OSCILLATIONS OF CALCIUM ION CONCENTRATIONS IN *PHYSARUM POLYCEPHALUM*

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The natural ebb and flow of cytoplasmic streaming inside the giant multinucleate plasmodia of *Physarum polycephalum* has been much studied (5, 10). Beause both actin and myosin can be extracted from *Physarum* (7), it is reasonable to suppose that streaming might be due to a calcium-sensitive contractile system similar to that found in muscle. In order to test this hypothesis, we injected the calcium-specific photoprotein, aequorin, into short single strands of *Physarum*. The aequorin method for the detection of changes in the ionized intracellular calcium level has previously revealed calcium transients associated with contraction in barnacle muscle (9) and calcium permeability changes in squid axons (1).

Our main conclusions are: first, that cyclic changes in the ionized intracellular calcium level do occur during streaming in *Physarum;* and second, that the polarity of the streaming is such that contraction occurs in the region of elevated Ca^{++}.

METHODS

Physarum polycephalum strain M3cV, a gift of Dr. Joyce Mohberg of the University of Wisconsin, Madison, Wis., was grown on an axenic medium (2) solidified with 2% agar. Small pieces of plasmodium were allowed to migrate on non-nutrient agar containing 1 mM NaCl, 1 mM $CaCl_2$ for the experiments reported here.

Microinjection of *Physarum* is difficult because of the efficiency of the surface precipitation, or wound-healing, response. We used beveled glass micropipets which had been washed with 20 mM EDTA then distilled water, and dried. Micropipets were filled with 0.2 μl of aequorin solution (see reference 1 for details), mounted on a Leitz micromanipulator, and inserted gently into a large strand of plasmodium. Then, small aliquots of the aequorin solution were forced into the strand with a low air pressure, after which the organism was allowed to recover for a few minutes.

After microinjection, the strand of plasmodium together with the supporting rectangular block of agar was placed on a glass slide that held Ag-AgCl electrodes (see Fig. 1 A). Next, two deep "V" cuts were made from opposite sides through the agar block, leaving only the strand of plasmodium bridging the gap at the junction of the two V cuts. The total volume of *Physarum* cytoplasm in our preparations was on the order of 3–5 μl.

The electrodes were coupled through a high-impedance differential voltage amplifier to a chart recorder. Although slightly different from the method of Kamiya (5), this set-up produced similar "electroplasmograms" (EPG) recording the differences in electrical potential between the two ends of a plasmodium. By observing the streaming movements of the aequorin-injected plasmodium with a compound microscope and simultaneously measuring the "electroplasmogram," we were able to verify that protoplasm flows away from the end of the strand which is more negative.

Fig. 1 B illustrates the experimental apparatus for recording Ca^{++} -mediated light emission (9) from an aequorin-injected strand of plasmodium. Light produced during the reaction between the injected aequorin and intracellular Ca^{++} was measured by an EMI 9635A

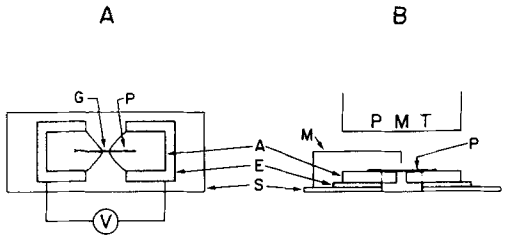

FIGURE 1 A, Top view of a *Physarum* strand (*P*) bridging the air gap (*G*) between two agar blocks (*A*) which are connected to silver-silver choloride electrodes (*E*) for recording the potential differences (*V*) that occur during free streaming. B, Side view of the preparation shows mask (*M*) covering one end, and photomultiplier tube (*PMT*) for recording calcium-mediated light output (calcium transient, *CT*) from the unmasked end.

photomultiplier tube (PMT). The dynode chain and other electrical details of the photomultiplier circuit were similar to those described before (1), except that the low-pass electrical filter had a time constant of about 300 ms. The changes in intracellular Ca^{++} concentration which accompany normal streaming occur relatively slowly and are not seriously distorted by this electrical filter. A black light-absorbing mask could be fitted over either end of the plasmodium so that only light emitted from the unmasked end reached the photomultiplier tube. For the experiments shown in Figs. 2 and 4, the entire preparation was placed in a light-tight steel box which provided the electrostatic, electromagnetic, and light shielding necessary to record successfully at these very low light levels. The output current of the PMT, which is directly proportional to light intensity, was amplified and displayed on a chart recorder. The approximate calibration factor (1) for converting output current to molecules of aequorin reacting with Ca^{++} per second is: 100 pA = 6×10^3 molecules of aequorin reacting per second.

RESULTS

Fig. 2 shows simultaneous recordings of electrical potential and Ca^{++} -mediated light output (calcium transient), both varying cyclically with time. As Kamiya described (5), the electrical recording shows a roughly sinusoidal variation superimposed on a slowly changing base line. The light recording also shows a cyclic variation which could be recorded even up to 24 h afterwards, over a steadily declining base line. Maximum calcium concentrations occur in the unmasked end when it is becoming electrically negative relative to the other end.

There is considerable cycle to cycle variation in these recordings. Fig. 3 illustrates the results of a statistical analysis of 50 consecutive cycles of free streaming. The electrical and light cycle lengths have similar means and standard deviations. Maximum Ca^{++} concentrations precede maximum external electrode electronegativity by a small fraction of a cycle. There is also a curious statistical correlation between the duration of the nth light cycle and the duration of the (n + 1)th electrical cycle ($P < 0.001$, t-test, $n = 49$, $r = 0.35$).

Besides its slow natural electrical activity, *Physarum* is known to show a rapid electrical response to various stimuli such as mechanical shock (3, 11, 12). Fig. 4 shows simultaneous electrical and light recordings during several responses to gentle mechanical shock. Both traces often, but not always show a fast response together. Because the rapid electrical response causes gelling of the cytoplasm (11), our finding of a dramatic increase of Ca^{++} concentration suggests a possible cause and effect relationship.

DISCUSSION

Our results in Figs. 2–4 lead to the following conclusions. First, there is a slow periodic fluctuation of the concentration of free Ca^{++} in the unmasked end of the *Physarum* strand. The mean frequency of this fluctuation is equal to that of the

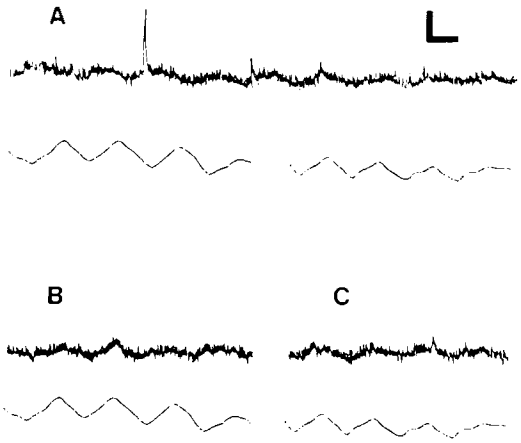

FIGURE 2 Simultaneous recording of aequorin light emission (upper trace) and EGP (lower trace). polarity: upper trace, increasing light in upwards direction; lower trace, increasing negativity of unmasked end in upwards direction. Records A–C were obtained 4.5, 3.5, and 5.5 h, respectively, after the aequorin injection. Calibration bar, vertical: upper trace, 225 pA; lower trace, 7 mV; horizontal: 1.24 min. Dark current = 190 pA. Temperature, 21°C.

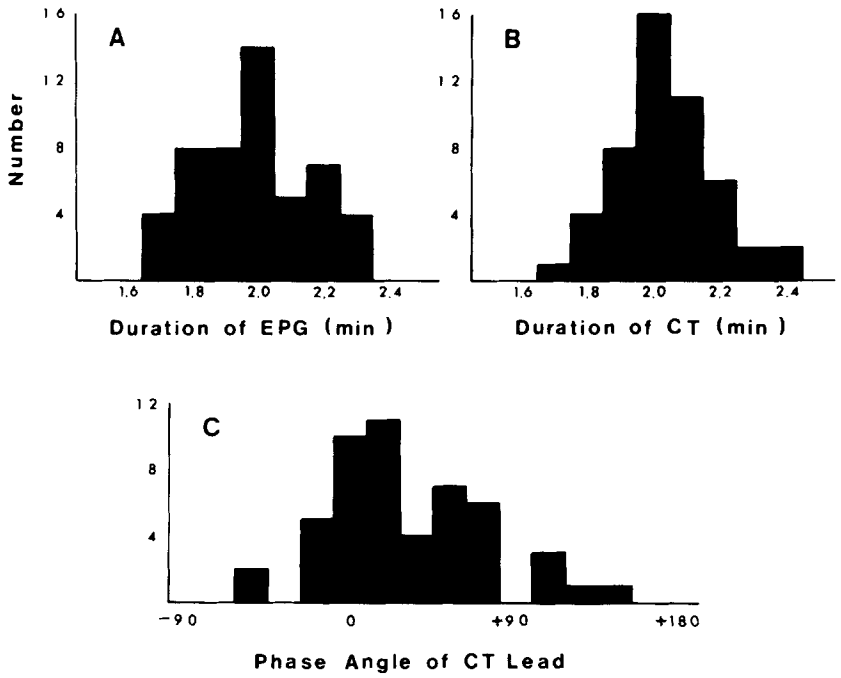

FIGURE 3 Statistical analysis of 50 consecutive streaming cycles, from the same aequorin-injected plasmodium as illustrated in Fig. 2. Measurements were taken at peak electronegativity in unmasked end and peak calcium signal. The slow shifts in the D.C. base line of the EPG were ignored. (*a*) Histogram of EPG cycle duration; mean, 1.99 min; standard deviation, 0.17 min; cell size, 0.1 min; *n*, 50; temperature, 21°C. (*b*) Histogram of calcium transient (*CT*) duration; mean = 1.99 min; standard deviation = 0.15 min; cell size = 0.1 min. (*c*) Histogram of phase angle between calcium and the EPG. Polarity positive for calcium lead over EPG. Mean, 13; standard deviation, 41; cell size, 18.

electrical fluctuation. Second, there is a rapid electrical event of the surface of *Physarum* which often, but not always leads to a dramatic increase in the Ca^{++} level. Third, Ca^{++} concentrations increase at the end which is depolarizing and contracting.

The third conclusion depends upon the observations of Kamiya and his colleagues (5, 6, 11) that variations in pressure, and hence streaming rate, precede electrical potential variations by up to a quarter of a cycle. Although this phase lead is somewhat puzzling, it must be remembered that the electroplasmogram is an extracellular record of the voltage difference between the two ends. Therefore, peak electronegativity does not necessarily correspond to peak intracellular depolarization of the contracting end, since voltages developed at the relaxing end must be considered as well. Our observations thus suggest that *Physarum* is essentially analogous with muscle, in accord with the fact that Ca^{++} has been shown to control the activity of *Physarum* actomyosin ATPase in vitro (8) and also by the effects of caffeine (4). Our basic interpretation is, therefore, that cytoplasmic streaming occurs as a result of pressure differences which are due to a fluctuating Ca^{++} concentration

FIGURE 4 Simultaneous recording of electrical activity (upper trace) and calcium transient (lower trace) induced by gentle mechanical stimuli (*s*). Normal cyclical changes in calcium (cf. Fig. 2) are not obvious here because this strand had a much lower aequorin concentration and also because the mechanical stimulus disrupts normal streaming. Polarity: upper trace, increasing negativity of the unmasked end in the downwards direction; lower trace, increasing light in the upwards direction. Calibration bar, vertical: upper trace, 7 mV; lower trace, 675 pA; horizontal, 1.3 min. Dark current = 190 pA.

via its effect on a Ca^{++}-sensitive actomyosin system.

Several possible causes of light fluctuations, apart from Ca^{++} concentration fluctuations, must also be considered. For example, if the injected aequorin were uniformly distributed throughout the *Physarum* protoplasm with a constant Ca^{++} level (presumably about 10^{-7} M), changes in light emission might arise simply because the amount of protoplasm varied at the unmasked end. In fact, however, light emission increased while protoplasm left the unmasked end, and the observed fluctuations (up to 30%, in Fig. 2) were much larger in size than any fluctuations in volume. Alternatively, light absorption might vary in different regions of the organism. However, the amplitude of the light variations was often too high to permit this explanation to be acceptable, and the rapid light responses took place with little simultaneous change in absorption or volume. Control experiments showed no significant light output in the absence of aequorin. While there is no absolute proof that aequorin is free in the cytoplasm during its light emission, there is little reason to suppose that it is inside small vesicles.

Our results do not reveal the manner in which Ca^{++} ions are released, nor the reactions controlling the motile oscillator in *Physarum*. However, they will make it possible to proceed with confidence towards answering these two important questions, and we believe that *Physarum* will continue to serve as a useful model for motility in all cells.

SUMMARY

Aequorin is a photoprotein which emits light in response to changes in free calcium concentration. When aequorin was microinjected into plasmodia of *Physarum polycephalum*, light emission varied in synchrony with the motile oscillations of the organisms. Therefore, movement is correlated with changes in the concentration of free calcium.

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