CELL STRUCTURE AND FUNCTION 17: 371–375 (1992)

Entrainment to External Ca^{2+} Oscillation in Ionophore-Treated *Physarum* Plasmodium

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Key words: *Physarum*/oscillation/entrainment/phase locking/calcium ion

ABSTRACT. **To elucidate the mechanism of mutual interaction between intracellular chemical rhythms in the *Physarum* plasmodium, external Ca^{2+} oscillation was applied to the ionophore-treated plasmodial strand and its response was measured as tension oscillation. (i) Tension oscillation is entrained and phase locked to the externally applied Ca^{2+} oscillation. (ii) Two kinds of stable phase relationship, in-phasic and anti-phasic ones, are observed between them. (iii) Transition between the two stable phase relationships is also observed. These results suggest that intracellular rhythms which control tension generation are mutually entrained by means of cytosolic Ca^{2+} oscillation in the organism and that their interactions have two kinds of stable phase relationships.**

The *Physarum* plasmodium migrates coordinately as one whole body even in a complicated environment. We recently clarified that such coordinative migration necessitates mutual entrainment between intracellular rhythms to process information from external environment (10). How do such rhythms interact with each other in the organism?

Tension oscillation which generates motive force of tactic migration is observed in every part of the plasmodium (19). Chemical substances, such as Ca^{2+} (7, 21), ATP (22) and H^+ (11), also oscillate in a fixed phase relationship to tension rhythm. Mechanical interaction is not required for spatial synchronization between tension oscillations (20). Propagation velocity of the oscillation is much higher than that of endoplasmic streaming (10). These facts suggest that the mutual interaction between intracellular rhythms controlling tension generation is mediated by some chemical reactions.

Ueda *et al.* reported that a wavy ATP pattern propagates from the front to the rear in the organism (17). We also found that the frequency of intracellular Ca^{2+} oscillation is modulated not only at stimulated sites but also in nonstimulated regions after applying a local attractive stimulus (12). However, the causal relationship between these chemical interactions and their characteristics still remain obscure.

The purpose of this report is to identify the key oscillation in such chemical interactions and to clarify their properties. As a candidate, we apply external oscillation of Ca^{2+} concentration to the ionophore-treated plasmodium and measure its responses as tension oscillation. Our results show that the intracellular rhythm which regulates tension development is entrained by means of Ca^{2+} oscillation and that the interaction has two kinds of stable phase relationships. The mechanism and function of such interaction are discussed in the last section.

MATERIALS AND METHODS

Organism. The plasmodium of *Physarum polycephalum* was cultured by the method of Camp (1) and stored as sclerotia. It was allowed to migrate on a 1.5% agar gel sheet overnight without feeding before use. A plasmodial strand about 15 mm in length and 0.6 to 0.8 mm in diameter was used for the experiments.

Regulation of Ca^{2+} concentration. The plasmodial strand is placed in a small measurement chamber (3 mm × 10 mm × 3 mm) as shown in Fig. 1. Then artificially controlled Ca^{2+} concentration oscillation is applied to the organism by the following methods. Ca^{2+} concentration is regulated by changing the mixing ratio between two kinds of solutions. One is a calcium-containing solution and the other is a calcium-free solution. The mixing ratio is continuously varied in the form of the sine function and the mixed solution flows through the measurement chamber.

The calcium solution (pCa 4.6) contains 2 mM $CaCl_2$, 2 mM EGTA, 10 mM MOPS, 13 mM KCl and 2 mM NaCl, and it was adjusted to pH 7.0 by adding 11.8 mM KOH. The calcium-free solution contains 2 mM EGTA, 10 mM MOPS, 17

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mM KCl and 2 mM NaCl, and it was adjusted to pH 7.0 by adding 7.8 mM KOH. Amphotericin B (Sigma) is applied to the both solutions as an ionophore at the final concentration of 2 μg/ml according to the method of Yoshimoto *et al.* (21). Since this ionophore has no Ca^{2+} selectivity as A23187, concentrations of sodium and potassium ions in each solution are approximately adjusted to that of the intracellular value of the plasmodium (6). To remove the slime layer around the organism, 0.1 μg/ml lysozyme (Sigma) is also added to both solutions. They are continuously oxygenated before mixing.

The mixed solution flows at the speed of 1.0 ml/min in the measurement chamber by means of a peristaltic pump (Minipuls2, Gilson). Temperature and pressure of the solution in the chamber are adjusted to 20°C and atmospheric pressure, respectively. To measure the Ca^{2+} concentration in the chamber, a calcium electrode (F2110Ca, Radiometer) and reference electrode are installed, as shown in the figure. By the use of an electromagnetic valve (Mixograd Valve, Gilson) controlled by microcomputer, the mixing ratio is regulated at 2 sec intervals. The concentration of Ca^{2+} oscillates between pCa 6.0 and 7.0, and its mean value is fixed at pCa 6.5. Before onset of Ca^{2+} oscillation, the concentration around the plasmodium is fixed at a constant level of pCa 6.5 for about 30 min. The period of external Ca^{2+} oscillation is approximately adjusted to the mean period of tension oscillation observed in this time interval in the organism.

Measurement of tension development. To measure the responses to external Ca^{2+} oscillation, tension oscillation in the plasmodium is recorded simultaneously by using the method of Tanaka *et al.* (16). The plasmodial strand is horizontally connected between the two extensions of two glass capillary tubes placed 5 mm apart, as shown in Fig. 1. By the use of a tension transducer (AE-801, Akers, Horten, Norway) which is attached to one of the extensions, longitudinal isometric ten-

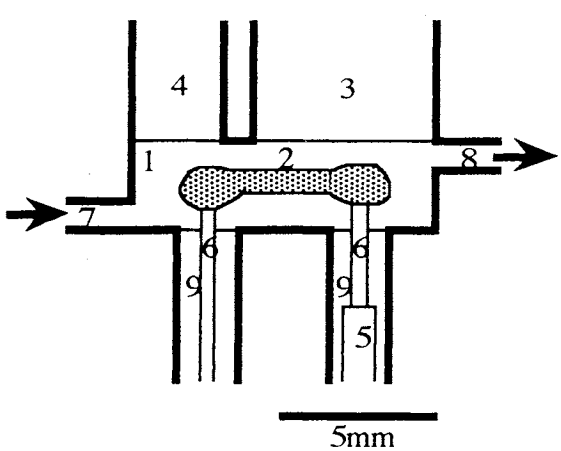

Fig. 1. Schematic illustration of the experimental apparatus (side-view). 1 measurement chamber, 2 plasmodial strand, 3 calcium electrode, 4 reference electrode, 5 tension transducer, 6 extension of glass capillary, 7 inlet of solution, 8 outlet of solution, 9 silicon oil. See text for details

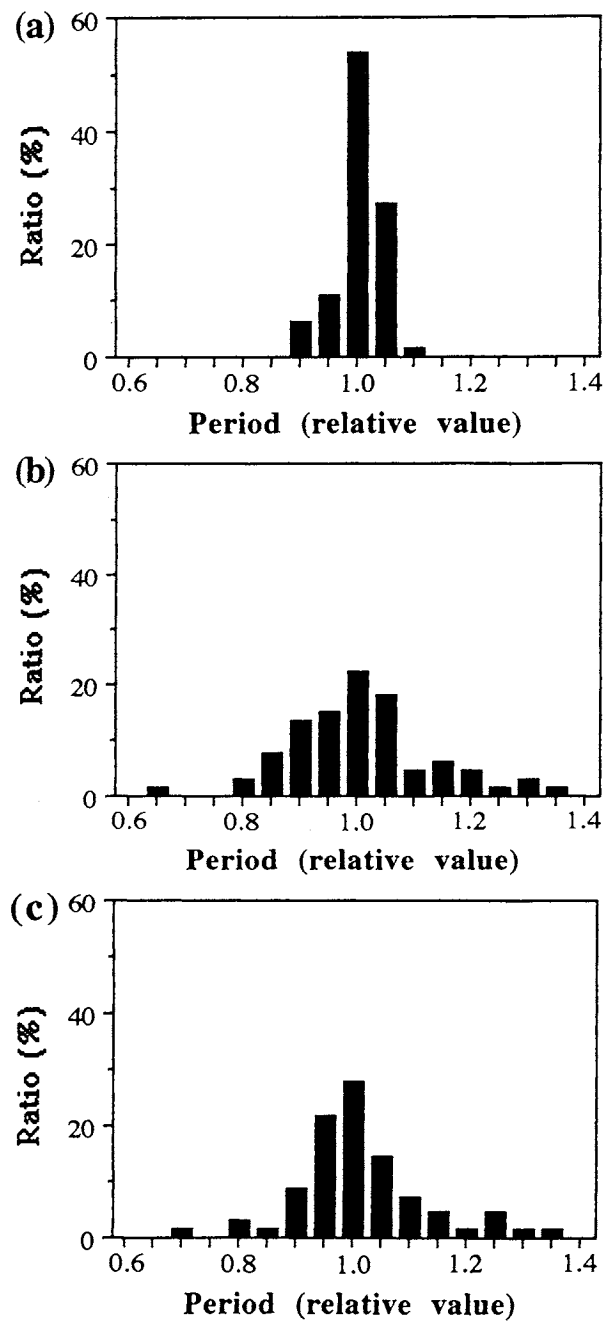

Fig. 2. Distribution of the period in tension oscillation. The period in each cycle was normalized by the mean period of tension observed before the onset of external Ca^{2+} oscillation. The distribution pattern was obtained from 17 cycles of each of 4 different plasmodia (n=68) and represented as a ratio in each column. (a) Distribution pattern under external Ca^{2+} oscillation. Mean period of tension oscillation and its standard deviation were 1.00 ± 0.04. (b) Distribution pattern under the constant concentration at pCa 6.5. Mean and SD were 1.02 ± 0.13. (c) Distribution pattern under external Ca^{2+} oscillation without ionophore treatment. Mean and SD were 1.01 ± 0.11.

sion of the plasmodial strand is measured. Its sensitivity is about 0.1 mg. To seal the transducer from the Ca^{2+} solution, the holes in which the transducer and extensions are placed are filled with silicon oil (Shinetsu).

Data analysis. The period of tension oscillation is defined as the time interval between two successive minimal peaks in the time course. The phase difference between external Ca^{2+} oscillation and tension oscillation is defined as follows. The time interval of the two corresponding maximal peaks between the tension and Ca^{2+} oscillation is divided by the period of Ca^{2+} oscillation and multiplied by 360°. Then it is assumed that the phase difference has a positive value when the tension oscillation has an advanced phase compared to that of Ca^{2+} oscillation.

RESULTS

Figure 2a shows a fluctuation of period in the time course of tension oscillation which was observed under the application of external Ca^{2+} oscillation. It clearly exhibits the sharp distribution pattern of the period and its mean value is 1.0, which means that the period of tension oscillation exactly coincides with that of external Ca^{2+} oscillation. However, as shown in Fig. 2b, the pattern of period distribution became rather broad when external Ca^{2+} concentration was kept constant at pCa 6.5. In addition, when the plasmodium was not treated by the ionophore, such phenomena in Fig. 2a were not observed even under the same Ca^{2+} oscillation as shown in Fig. 2c. These results indicate that the intrinsic fluctuation of tension oscillation is suppressed by external Ca^{2+} oscillation and that the Ca^{2+} oscillation directly affects the intracellular chemical oscillations. Thus, it is shown that intracellular oscillator controlling tension generation is entrained to external Ca^{2+} oscillation.

Figure 3a shows an example of the time course of tension development, external Ca^{2+} concentration and the phase difference between the two oscillations, respectively. It is clearly shown that tension generation oscillates in the same period and in fixed phase relationship to external Ca^{2+} oscillation. The stable phase relationship between them was an anti-phasic state in this case. When the phase of external Ca^{2+} oscillation was shifted to $-90°$ at the point indicated by the arrow in the figure, such an anti-phasic relationship recovered after some

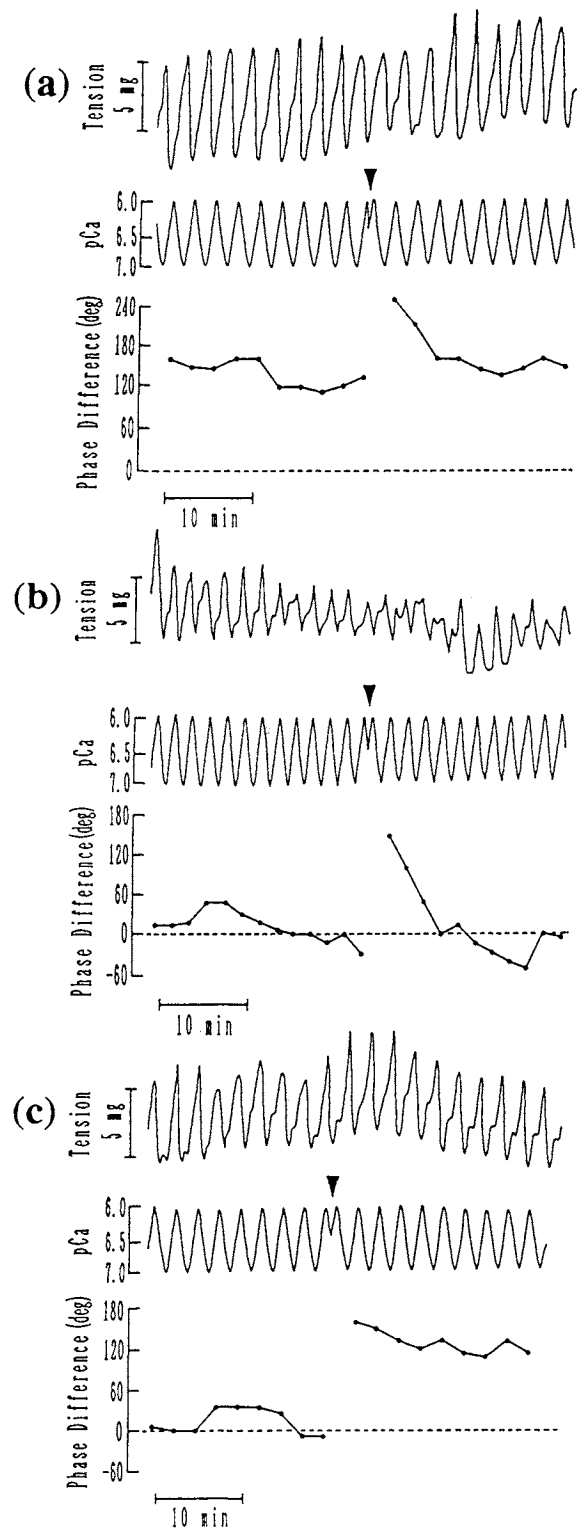

Fig. 3. Time courses of tension development, external Ca^{2+} concentration and the phase difference between them. Arrow indicates the timing of phase shift of external Ca^{2+} oscillation. (a) Stable antiphasic relationship between the two oscillations. This example was obtained under the phase shift of $-90°$. The period of external Ca^{2+} oscillation was fixed at 150 sec. (b) Stable in-phasic relationship. This was observed under the phase shift of 180° and its period was fixed at 120 sec. (c) Transition from the stable in-phasic state to the stable anti-phasic state. This was observed under the phase shift of 180° and its period was fixed at 150 sec.

transition cycles of tension oscillation. These results mean that the intracellular oscillator controlling tension generation is phase locked to external Ca^{2+} oscillation under the entrained state.

Figure 3b shows another example of phase locking under a stable entrained state between the two oscillations. Before phase shifting, the stable phase relationship was an in-phasic state. After 180° phase shift of external Ca^{2+} oscillation, the in-phasic phase relationship also recovered within some transition cycles of tension oscillation. Other details were the same as in the above case. Figure 3c exhibits an example which shows the transition between the two stable phase relationships. Before phase shifting, the relationship was kept to an in-phasic state. After the 180° phase shift of Ca^{2+} oscillation, it changed to the stable anti-phasic state. Thus, it is suggested that not only the anti-phasic relationship but also the in-phasic one are stable entrained states and that they dynamically change with one another.

Therefore, these experimental results are summarized as follows. (i) Tension oscillation in the ionophore-treated plasmodial strand is entrained and phase locked to externally applied Ca^{2+} oscillation. (ii) Two kinds of stable phase relationship, in-phasic an anti-phasic ones, are observed between them. (iii) Transition process between the two stable phase relationships is also observed.

DISCUSSION

These results indicate that tension oscillation measured in this experiment represents not the direct response of the contractile system to external Ca^{2+} oscillation but the dynamic response of the intracellular chemical oscillator controlling tension generation. This is supported by the following two results. Although tension development monotonously changes according to the increase of Ca^{2+} concentration from pCa 7.0 to 6.0 in the plasmodium (7, 23), both in-phasic and anti-phasic relationships between the two oscillations were simultaneously observed in the same region of Ca^{2+} concentration. In addition, the transition process of tension oscillation for the re-entrainment was observed after the phase shift of external Ca^{2+} oscillation. Therefore, the following two findings are obtained.

One is the identification of the key oscillation in intracellular mutual entrainment. Since the intracellular rhythm which controls tension generation is entrained to external Ca^{2+} oscillation, we could conclude that such rhythms interact by means of the cytosolic Ca^{2+} oscillation in the organism. This conclusion is further supported by the following two reports. Satoh *et al.* clarified that the primary oscillator controlling tension generation is mitochondria, especially the Ca^{2+} up-take process (14). We also reported that coordinated tactic migration as a whole body disappears under anaerobic conditions in which mitochondrial activity is suppressed (16). However, these do not mean that the possibility of ATP oscillation as a mediator should be excluded. On the other hand, we have recently found that a global gradient of Ca^{2+} concentration exists from the front to the rear in the plasmodium and that its polarity always coincides with migratory direction (12). Since the intracellular rhythms and this spatial gradient have a common mediator, Ca^{2+}, it is suggested that they interact with each other in the process of coordinative migration. As for a possible mechanism governing it, the model of cytosolic Ca^{2+} oscillation using Ca^{2+}-induced Ca^{2+} release from intracellular stores (2, 18) could be proposed.

The other important point is the variety of the phase relationships generated by such mutual entrainment. Since our results show two kinds of stable relationships and the transition between them, it is suggested that various phase patterns could be dynamically organized under the entrained state in the coupled-oscillator system in the plasmodium. This kind of phenomenon has been found and theoretically investigated in some biological systems, such as the circadian pacemaker (5, 13) and cooperative human hand movement (3, 15). However, in the plasmodium, simple and steady patterns of phase gradient have been studied mainly in correlation with the information processing mechanism in tactic response (4, 8, 9, 16). Kuroda *et al.* reported that abrupt irregular change of the phase relationship among local intracellular Ca^{2+} oscillations is observed in the microplasmodium (7). Yoshimoto also showed a possibility of such fluctuations in the plasmodium (24). Our previous report further indicated that the fluctuation of local phase relationship between endoplasmic and ectoplasmic regions decreases after environmental stimulus (10, 16). Thus, such fluctuations might have a close correlation with the flexible interaction between recognizing process of environmental conditions and other information processing processes in taxis. These mechanisms should be further investigated experimentally and theoretically.

Acknowledgments. The authors are grateful to Dr. Y. Yamaguchi, Mr. Y. Kawato, Mr. K. Natsume, Mr. T. Isono and Mrs. Y. Miyake for their helpful discussion and assistance.

REFERENCES

1. Camp, W.G. (1936). A method of cultivating myxomycete plasmodia. *Bull. Torrey. Bot. Club,* **63**: 205–210.
2. Goldbeter, A., Dupont, G. and Berridge, M.J. (1990). Minimal model for signal-induced Ca^{2+} oscillations and for their frequency encoding through protein phosphorylation. *Proc. Natl. Acad. Sci. USA.*, **87**: 1461–1465.
3. Haken, H., Kelso, J.A.S. and Bunz, H. (1985). A Theoreti-

cal model of phase transition in human hand movements. *Biol. Cybern.*, **51**: 347–356.
4. Hejnowicz, Z. and Wohlfarth-Bottermann, K.E. (1980). Propagated waves induced by gradients of physiological factors within plasmodia of *Physarum polycephalum*. *Planta,* **150**: 144–152.
5. Kawato, M. and Suzuki, R. (1980). Two coupled neural oscillators as a model of the circadian pacemaker. *J. Theor. Biol.,* **86**: 547–575.
6. Kuroda, H. and Kuroda, K. (1980). Calcium accumulation in vacuoles of *Physarum polycephalum* following starvation. *J. Cell Sci.,* **44**: 75–85.
7. Kuroda, R., Hatano, S., Hiramoto, Y. and Kuroda, H. (1988). Change of cytosolic Ca-ion concentration in the contraction and relaxation cycle of *Physarum* microplasmodia. *Protoplasma*, [Supl **1**]: 72–80.
8. Matsumoto, K., Ueda, T. and Kobatake, Y. (1986). Propagation of phase wave in relation to tactic responses by the plasmodium of *Physarum polycephalum*. *J. Theor. Biol.,* **122**: 339–345.
9. Matsumoto, K., Ueda, T. and Kobatake, Y. (1988). Reversal of thermotaxis with oscillatory stimulus in the plasmodium of *Physarum polycephalum*. *J. Theor. Biol.,* **131**: 175–182.
10. Miyake, Y., Yano, M. and Shimizu, H. (1991). Relationship between endoplasmic and ectoplasmic oscillations during chemotaxis of *Physarum polycephalum*. *Protoplasma,* **162**: 175–181.
11. Nakamura, S., Yoshimoto, Y. and Kamiya, N. (1982). Oscillation in surface pH of the *Physarum* plasmodium. *Proc. Japan Acad.,* **58**: ser. B, 270–273.
12. Natsume, K., Miyake, Y., Yano, M. and Shimizu, H. (1992). Development of spatio-temporal pattern of Ca^{2+} on the chemotactic behavior of *Physarum polycephalum*. *Protoplasma,* **166**: 55–60.
13. Pavlidis, T. (1971). Populations of biochemical oscillators as circadian clocks. *J. Theor. Biol.,* **33**: 319–338.
14. Satoh, H., Ueda, T. and Kobatake, Y. (1982). Primary oscillator of contractional rhythm in the plasmodium of *Physarum polycephalum*: Role of mitochondria. *Cell Struct. Funct.,* **7**: 275–283.
15. Schoner, G. and Kelso, J.A.S. (1988). Dynamic pattern generation in behavioral and neural systems. *Science,* **239**: 1513–1520.
16. Tanaka, H., Yoshimura, H., Miyake, Y., Imaizumi, J., Nagayama, K. and Shimizu, H. (1987). Information processing for the organization of chemotactic behavior of *Physarum polycephalum* studied by micro-thermography. *Protoplasma,* **138**: 98–104.
17. Ueda, T., Nakagaki, T. and Yamada, T. (1990). Dynamic organization of ATP and birefringent fibrils during free locomotion and galvanotaxis in the plasmodium of *Physarum polycephalum*. *J. Cell Biol.,* **110**: 1097–1102.
18. Yamaguchi, Y., Komatsu, Y. and Shimizu, H. (1985). Theoretical study of wave propagation and oscillation of contraction in skinned fibers of skeletal muscle. *Jikeikai Med. J.,* **32**: 189–209.
19. Yoshimoto, Y. and Kamiya, N. (1978a). Studies on contraction rhythm of the plasmodial strand. I. Synchronization of local rhythms. *Protoplasma,* **95**: 89–99.
20. Yoshimoto, Y. and Kamiya, N. (1978b). Studies on contraction rhythm of the plasmodial strand. II. Effect of externally applied force. *Protoplasma,* **95**: 101–109.
21. Yoshimoto, Y., Matsumura, F. and Kamiya, N. (1981a). Simultaneous oscillations of Ca^{2+} efflux and tension generation in the permealized plasmodial strand of *Physarum*. *Cell Motility,* **1**: 433–443.
22. Yoshimoto, Y., Sakai, T. and Kamiya, N. (1981b). ATP oscillation in *Physarum* plasmodium. *Protoplasma,* **109**: 159–168.
23. Yoshimoto, Y. and Kamiya, N. (1984). ATP-and calcium-controlled contraction in a saponin model of *Physarum polycephalum*. *Cell Struct. Funct.,* **9**: 135–141.
24. Yoshimoto, Y. (1991). Ca^{2+} and ATP oscillations in *Physarum* plasmodium. *J. Dep. Liberal Arts Kansai Med. Univ.,* **13**: 9–22.

(Received for publication, September 11, 1992
and in revised form, October 27, 1992)