP phosphodiesterase and cyclic GMP phospodiesterase in this cell system. Figure 2 shows these two enzyme activities measured in the same synchronous culture. There were two distinct differences between these enzymes. Cyclic AMP phosphodiesterase activity remained approximately the same throughout the hypoxic period, whereas cyclic GMP phosphodiesterase falls dramatically within the first 72 min of hypoxia. Immediately following the reshaking, cyclic GMP

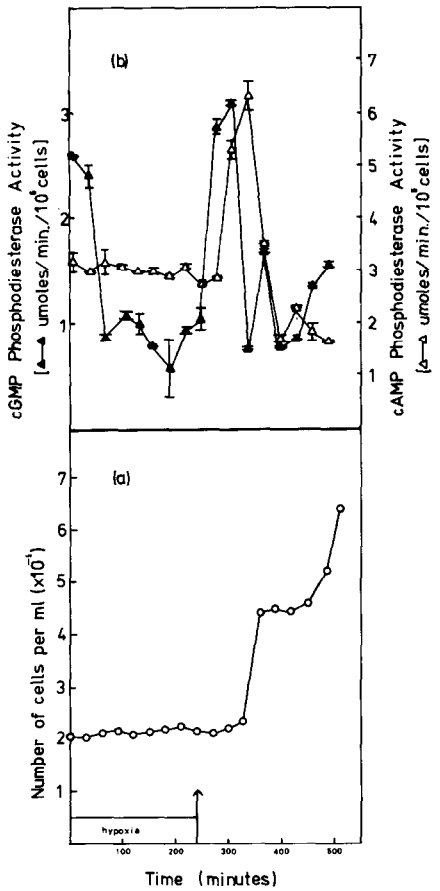

Fig.2. The time course of a typical culture of *Tetrahymena pyriformis* strain W synchronized by a 240 min hypoxic shock. The arrow indicates the point at which reshaking started. (a) Cell density. (b) Cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase activities. These results represent the mean of at least 4 experiments.

phosphodiesterase rises to maximum at 312 min. Cyclic AMP phosphodiesterase rises later than cyclic GMP phosphodiesterase (the cyclic AMP phosphodiesterase maximum is 30 min later at 342 min). The cyclic GMP phosphodiesterase maximum occurs at the very onset of cell division, whereas the cyclic AMP phosphodiesterase maximum is at the completion of cell division.

In the natural cell cycle of *Tetrahymena pyriformis* cyclic AMP phosphodiesterase is maximal before cyclic GMP phosphodiesterase, both occurring before cell division [1,2]. In hypoxia-synchronized cells the order of appearance of maximum activities of the two enzymes is reversed. Use of this synchronization technique represents a means of manipulating the two phosphodiesterases in order to explore their role in cyclic nucleotide control of the cell cycle. A further approach to this problem is to attempt a chromatographic separation of the two enzymes. Preliminary results from this laboratory indicate that the two enzymes can be physically separated by gel filtration on Sephadex G-200.

Acknowledgement

J. R. D. is grateful to the Medical Research Council for the award of a Research Studentship.

References

[1] Dickinson, J. R., Graves, M. G. and Swoboda, B. E. P. (1976) FEBS Lett. 65, 152–154.
[2] Graves, M. G., Dickinson, J. R. and Swoboda, B. E. P. (1976) FEBS Lett. 69, 165–166.
[3] Dickinson, J. R., Graves, M. G. and Swoboda, B. E. P. (1977) Eur. J. Biochem. in press.
[4] Krishnan, N. and Krishna, G. (1976) Anal. Biochem. 70, 18–31.
[5] Ashman, D. E., Lipton, R., Melicour, M. M. and Price, T. D. (1963) Biochem. Biophys. Res. Commun. 11, 300–334.
[6] Goldberg, N. D., O'Dea, R. F. and Haddox, M. K. (1973) in: Adv. Cyclic Nucleotide Res. 3, (Robinson, G. A. and Greengard, P. eds) pp. 155–223, Raven Press, New York.
[7] Seifert, W. and Rudland, P. S. (1974) Nature 248, 138–140.
[8] Miller, Z., Lovelace, E., Gallo, M. and Pastan, I. (1975) Science 190, 1213–1215.