Variations in Intracellular Nucleotide and Cyclic Nucleotide Concentrations in Relation to Induction and Commitment to Rhythmical Sporulation in the Myxomycete *Physarum polycephalum*

Tatsuo Akitaya, Tetsuo Ueda and Yonosuke Kobatake

Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo 060, Japan

ABSTRACT. Times of the induction and of commitment to sporulation and variations in intracellular nucleotide (ATP, ADP, AMP, CTP, CDP, UTP, UDP) and cyclic nucleotide (cAMP, cGMP) concentrations were determined from the time at which starved plasmodia were exposed to continuous light until sporulation about 10 h after illumination. Induction took place after about 1 h, a burst of cAMP and cGMP concentrations occurring at about the same time. Plasmodia acquired the commitment after about 4.5 h, which corresponded to the second-rising phase of the oscillating nucleotide concentrations. All the nucleotides studied oscillated virtually in phase, evidence that oscillation is related to conversion between monomeric and polymeric nucleotides. Anaerobic conditions suppressed both sporulation and oscillation of the intracellular nucleotide concentrations. The rate of O₂ uptake decreased on illumination, but did not oscillate. Sporulation is discussed in terms of nonlinear dynamics as a bifurcation from stable to oscillatory branches in an open nonequilibrium system.

Sporulation in *Physarum polycephalum* is the differentiation process a starving plasmodium undergoes after illumination (3, 4, 5, 8). The plasmodium becomes able to sporulate after approximately 3 days of starvation. The competent plasmodium no longer synthesizes DNA (2). Therefore, the differentiation process is related to the time of illumination. About 10 h after illumination, at the earliest, then at 5 or 10 h intervals, pillars are formed that develop sporangia in the apical region. Oscillations in the intracellular ATP, cAMP and cGMP concentrations are related to this rhythmical sporulation (1).

Before visible morphogenesis takes place, a light-requiring step (induction) and an irreversible transition to sporulation (commitment) can be delineated. We show that both induction and commitment take place in particular phases during variations in the intracellular nucleotide and cyclic nucleotide concentrations. A burst of cyclic nucleotide concentrations coincides with induction, and the second-rising phase of the nucleotide oscillations with commitment. Based on the relation between the onset of oscillation and ability to sporulate, sporulation is described as a bifurcation from stable to unstable branches in an open nonequilibrium system.

MATERIAL AND METHODS

Organism. The plasmodium (HU 195 $\times$ HU 200) of *Physarum polycephalum* was cultured by feeding it oatflakes on 1% agar gel. The organism was starved by placing it on moist filter paper (Whatman, No. 1) or on 1% water agar (Difco, Bactoagar) for more than 3 days in a trough (30 cm $\times$ 40 cm wide) in a dark place.

Determination of induction and commitment to sporulation. A large, starved plasmodium migrating on moist filter paper was cut into strips (ca. 1 cm $\times$ 1 cm) together with the filter paper, each strip was transferred to moist filter paper (5 sheets, Whatman, No. 1) in a plastic petri dish (ϕ =9 cm). About 100 plasmodia were exposed to continuous light (30 W $\times$ 2 fluorescent tubes, 30 cm above) (1), ten at a time being placed in the dark at 30 min intervals. The number of organisms which had sporulated 10 h after the illumination were scored, induction being determined as a function of time.

A large, starved plasmodium migrating on agar gel was exposed to continuous light as described above. Tip portions (about 5 $\times$ 5 mm², 10 at a time) of the plasmodium were cut off and placed on moist oatflakes in the dark at 30 min intervals. Sporulation frequency was determined as above, commitment in this case being given as a function of time.

Determination of intracellular nucleotide concentrations during sporulation. Small pieces (about 5 $\times$ 5 mm²) of the illuminated plasmodium were sampled, and their nucleotides extracted by treatment in 4 ml of hot buffer (10 mM Tris/HCl, pH 7.0, 90–95°C) for 10 min as reported elsewhere (1). After centrifugation (15,000 $\times$ g, 20 min), the protein in the pellet was assayed by the method of Lowry *et al.* and the filtered supernatant (Millipore, pore size, 0.45 μ m) assayed for nucleotides by high performance liquid chromatography (Pharmacia Fine Chemicals, type FPLC, Sweden) according to the manufacturers instructions: sample volume; 50 μ l, column; Polyanion SI HR5/5, flow rate: 1 ml/min, buffer A; 10 ml (NH₄)₂HPO₄/NH₄H₂PO₄, pH 7.0, buffer B; 850 mM buffer, gradient, linear; 0–100%

Fig. 1. Elution pattern of intracellular nucleotides extracted from the *Physarum* plasmodium.

B/15 min. The typical elution pattern is shown in Fig. 1. Each nucleotide in the pattern was assigned based on comparisons with external standards (Boehringer-Manheim-Yamanouchi K.K.). The amounts of nucleotides were determined by integrating each peak in the elution patterns by the half-width method. Concentrations of nucleotides were determined by dividing the amount of nucleotides by the amount of proteins. Results of ATP analysis agreed with results from bioluminescence determined with the firefly lantern (1).

Measurements of intracellular cAMP and cGMP concentrations. Frontal pieces (about 5×5 mm²) of the plasmodium were excised at 5 min intervals then immersed in isopentane cooled by liquid N₂. Each piece then was transferred to 1 ml of ice-cooled 1 M HCl and homogenized. The homogenate was washed twice with 0.5 ml 1 M HCl after which it was centrifuged ($15,000 \times g$, 20 min). The pellet was used for the protein assay, and the supernatant for the cAMP and cGMP assays. The radioimmunological assays of cAMP and cGMP used commercial kits (Yamasa Shoyu K.K.) (1). Protein was determined by the method of Lowry *et al.*

Measurements of O₂ uptake during morphogenesis. Variations in the rate of O₂ uptake before and after illumination were measured with a Warburg manometer (inset, Fig. 4B). Each experiment was repeated more than three times for each experimental condition. The results reported are typical.

RESULTS

Variations in intracellular nucleotide concentrations following illumination in relation to the induction and of commitment to sporulation. Time courses of the induction and of commitment to sporulation (A), intracellular ATP, ADP and AMP concentrations (B), intracellular CTP and CDP concentrations (C), and intracellular UTP and UDP concentrations (D), when starved plasmodia were exposed to continuous light from time zero onward to sporulation at about 10 h, are shown in Fig. 2. All the nucleotide concentrations began oscillating upon illumination (two cycles in this case). The first-rising phase corresponds to induction and the second to commitment to sporulation (compare Fig. 2B, C and D with A). When sporulation occurred at 5 h intervals rather than at 10 h, induction and commitment tended to occur at correspondingly later times (data not shown).

The fact that the ATP and ADP concentrations oscillate in phase (Fig. 2B) rules out the possibility that ATP oscillation is a consequence of energy balance; if it were, the ATP and ADP concentrations would have oscillated in opposite phase. Instead, all the nucleotides basically oscillate in phase (B, C and D), evidence that nucleotide oscillation is the transformation between monomeric and polymeric nucleotides, possibly related to RNA synthesis.

A burst of intracellular cyclic nucleotide concentrations during the early stages of sporulation. Cyclic AMP and cyclic GMP oscillated with a 5 h period in phase with ATP (1). A similar analysis was used to examine the early stages of sporulation by sampling at 5 min intervals. Typical time courses of intracellular cAMP and cGMP concentrations are shown in Fig. 3. About 1 h after illumination, both cyclic nucleotides had increased more than 3-fold for several minutes. The timing of this burst in cyclic nucleotide concentrations agrees with the onset of the induction of sporulation (Fig. 2A). This suggests that the cyclic nucleotides act as inducers for sporulation in the *Physarum* plasmodium.

Effect of oxygen tension on ATP variation and O₂ uptake during early stages of

Fig. 2. Time courses of induction, commitment and intracellular nucleotide concentrations in the *Physarum* plasmodium after illumination. The organism was starved for 100 h then exposed to light at time zero; it sporulated after about 10 h. A: induction at 1.5 h and commitment at 4.5 h. B: adenine nucleotides. C: cytidine nucleotides. D: uridine nucleotides. All the nucleotides oscillate with a period of 4–5 h, which corresponds to that of rhythmical sporulation [1].

Fig. 3. Time courses of intracellular cAMP and cGMP concentrations during early stages of sporulation. The plasmodium was starved for 120 h then exposed to light at time zero. The burst of cAMP and cGMP after 50 min, corresponded to induction (see Fig. 2A).

Fig. 4. Effect of oxygen tension on light-induced ATP variations (A) and O_2 uptake during early stages of sporulation (B). Plasmodia were starved for 120 h then exposed to light at time zero. (A) Anaerobic conditions suppressed light-induced ATP variations and sporulation. (B) The rate of O_2 uptake was reduced by illumination, but did not oscillate. The inset shows a Warburg manometer; P: plasmodium, A: 4.5 M KOH, ΔV : volume change.

morphogenesis. No sporulation took place under anaerobic conditions, and at that time, oscillatory variations in the intracellular ATP concentration also were suppressed (Fig. 4A). The uptake of O_2 was reduced by illumination, but respiration, itself, showed no oscillation (Fig. 4B).

Oscillatory respirations have been shown for *Acanthamoeba*, *Tetrahymena*, *Schizosaccharomyces*, etc. (6), but respiration in the *Physarum* plasmodium during sporulation takes place at a constant rate.

DISCUSSION

Sporulation as a bifurcation phenomenon in an open nonequilibrium system. We have used nonlinear dynamics in an open nonequilibrium system to clarify the nature of sporulation. Our experimental findings on the sporulation of *Physarum polycephalum* are as follows: (a) Sporulation is rhythmic, occurring at 5 or 10 h intervals 10 h after illumination (1); (b) The ability to sporulate corresponds to the onset of oscillations in the intracellular nucleotide concentrations (Fig. 2); (c) O_2 is a requisite for sporulation, but the rate of respiration is constant during morphogenesis (Fig. 4B); (d) Sporulation is induced by continuous light above threshold

Fig. 5. Diagram of the thermodynamic state of the *Physarum* plasmodium during sporulation. Sporulation is a bifurcation in nonlinear dynamics.

strength, but too strong a light suppresses sporulation.

These results are shown graphically in Fig. 5, in which the x axis gives the rate of O_2 uptake (or O_2 pressure) and the y axis the intensity of the light. The shaded area is the region in which intracellular nucleotide concentrations oscillate and sporulation occurs. The system (cell) has a constant supply of O_2 and light (upper trace, Fig. 5), yet the intracellular components (e.g., the x_1, x_2 , etc. standing for nucleotides) oscillate. This rhythmical sporulation is a typical dissipative structure found in an open nonequilibrium system far from equilibrium (7). Nonlinear dynamics characterizes sporulation as a bifurcation from stable to oscillatory branches. Further study is necessary to determine detailed molecular mechanisms.

Acknowledgments. This research was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan. One of us (A.T.) thanks Dr. H. Tanabe for teaching him the HPLC method.

REFERENCES

1. AKITAYA, T., S. OHSAKA, T. UEDA and Y. KOBATAKE. Oscillations in intracellular ATP, cAMP and cGMP concentration in relation to rhythmical sporulation under continuous light in the myxomycete *Physarum polycephalum*. *J. Gen. Microbiol.* **131**, 195–200, 1985
2. CHAPMAN, A. and J.G. COOTE. Sporulation competence in *Physarum polycephalum* CL and the

- requirement for DNA replication and mitosis. *J. Gen. Microbiol.* **128**, 1489–1501, 1982
3. DEE, J. Slime moulds in biological research. *Sci. Prog.* **62**, 523–542, 1975
 4. GOODMAN, E.M. *Physarum polycephalum*: a review of a model system using a structure-function approach. *International Rev. Cytol.* **63**, 1–58, 1980
 5. GORMAN, J.A. and A.S. WILKINS. Developmental phases in the life cycle of *Physarum* and related myxomycetes. in *Growth and Differentiation in Physarum polycephalum*, ed. Dove, W.F. and Rusch, H.P., Princeton Uni. Press, Princeton, pp. 157–202, 1980
 6. LLOYD, D. and S.W. EDWARDS. Epigenetic oscillations during the cell cycles of lower eukaryotes are coupled to a clock. In *Cell Cycle Clocks*, ed. Edmunds, L.N., Jr., Marcel Dekker, New York, pp. 27–46, 1984
 7. NICOLIS, G. and I. PRIGOGINE. *Self-organization in Nonequilibrium Systems*, John Wiley & Sons, Inc., 1977
 8. SAUER, H.W. *Developmental Biology of Physarum*, Chap. 6, Cambridge Univ. Press, Cambridge, 1982

(Received for publication, October 28, 1985)