Model of the Ca^{2+} oscillator for shuttle streaming in *Physarum polycephalum*

D. A. Smith and R. Saldana
Department of Physics, Monash University, Clayton 3168, Australia

ABSTRACT We propose a mechanism for the cytoplasmic Ca^{++} oscillator which is thought to power shuttle streaming in strands of the slime-mold *Physarum polycephalum*. The mechanism uses a phosphorylation-dephosphorylation cycle of myosin light chain kinase. This kinase is bistable if the kinase phosphorylation chain, through adenylate cyclase and cAMP, is activated by calcium. Relaxation oscillations can then occur if calcium is exchanged between the cytoplasm and internal vacuoles known to exist in physarum. As contractile activity in physarum myosin is inhibited by calcium, this model can give calcium oscillations 180° out of phase with actin filament tension as observed. Oscillations of ATP concentration are correctly predicted to be in phase with the tension, provided the actomyosin cycling rate is comparable with ATPase rates for phosphorylation of the myosin light chain and its kinase.

INTRODUCTION

Rhythmic motility, particularly shuttle streaming, in the slime-mold *Physarum polycephalum* has been studied extensively (e.g., Kamiya, 1955, 1978, 1981; Kessler, 1982; Nakamura et al., 1982; Satoh et al., 1984, 1985; Tyson, 1982; Ueda et al., 1987; Wohlfarth-Botterman, 1979; Yoshimoto and Kamiya 1978*a*, *b*, *c*, *d*) but the mechanism remains obscure. *Physarum* is in some ways a model system to test theories of crawling motility, because of the apparent simplicity of shuttle streaming and because its membrane is effectively impermeable (Kamiya et al., 1981; Yoshimoto et al., 1981*a*). Its motility is thought to be due to actin filaments in the presence of myosin, rather than microtubules (Kessler, 1982). Shuttle streaming in *Physarum* is known to coexist with the cyclic assembly and disassembly of cytoplasmic actin filaments, which are dense in the ectoplasmic (gel) layer just inside the membrane but also occur in the endoplasm (Isenberg and Wohlfarth-Botterman, 1976; Ishigami et al., 1987; Nagai and Kato, 1975; Nagai et al., 1978). Theories of motility based on these processes all contain a chemo-mechanical coupling, either to cytoplasmic convection (Dembo et al., 1986) or to strand tension or strain (Odell and Frisch, 1975; Oster and Odell, 1984*a*, *b*), as part of the oscillatory feedback mechanism. However, regular Ca^{2+} oscillations of the same period as shuttle streaming (100–200 s) have been observed in a *Physarum* homogenate (Yoshimoto and Kamiya, 1982), which strongly suggests (Ueda et al., 1986) that shuttle streaming is driven by an autonomous field of chemical oscillators in the cytoplasm, which function independently of tension generation or fluid flow.

In *Physarum*, the tension and ATP concentration oscillate in phase (Kamiya et al., 1981; Yoshimoto et al., 1981*b*), but in antiphase with oscillations in the cytoplasmic free calcium level (Yoshimoto et al., 1981*a*). The fractional amplitude of ATP oscillations is smaller than that of Ca^{2+}. There are large-amplitude oscillations in cAMP (Ueda et al., 1986) whose phase is close to that of the calcium oscillation. These authors also found that cGMP is present but does not oscillate.

Wohlfarth-Botterman and Fleischer (1976) have concluded that the cyclic transformations in actin polymerization that accompany shuttle streaming are part of the contraction-relaxation cycle of cytoplasmic actomyosin, from which it might easily be inferred that these changes are an essential part of the *Physarum* oscillator. Cyclic actin polymerization has been shown (Dembo, 1986) to be the cause of "flare" (Taylor et al., 1973) and "rosette" (Kuroda, 1979) flow patterns observed in demembranated cytoplasm. The toxin phalloidin, which is known to block actin depolymerization but not actomyosin ATP-ase activity, stops shuttle streaming (Stockem et al., 1978; Gotz van Olenhusen and Wohlfarth-Botterman, 1979). However, it is not known if phalloidin stops the calcium oscillator, so it is quite possible that the actin assembly/disassembly cycle is simply driven by an independent oscillator and that shuttle streaming requires periodic depolymerization of F-actin to lower the viscous resistance to flow. The time scale of $F \rightleftharpoons G$ actin conversion is quite fast, ~ 10 s (Kuroda, 1979). Similar comments apply to theories based on cyclic gel-sol conversion (Hellewell and Taylor, 1979) in which solation is triggered by micromolar calcium (Condeelis and Taylor, 1977). In support of this view, Kamiya and

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Nakajima (1955) found that chemical oscillations persist when shuttle streaming was blocked by carbon dioxide gas, which prevented solation of the gel state.

The impermeability of the *Physarum* membrane considerably reduces the number of possible mechanisms for oscillation. There is probably no similarity with the ATP-cAMP-adenylate cyclase oscillator in *Dictyostelium discoideum,* whose membrane is permeable to cyclic AMP. In fact, ATP is now thought to play only a subsidiary role in the *Dictyostelium* oscillator (Martiel and Goldbeter, 1987; Othmer et al., 1985; Rapp et al., 1985) and in both genera the amplitude of ATP oscillations is quite small, possibly because the mitochondrial ATP supply is buffered by phosphocreatine. Also, we have attempted, without success, to devise a model oscillator based on actin filament assembly/disassembly, which requires the involvement of ATP. For these reasons, ATP will not be considered as an essential oscillatory variable. Other possible mechanisms are considered in the discussion section.

We suggest that the following mechanism, also speculative, is worthy of consideration. Myosin in smooth muscle has two regulators of its ATPase activity with actin. The first is phosphorylation of its P-light chain which is regulated by myosin light chain kinase in the presence of Ca_4-calmodulin (Hartshorne, 1987; Kamm and Stull, 1985): there is evidence for kinase activity in physarum (Kohama, 1987). The second is a down modulation of the first process by phosphorylation of the kinase, which leads to an increase in free calcium level (and therefore, a decrease in light chain phosphorylation) because of the decreased affinity of calcium-calmodulin for the kinase when the latter is phosphorylated (Adelstein et al., 1978; Conti and Adelstein, 1981). Phosphorylation of myosin LC kinase is activated by the catalytic part of cAMP-dependent protein kinase (PKA) (Kamm and Stull, 1989; Swillens and Dumont, 1977) so it can ultimately be regulated by calcium through adenylate cyclase and cAMP production (Birnbaumer, 1977). The kinase phosphorylation rate could be 0.1–1.0 s^{-1}, depending on the concentration of PKA (Stull et al., 1986). We assume that calcium binding to adenylate cyclase activates the AC-cAMP-PKA chain for LC kinase phosphorylation. Then, an assembly of LC kinases bound to their myosin S1 units can exist in either of two stable states with the same total calcium content, but with different free calcium levels and different kinase phosphorylation fractions. This is true over a limited range of total calcium concentrations. A transition from the low free calcium-low phosphorylation state to the high free calcium-high phosphorylation state can be made by raising the cytoplasmic calcium level; this will phosphorylate more LC kinase, and throw off more free calcium because of the decreased affinity of calcium-calmodulin for the kinase. This process constitutes a positive feedback loop, somewhat analogous to calcium-stimulated calcium release. It works only at micromolar calcium levels, because at higher calcium levels the binding of calcium-calmodulin to all light chains is saturated, and kinase phosphorylation can only take place in the absence of bound calcium-calmodulin (Conti and Adelstein, 1981; see also Ikebe and Reardon, 1990).

By coupling this bistable calcium-myosin light chain-cAMP system with an (ATP-driven) calcium pump to leaky vacuoles, a relaxation oscillator can be made (Fig. 1), in which calcium leaks out of the vacuoles and binds to myosin light chains, eventually rising to a level sufficient to trigger kinase phosphorylation. This provokes an additional rise by calcium dissociating from the kinase-phosphorylated myosin. As extra calcium is slowly pumped away to the vacuoles, the calcium level drops until the kinase is dephosphorylated, soaking up more calcium until the whole process starts again.

However, the following modifications need to be made for *Physarum* myosin. Kohama and others (Kohama, 1987) have shown that *Physarum* myosin does not bind calmodulin via a kinase and indeed each S1 unit has a calmodulinlike sequence on its calcium-binding light chain (denoted by LC_2). Whereas smooth muscle myosin can only have its kinase phosphorylated if calmodulin (with or without bound calcium) is absent, physarum myosin has its calmodulinlike sequence built in, so this restriction must be ignored if the kinase is to be phosphorylated at all. It is assumed that kinase phosphorylation can take place for any number of calcium ions bound to LC_2, up to the maximum number of two.

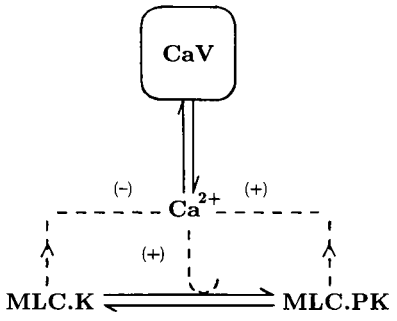

FIGURE 1 Feedback loops in the proposed *Physarum* oscillator. MLC.K (myosin light chain plus kinase) lowers cytoplasmic Ca^{2+} by binding it strongly, whereas the phosphorylated kinase MLC.PK can raise it by binding only weakly. However, calcium can itself trigger the high calcium state by activating the phosphorylation of the kinase (through adenylate cyclase and production of cAMP and cAMP-dependent protein kinase [PKA]). Slow changes in calcium level are caused by calcium transfer with internal vacuoles (*circled*).

Kohama and colleagues have also found that calcium inhibits actomyosin ATPase activity with physarum myosin (Kohama, 1981, 1987), which is the reverse of what happens in smooth muscle (Kamm and Stull, 1985) and directly regulated muscle, e.g., scallop muscle (Okagaki et al., 1989). If we assume that contractile activity is regulated solely by phosphorylation of the P-light chain (denoted LC_1), then it is this step which must be switched off when a sufficient number of calcium ions are bound to LC_2.

MATHEMATICAL FORMULATION

Fig. 2 shows the complete set of reactions for the proposed oscillator. Many rapid steps can be omitted to produce the simplest mathematical description capable of oscillations, as follows. All calcium binding steps are assumed to be in rapid equilibrium. The calcium-adenylate cyclase-cAMP-PKA chain for activating kinase phosphorylation is contracted to an immediate dependence of the phosphorylation rate $k_Q(n_c)$ on the cytoplasmic Ca^{2+} concentration n_c. The rates of phosphorylation and dephosphorylation, k_p and k_D, of the P-light chains LC_1 of myosin are fast enough to establish an instantaneous steady-state distribution between the left- and right-hand blocks in Fig. 2 on the time scale of the oscillator. The rates $k_Q(n_c)$ and k_E of the vertical transitions are assumed to be the same for the left and right blocks, so that one oscillatory variable is the probability $\phi(t)$ that a LC-kinase is phophorylated (occurrence of the top blocks in Fig. 2). The other variable must be the total calcium concentration $c(t)$ outside vacuoles, including calcium bound to myosin and adenylate cyclase. The reason for this choice is that fast reversible calcium binding events to these substances do not change the value of $c(t)$, which is affected only by calcium exchange with the vacuoles.

Hence, the equations of the oscillator are,

$$\dot{c}(t) = k_L(N_c - c(t)) - k_V n_c(t) \quad (1)$$

$$\dot{\phi}(t) = k_Q(n_c(t))(1 - \phi(t)) - k_E\phi(t), \quad (2)$$

where the free calcium level n_c is to be regarded as a function $n_c(c, \phi)$ of $c(t)$ and $\phi(t)$, determined by a conservation law,

$$c = n_c + N_M \sum_{k=1}^{2} k[q_{ka}(n_c)(1 - \phi) + q_{kb}(n_c)\phi], \quad (3)$$

for cytoplasmic calcium. The second term on the right-hand side is the amount of calcium bound to the Ca-light chains of myosins. N_M is the concentration of myosin S1 units, and q_{ka} and q_{kb} are the fractions of unphosphorylated and phosphorylated myosin kinases with k bound calcium ions, $k = 1, 2$. Assuming that the two calcium binding sites act independently with the same affinity, which is K_a or K_b, then,

$$q_{ka}(n_c) = \frac{(K_a n_c)^k}{(1 + K_a n_c)^2} \quad (4)$$

$$q_{kb}(n_c) = \frac{(K_b n_c)^k}{(1 + K_b n_c)^2}.$$

The kinase phosphorylation rate in Eq. 2 is chosen to be

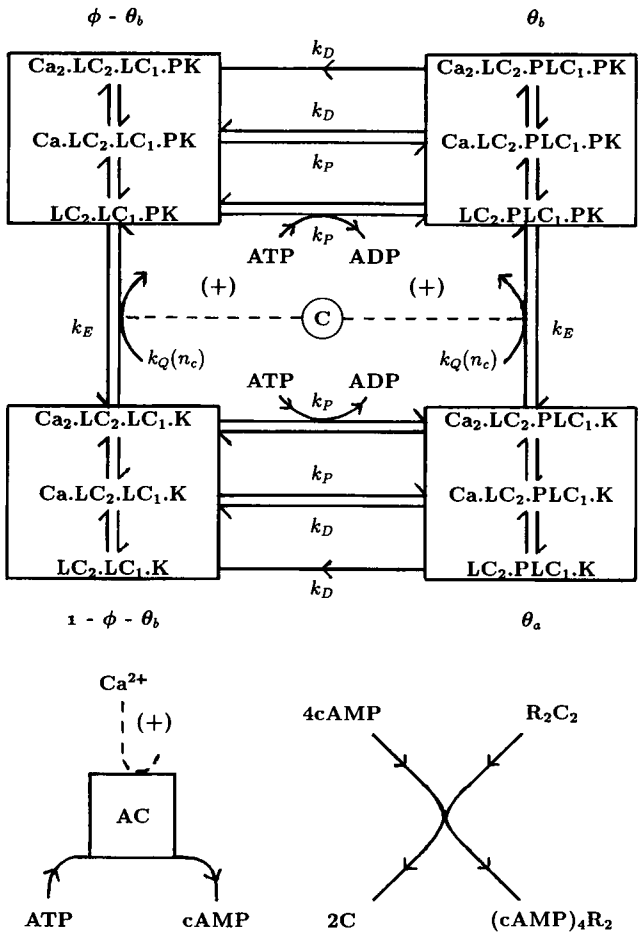

FIGURE 2 The complete set of reactions for bistability in *Physarum*. The four blocks represent the phosphorylation states of LC_2 (the calcium-binding light chain) and LC_1 (the phosphorylable light chain) of myosin with an attached kinase K, which can also be phosphorylated). The probabilities of these stats are θ_a, $\phi - \theta_a$, $1 - \phi - \theta_b$ and θ_b, going clockwise from bottom right. R_2C_2 is cAMP-dependent protein kinase (PKA), C is its regulatory subunit and AC is adenylate cyclase. All steps are taken to be in rapid equilibrium or at steady-state conditions except the vertical transitions (kinase phosphorylation and dephosphorylation). Calcium exchange with vacuoles is not shown.

an increasing function,

$$k_Q(n_c) = k_Q\left(\frac{K_* n_c}{1 + K_* n_c}\right)^\beta, \qquad (5)$$

of the free calcium level n_c with a Michaelis-Menten constant K_* and a Hill coefficient β. This form can be justified from the adenylate cyclase-cAMP-PKA activation chain in restricted circumstances, but the value of K_* will be treated as adjustable. It can be shown that the value of the Hill coefficient β lies between 4 and 12, increasing with the degree of irreversibility in the production of protein kinase A, if three bound calcium ions are required to activate one adenylate cyclase molecule for cAMP production (Brostrom et al., 1978). Eq. 3 does not include calcium bound to adenylate cyclase, but the concentration of the latter is probably too small to affect the calcium balance. Table 1 lists values of all constants used in the computing.

Eqs. 1–3 for $c(t)$, $\phi(t)$ and $n_c(t)$ were solved numerically on a main frame computer using a standard fourth-order Runge-Kutta routine, and solutions of Eq. 3 for n_c by bracketing and bisection (program CAOSC). There is a domain of parameter values giving oscillatory solutions, which is too small to be determined by trial and error but can always be located by the following recipe. An auxiliary program CAROOT was written to plot the right-hand side of Eq. 2 for $\dot{\phi}$ as a function of ϕ for a given value of c, using Eq. 3 to evaluate the function $n_c(c, \phi)$. This is an increasing function of both variables, so that as ϕ increases from 0 to 1, the time derivative of ϕ, after going negative, can become positive again if kinase phosphorylation occurs in the middle of the range of ϕ values. This will occur if the threshold value n_{c*} for kinase phosphorylation, which is inversely related to K_*, lies between the free calcium levels $n_{ca}(c)$, $n_{cb}(c)$ corresponding to $\phi = 0$ and 1, respectively. The program calculates these limits for a given value of c, then prompts for n_{c*}. Experience shows that it is best to choose a value close to the upper limit n_{cb}.

CAROOT can also be used to discover the range of values of c giving bistability, as in Fig. 3. The dashed curve has three fixed points (intersections with the horizontal axis), of which the outer two are necessarily stable and the middle one unstable, as follows from the signs of the derivative on either side of the crossings.

TABLE 1 Parameter values used

$k_P = 0.5\ s^{-1}$	$k_D = 0.2\ s^{-1}$	$k_Q = 1.0\ s^{-1}$	$k_E = 0.1\ s^{-1}$
$k_V = 0.08\ s^{-1}$	$k_L = 0.004\ s^{-1}$	$K_A = 3.0\ s^{-1}$	$K_B = 0.15\ \mu M^{-1}$
$K_* = 1.5\ \mu M^{-1}$	$N_c = 25\ \mu M^{-1}$	$N_M = 10\ \mu M^{-1}$	$\beta = 4$

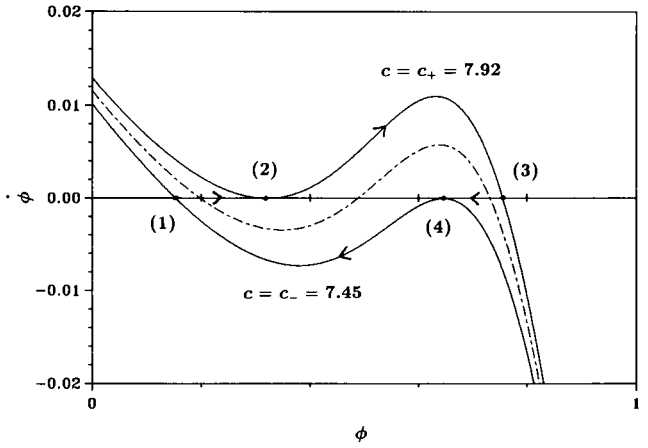

FIGURE 3 Computed form of the right-hand side of Eq. 2 as a function of the kinase phosphorylation fraction ϕ for the limiting values c_+, c_- of total calcium concentration giving bistability, and an intermediate value (*dashed curve*). The fixed points labelled 1,2,3,4 define the locus and direction of the oscillator pathway in the limiting case where calcium exchange with vacuoles is infinitely slow.

The limits of bistability are given by the full curves, with calcium levels $c_+ = 7.92$ and $c_- = 7.45$ μM. This was achieved with $K_* = 1.5$ and $K_a = 3.0$, $K_b = 0.15$ in units of μM^{-1}. The ratio K_a/K_b is 20 as found for smooth muscle myosin (Conti and Adelstein, 1981). The values ϕ_i of the four fixed points for these limiting values of c were then read off the data files. A second auxiliary program CAPLOT was used to generate the free calcium level $n_c(c, \phi)$ against c for each of these values of ϕ, giving the curves shown in Fig. 4.

For oscillations to occur, the calcium pump must be slower than the leak rate from the vacuoles at fixed point 1 of Fig. 3, so cytoplasmic calcium as measured by c slowly rises and the fixed point moves to 2. When c rises above this level, the left-hand fixed point vanishes and kinase phosphorylation occurs finishing at the right-hand fixed point 3. The value of the free calcium level n_c in the cytoplasm has now risen abruptly, because the light chain with phosphorylated kinase does not bind calcium so well. Because of this increase, the calcium pump operates at above the leak rate and reduces cytoplasmic calcium, taking the fixed point to 4. At that stage, the kinase is dephosphorylated and the cycle is complete. These operations of the calcium pump are assured if its load line,

$$n_c = \frac{k_L}{k_V}(N_c - c), \qquad (6)$$

(for which $\dot{c} = 0$) lies between the fixed points 2 and 4 as marked on Fig. 4. We used this condition to set an

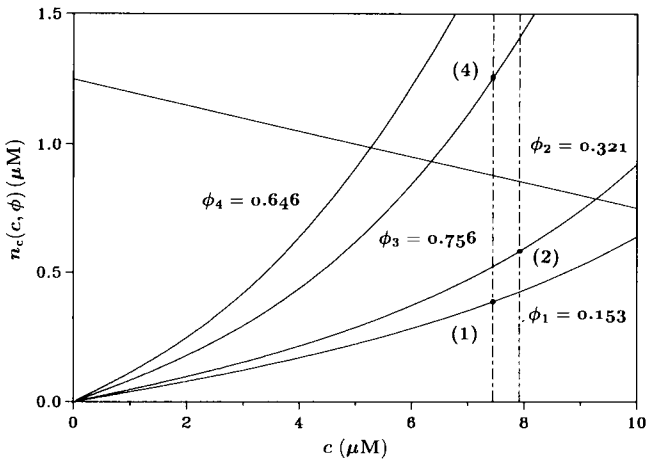

FIGURE 4 Computed curves of free calcium level n_c versus total calcium level (free or bound to myosin light chains) c at the four values of ϕ corresponding to the fixed points in Fig. 3. The chosen load line of the calcium pump to vacuoles (Eq. 6) is shown, intersecting the vertical axis at $n_c = (k_L/k_V)N_c$ and the horizontal axis at $c = N_c$. For the bistable kinase to give oscillations, the load line must pass between the points 2 and 4.

appropriate value of k_v, given a suitable choice of the total calcium concentration N_c in *Physarum* cytoplasm, including vacuoles. The leak rate k_L then determines the frequency of the oscillator.

In this description, the fixed points of Eq. 2 are calculated at constant c, and can be made to come and go by changing the value of c. However, c is not a parameter but a variable in time, so that this one-dimensional stability analysis becomes exact in the limit in which $c(t)$ varies very slowly on the time scale of kinase phosphorylation, i.e., k_V, $k_L \ll k_Q$, k_E. Kinase phosphorylation and dephosphorylation events then occupy a very small fraction of the oscillation cycle. This analysis is simple and provides a reliable guide to the conditions for oscillation when k_L and k_V are small but finite, although the waveforms then become smoother. There is no need for a full stability analysis of both equations of motion.

There is some flexibility in calculating filament tension, as various assumptions can be made about how calcium inhibits the contractile activity of actomyosin. It is now known (Okagaki et al., 1989) that regulation in *Physarum* is indirect via phosphorylation of the P-light chain (LC_1) of myosin, as it is in smooth muscle. There is a choice: two bound calcium ions per light chain may be required to inhibit contractility, or only one may be sufficient. We assumed the former. Then steady-state conditions for the phosphorylation-dephosphorylation reactions of LC_1 give the occupation fractions θ_a, θ_b of the states PLC_1.K and PLC_1.PK in Fig. 2, viz.

$$\theta_a = \frac{k_P(1 - q_{2a}(n_c))}{k_P(1 - q_{2a}(n_c)) + k_D}(1 - \phi) \quad (7)$$

$$\theta_b = \frac{k_P(1 - q_{2b}(n_c))}{k_P(1 - q_{2b}(n_c)) + k_D}\phi, \quad (8)$$

and the quantity $\theta_a(t) + \theta_b(t)$ is taken as a measure of the tension $T(t)$. It is possible that bound calcium on LC_2 regulates contractility directly, as in scallop muscle, but in an inhibitory fashion. The tension will still be a decreasing function of n_c, but light-chain phosphorylation will not be part of the total ATP-ase rate. The contributions to ATP-ase are considered in the next section.

Table 2 lists and defines all mathematical symbols used in the main text of this paper.

RESULTS

Fig. 5 shows the computed waveforms for the parameters of Table 1, starting from the values $c = 7.7$, $\phi = 0.5$. No waveform is strictly sinusoidal, although the $\phi(t)$ waveform is closest. The tension has sharply defined minima whose phase is within 10° of the maxima in free

TABLE 2 Compendium of mathematical symbols used

c	total cytoplasmic Ca^{2+} concentration outside vacuoles
k_P	phosphorylation rate const. of myosin P-light chain
k_D	dephosphorylation rate const. for above
k_Q	maximal phosphorylation rate const. for myosin LC kinase
k_E	dephosphorylation rate const. for above
k_V	rate const. for Ca^{2+} pump to vacuoles
k_L	Ca^{2+} leak rate const. from vacuoles
K_A	Ca^{2+} affinity for Ca-LC with unphosphorylated kinase
K_B	as above with phosphorylated kinase
K_*	effective activation constant for Ca^{2+}-AC-cAMP-PKA chain
n_c	free cytoplasmic Ca^{2+} concentration
n_{c*}	value of n_c for 50% activation in Eq. 5.
n_{ca}	value of n_c for unphosphorylated kinase
n_{cb}	value of n_c for phosphorylated kinase
N_c	total calcium concentration in cytoplasm, incorporating vacuoles
N_M	total myosin S1 concentration
β	Hill coefficient for Ca^{2+}-AC-cAMP-PKA chain
ϕ	fraction of S1's with phosphorylated kinases
q_{2a}	fraction of S1's with two bound Ca^{2+} ions, given an unphosphorylated kinase
q_{2b}	as above, given phosphorylated kinase
R	total ATP-ase rate per unit volume
T	tension per actin filament
θ_a	fraction of S1's with unphosphorylated P-LC but phosphorylated kinase
θ_b	fraction of S1's with P-LC and kinase both phosphorylated
θ	$\theta_a + \theta_b$

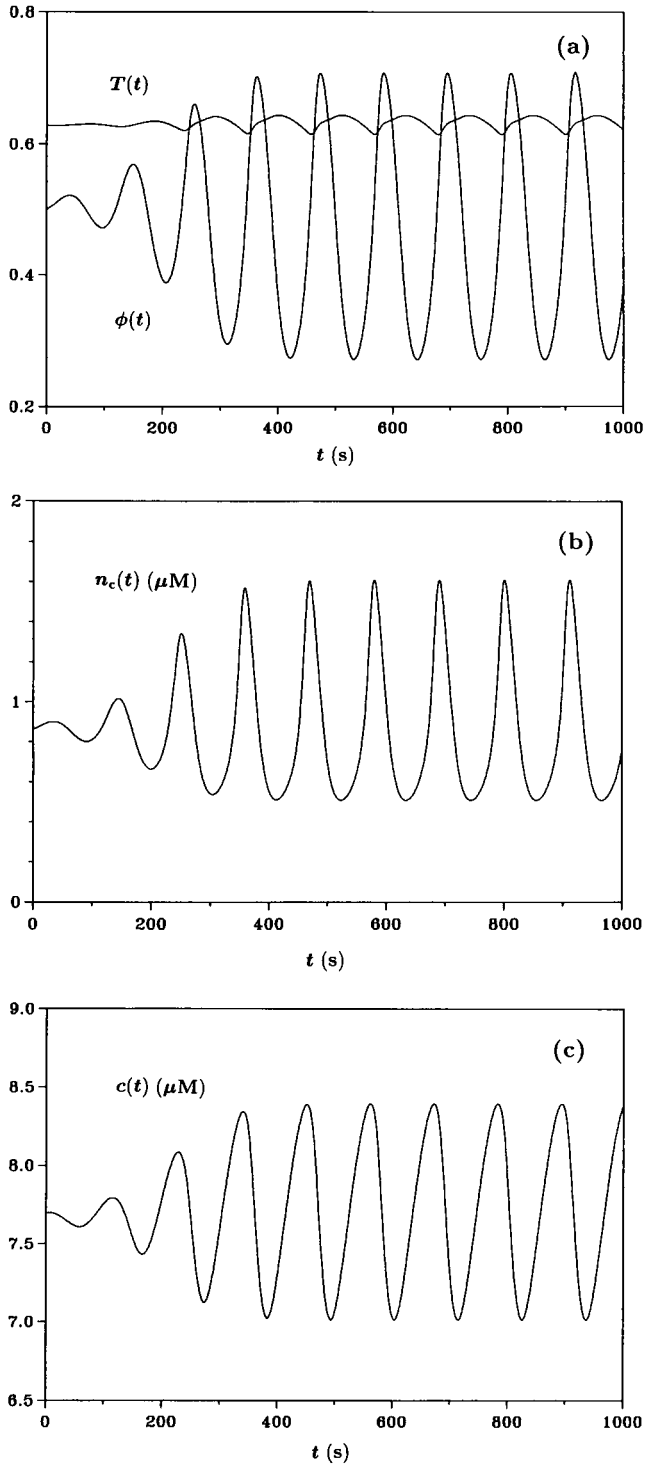

FIGURE 5 Predicted oscillations in (*a*) tension T and the kinase phosphorylation fraction ϕ, (*b*) free calcium concentration n_c and (*c*) total calcium concentration c outside vacuoles. The tension is measured per actin filament in units of the expected tension per myosin head, of order 3×10^{-12}N (Huxley, 1957).

calcium oscillations in Fig. 5 *b*. The model does not guarantee an exact antiphase relation between tension and calcium, because of the contribution from θ_b: although θ_a and θ_b are decreasing functions of n_c, the latter is an increasing function of ϕ, whose oscillations are almost exactly in phase with free calcium as expected. There is no reason to expect that the kinase-phosphorylated state PLC_1.PK will not contribute to tension, although no direct evidence is available. Making the assumption that only one bound calcium is sufficient to inhibit contractility gave an unacceptable tension waveform. The tension and free calcium waveforms are in reasonable qualitative agreement with those observed in *Physarum*. No attempt was made to compare the absolute amplitude of the tension oscillation with experiment, because of uncertainties in the areal density of tension-bearing actin filaments in a strand. The average free calcium level found in Fig. 5 (1.1 μM) is higher than published values of 0.2–0.4 μM (Ueda et al., 1978), but this can be rectified by increasing the myosin content N_M. The fractional amplitude of the simulated tension oscillations (6% p-p) is similar to that observed by Yoshimoto et al. (1981) (Fig. 6 *a*). The fractional amplitude of oscillations in the free calcium level n_c is larger than that observed externally by the above authors using an ionophore, while similar to oscillations observed in a homogenate at micromolar Ca^{2+} levels (Yoshimoto and Kamiya, 1982). There is qualitative agreement with the shape of the waveforms, which are sharpened sinusoids.

In general, the parameter values used (Table 1) were carefully chosen to correspond with available biochemical data. The values of k_P and k_D are taken from the smooth muscle literature (Hai and Murphy, 1988). K_a is similar to values quoted by Kohama (1987), and the ratio K_a/K_b was set at 20 as quoted by Conti and Adelstein (1981) for the analogous case of Ca_4-calmodulin in smooth muscle. Adenylate cyclase must be present in physarum, because it is the only source of cAMP (Rasmussen, 1981), which is present (Ueda et al., 1986). Usually, adenylate cyclase is bound into the membrane but this may not be so in physarum because there is no lipid cell wall for physarum strands, only a slime layer (Kessler, 1982). The total myosin concentration must also be very low; the molar actin:myosin ratio can be as high as 200 compared with 10 or so in smooth muscle (Kessler, 1982). A notional value of 10 μM for N_M seems reasonable by comparison with smooth muscle. For simplicity, we have assumed that each myosin S1 unit has an attached kinase. This is probably not the case, as kinase concentrations in smooth muscle can be of the order of 1 μM (Marston, 1982). However, Marston has shown that the kinase-calmodulin complex in smooth muscle is capable of phosphorylating many myosin light chains. It can be shown that the structure of Eqs. 1 and 2

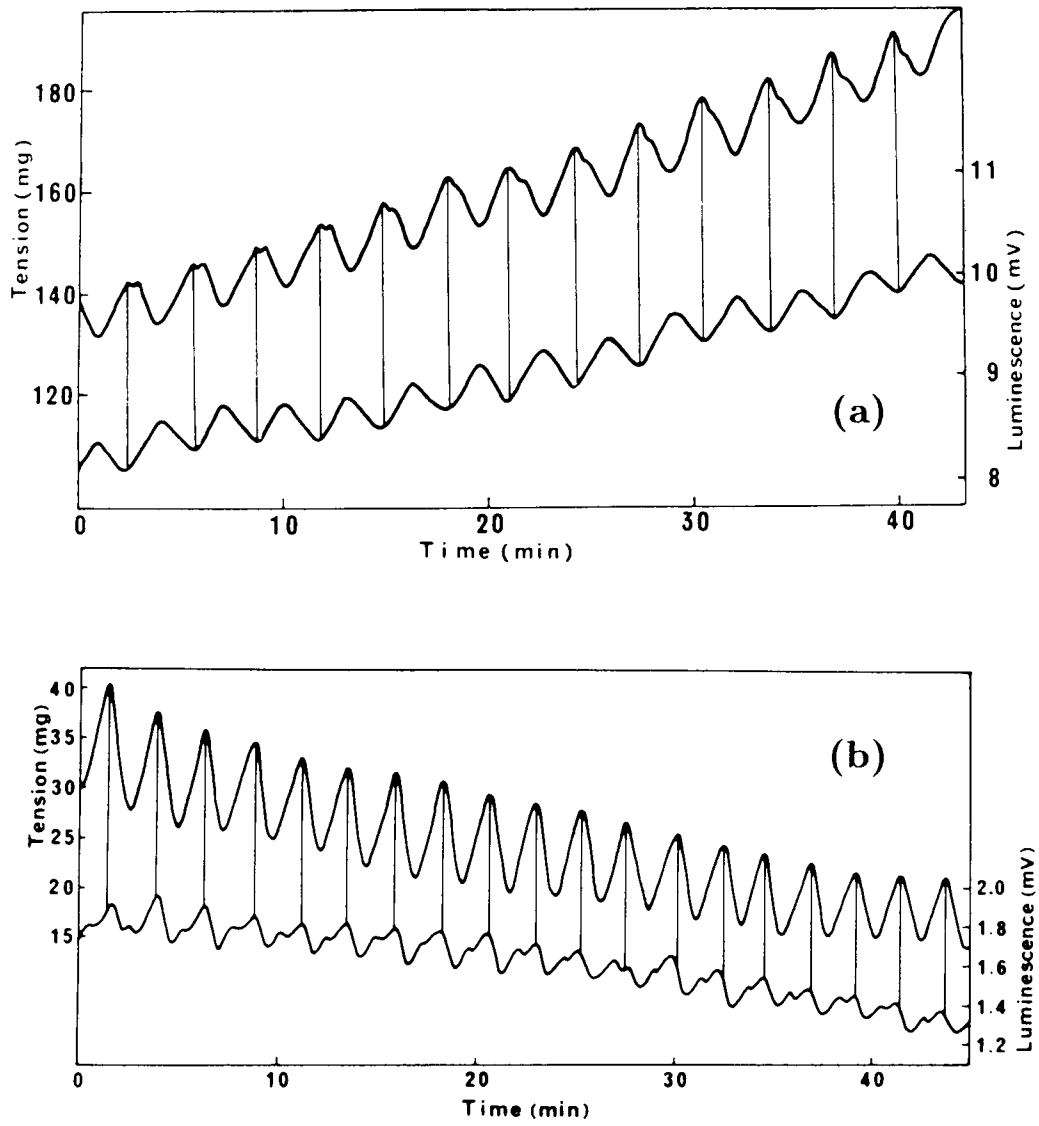

FIGURE 6 Published experimental records on physarum: (*a*) Tension (*top trace*) and free calcium (*bottom trace*) as measured externally by aequorin luminescence in a permeabilized strand (Yoshimoto et al., 1981), (*b*) Tension (*top trace*) and ATP concentration (*bottom trace*) observed with luciferin-luciferase (Yoshimoto et al., 1981). Reproduced with permission from Chapman and Hall, London.

is preserved when the kinase is in rapid equilibrium with myosin.

The parameters of the calcium pump are comparable with existing data (Kato, 1979). Its rate constant is k_V = 0.08 s^{-1} with an average free calcium level of 1.1 μM (Fig. 5 *b*) giving a pump rate (for Ca^{2+}) of 0.088 μM.s^{-1} compared with 0.071 M.s^{-1} from Kato's results using an ATP concentration of 20 μM (Kamiya et al., 1981). The calcium storage capacity of Kato's vacuoles was 24 μM, so N_c must be above this value. Choosing N_c = 25 μM requires a leak rate k_L of ~0.004 s^{-1} (Table 1) to position the load line for oscillations, but the equivalent value which fits Kato's data is 0.02 s^{-1}. It is not possible to raise our chosen value without making N_c lower than Kato's estimate or reducing the period of oscillation. However, the kinetics assumed in Eq. 1 are probably too simple to describe the pump's behavior exactly. Only K_* was adjusted without reference to published data, but the final value used (1.5 μM^{-1}) seems plausible.

The occurrence and nature of the oscillations are sensitive to some parameters. There is a narrow window of values of K_* giving bistability and hence oscillations, in this case from 1.14 to 1.89 μM^{-1}. For the given value of N_c, the limits of the load-line in Fig. 3 require the pump rate k_V to lie between 0.056 and 0.12 s^{-1}. Pushing the myosin concentration up to 100 μM increases the

amount of gain in the oscillatory feedback loop, and produces pronounced upward spikes in the waveforms for free calcium and kinase phosphorylation fraction $\phi(t)$, due to fast kinase phosphorylation. The total calcium concentration $c(t)$ does not change so rapidly and assumes a sawtooth waveform. In principle, these waveforms could be smoothed out by reducing the values of k_Q and k_E, but if this is done the system usually drops out of oscillation before substantial smoothing can be achieved. However, at a myosin concentration of 10 μM, the waveforms are sufficiently close to what is observed that further smoothing appears to be unnecessary.

Although ATP concentration in the cytoplasm is known to oscillate, it has not been computed explicitly because this requires some knowledge of how it is regulated by the mitochondria. There is an inverse relationship between ATP concentration and the activated demand for ATP, as measured by the net rate R of ATP hydrolysis. This follows if it is assumed that the mitchondria adjust instantaneously to changes in the ATP consumption rate; the proof is elementary and will not be given here. Because ATP concentration and tension are observed to be in phase (Fig. 6 *b*), the ATPase rate must oscillate 180° out-of-phase with the tension. By comparison with striated or smooth muscle, this is as unexpected as the out-of-phase relation between tension and calcium. It cannot just be due to the inhibitory nature of physarum myosin with respect to calcium, since the contractile ATPase rate and tension generation are both part of the actomyosin contraction cycle which is probably common to all motile systems driven by these proteins (Marston, 1982). This phase problem can be resolved if the actomyosin ATP-ase rate is dominated by another ATP-ase which increases with free cytoplasmic calcium level n_c. Fig. 7 shows the ATPase rate calculated from four independent sources present in our model, which combine to give a total ATPase rate oscillating in-phase with free calcium as required. The figure shows that the oscillatory part, although not the average level, of the contractile ATP-ase is dominated by oscillations in the rates of vacuole pumping and kinase phosphorylation, both of which hydrolyse ATP. This state of affairs is delicately balanced and can be upset by changing the values of k_P, k_D, k_Q, or the actin-myosin cycling rate. The curves in Fig. 7 were computed from the Appendix, which lists and estimates all likely sources of ATP-ase in physarum. It should be noted that assembly and disassembly of actin filaments, which in our model is driven by independent oscillations in calcium level, is apparently not a major source of ATPase as the rate-limiting step in this cycle

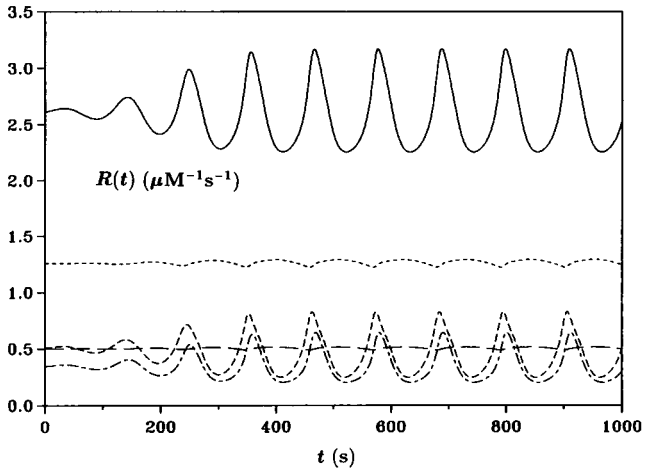

FIGURE 7 Time dependence of the ATP-ase rate (*full line*) predicted as in the Appendix from the following contributions: (*a*) the calcium pump to vacuoles (*dash-dot curve*), (*b*) myosin light chain phosphorylation (*short dash*), (*c*) myosin LC kinase phosphorylation (*medium dash*), (*d*) actomyosin ATP-ase with Huxley parameters $f = 0.4, g = 0.1$ s^{-1} as for smooth muscle (Hai and Murphy, 1988) (*long dash*). The resultant ATP-ase rate is 180° out of phase with the tension (Fig. 5 *a*), so that oscillations in ATP concentration must be in phase with tension (see text).

appears to be the dissociation of ADP from actin monomers (Pollard and Cooper, 1986).

The phase and amplitude of the chemical oscillator in physarum can be locally reset by injecting a higher concentration of ATP, calcium, et cetera. Excess ATP stimulates tension oscillations in physarum strands (Ueda et al., 1978) and isometric tension in an nonoscillating saponin model (Yoshimoto and Kamiya, 1984). These authors also find that the effect of adding cytoplasmic calcium is biphasic for tension, producing maximum tension at pCa = 7, whereas Ueda et al. (1978) find that extra calcium increases both the average tension and the amplitude of its oscillations, suggesting that calcium is also regulating factors extrinsic to the chemical oscillator. Computational resetting experiments were performed on the model oscillator, by raising the value of one parameter at a time by 10% after periodic oscillations had been established (at t = 1,000 s). Changes in amplitude, phase, period, and average level of the variables n_c, c, ϕ and T were measured and recorded in Table 3, which also lists which parameters are shifted by ATP, calcium, cAMP, and PKA. Because ATP affects many parameters of the model to various degrees, its net effect is unclear but changing its effector K_* has the largest effect on the oscillations. Note that the oscillations are biphasic in K_*, as there is a window of values giving bistability. The effect of raising the total cytoplasmic Ca^{2+} level (and hence N_c by the same amount) is to

TABLE 3 Resets in amplitudes, phases, periods and average levels

10% increase	Effector/s	Shifts in amplitude, phase, period and average level			
		n_c	c	ϕ	T
K_*	ATP,cAMP	−12%	−19%	−15%	−22%
	AC,PKA	−2°	5°	4°	2°
		−7.9%	−7.4%	−7.4%	−7.7%
		−0.02	−0.4	0.04	0.005
k_V	ATP	−13%	−0.5%	−0.3%	0.9%
		15°	4°	12°	0.6°
		4.5%	4.3%	4.7%	4.3%
		−0.1	−0.02	−0.03	0.002
k_Q	ATP	11%	6%	7%	3%
		6°	3°	5°	6°
		−3.3%	−3.2%	−3.2%	−3.2%
		0.04	−0.2	0.02	0.003
k_P	ATP				0.1%
					0°
					0%
					0.02
c,(N_c)	Ca^{2+}	0.6%	−2.5%	−5%	−5%
		13°	12°	6°	19°
		−5%	−4%	−5%	−4%
		0.03	0.007	0.02	−0.001
N_M	myosin	12%	15%	12%	15%
		−102°	−107°	−108°	−112°
		9.25%	9.5%	9.25%	9.25%
		0.02	0.7	−0.01	0.001

The responses to a 10% increase in the values of each parameter of the first column are described by an amplitude shift (as percentage), a phase shift (in degrees), the change in period (percentage) and the absolute shift in average level (units of μM, et cetera.) All parameters in the first column are increasing functions of their effectors. A transient period of 5–7 cycles was allowed before sampling the new periodic waveform. Differences in the phase shifts as established from maxima and minima respectively are due to a change in shape of the waveform and were averaged out. All entries in the same row for the change in period should be identical. Increasing k_D has the same effect as decreasing k_P (Eqs. 7, 8). For all four variables, the amplitude of oscillation and the period are decreased by adding free calcium, cAMP or PKA but are both increased by adding myosin. A myosin increase also introduces a phase lag of ~100°. Note that when the period changes, the phase shift is sensitive to the time of re-setting, which for this purpose was taken as zero.

reduce both steady and oscillatory tension as expected. However, Table 3 is intended solely for the chemical oscillator in *Physarum.*

One phase-resetting experiment which need not be modeled is the alternation of isometric and isotonic conditions (Kamiya, 1978; Nagai et al., 1978), because there is a straightforward interpretation. These authors found that on changing from constant length to constant tension, the filament length oscillated 180° out of phase with the preceding tension, the analogous relationship occurring when isometric conditions were restored. This implies that the contractile tension is a function of filament length rather than filament velocity, i.e., the velocity dependence expected from the sliding filament model of muscle contraction (Huxley, 1957) is overshadowed by a length dependence, possibly arising from passive elastic elements. A formal proof can be given by writing the tension as $T(\theta, l)$, an increasing function of both the degree of activation $\theta = \theta_a + \theta_b$ and filament length l. Under isometric conditions T varies only with θ, to which it is proportional, but in the isotonic case T = constant, forcing l to vary in the opposite sense as θ. An alternative explanation has been proposed by Kamiya et al., (1988), who point out that a catch mechanism could convert the 90° tension-length phase shift arising from a velocity-dependent tension to a 180° phase shift.

DISCUSSION

Although the model presented in this paper agrees with most properties of the endogenous chemical oscillator in *Physarum,* we do not believe that enough information is available to rule out other mechanisms. In fact, there are many possibilities. Our perspective is summarized by the following discussion.

Some of the likely ingredients of the *Physarum* oscillator are: (*a*) a cycle of actin filament polymerization driven by ATP (Pollard and Cooper, 1986) and filament splitting perhaps by fragmin and regulated by calcium (Hasegawa et al., 1980; Furuhashi and Hatano, 1989), (*b*) calcium-regulated production of cAMP through adenylate cyclase bound to the membrane, (*c*) accelerated pumping of Ca^{2+} into vacuoles by cAMP (Hicks et al., 1979), (*d*) calcium-stimulated calcium release (Fabiato, 1985) from vacuoles known to exist in *Physarum* and (*e*) regulation of actin-myosin ATPase by calcium. The apparent lack of channels in the membrane and of cGMP oscillations (Ueda et al., 1986) appear to rule out mechanisms involving inositol triphosphate (Berridge and Irvine, 1984).

The existence of chemical oscillations requires either (1) a negative feedback loop with a large number of slow reaction steps (Rapp and Berridge, 1977), (2) a bistable component which can then be switched by a slow reaction in one of its parameters, or (3) an autocatalytic step, such as (*d*). An oscillator mechanism based on (*a*), plus Ca^{2+} exchange with leaky vacuoles by an ATP-driven calcium pump constitutes a feedback loop of type I only if ATP oscillations are part of the mechanism. Only small-amplitude oscillations in ATP level are observed, perhaps because of buffering by creatine phosphate as in muscle cells. We have not been able to construct a negative feedback loop oscillator based on (*a*) and vacuoles, using ATP, cytoplasmic calcium and the proportions of F and G actin filaments as oscillatory

variables. In view of the very stringent oscillation conditions for oscillators of type I (Rapp, 1976), this is not surprising. Processes (*b*) and (*c*) give mechanism I if calcium activates adenylate cyclase activity, so the feedback loop is Rapp and Berridge's loop B. Again, the existence of oscillations requires many additional slow steps in the feedback loop. Conversely, if calcium inhibits adenylate cyclase, (*b*) and (*c*) can give a bistable calcium pump, but only if the pump could be almost completely switched off in the absence of cAMP, which may be so in physarum but is not the case where the cAMP effect has been studied (Hicks et al., 1979). Processes (*b*) and (*d*) will give a negative feedback loop with an autocatalytic step (mechanism III) provided calcium inhibits adenylate cyclase activity. However, it is doubtful if calcium-stimulated calcium release will function as the autocatalytic step for the slow changes in calcium level that occur in *Physarum,* because only rapid calcium changes (<1 s) provoke stimulated calcium release from the sarcoplasmic reticulum in cardiac muscle (Fabiato, 1985). Our general impression, reinforced by the atlas of Gurel and Gurel (1983), is that phase shift oscillators with one negative feedback loop are so difficult to establish that multiple feedback loops, leading either to bistability or the equivalent of an autocatalytic step, are a priori more likely for oscillating chemical systems in nature. The "two-pools" hypothesis used currently to describe calcium oscillators regulated by inositol triphosphate (Berridge, 1990) is of this kind.

Our proposed mechanism does require that adenylate cyclase is activated by calcium to produce bistability in myosin light-chain kinase. The older work on cyclic nucleotides suggests that calcium can act either as inhibitor or activator under different conditions (Birnbaumer, 1977). Brain adenylate cyclase is inhibited by calcium at concentrations >100 μM (Brostrom et al., 1978). Inhibitory behavior also appears at lower calcium levels unless calmodulin is present, in which case calcium shows an activating effect at concentrations below 0.1 μM (MacNeil et al., 1984). It seems possible that the upper limit of the activating range of calcium concentrations can be raised >1 μM by saturating adenylate cyclase with calmodulin, thus giving activatory response in the free calcium range found here for the physarum oscillator. Calmodulin is present in the cytoplasm of *Physarum* (Kuznicki and Drabikowski, 1979; Kuzncicki et al., 1979).

cAMP oscillations have been observed in physarum, and appear to lag behind the tension by $\sim 120°$ (Ueda et al., 1986), making them $\sim 60°$ ahead of the calcium waveform. This behavior cannot be produced by any model in which adenylate cyclase is activated by calcium, because this would cause cAMP to lag slightly, if at all, behind calcium. We have not been able to construct an oscillator for *Physarum* utilizing calcium inhibition of adenylate cyclase. It should be noted that in smooth muscle cells cAMP generally relaxes muscle tension (Rüegg, 1988), and this is thought to be due to the stimulation of an intracellular calcium pump rather than through any action of cAMP on myosin light chain kinase. An alternative mechanism which might avoid these difficulties is for contractility to be down regulated by phosphorylation of the heavy chain of physarum myosin (Korn and Hammer, 1988) rather than myosin kinase, although not much is known about how this process is regulated.

Shuttle streaming in *Physarum* filaments is to be regarded as a consequence of its endogenous calcium oscillator, but has not been investigated in this paper. Many aspects of shuttle streaming could remain problematic even after the mechanism of its oscillator is settled, notably the location of tension-generating and tension bearing elements in each strand, the old controversy over whether streaming is driven from the front or the back end (Allen, 1973; Yoshimoto and Kamiya, 1978*d*; Kamiya, et al., 1988) and the role of dynamic polymerization or cross-linking of actin filaments. With the availability of an appropriate local mechanism for the driving oscillator, these issues can be addressed through computer simulations, and future papers are planned to this end.

CONCLUSION

A working mechanism is proposed and confirmed by computer modeling for the endogenous calcium oscillator in filaments of the slime-mold *Physarum Polycephalum.* It produces quasisinusoidal in-phase oscillations in tension and ATP concentration, with out-of-phase oscillations in the concentrations of free calcium and cAMP. The mechanism requires calcium to inhibit actomyosin contractility in physarum myosin as discovered by Kohama and others, and also to activate the phosphorylation of myosin light chain kinase. It is suggested that the latter process occurs through calcium activation of the production of cAMP-dependent protein kinase through the membrane-bound protein adenylate cyclase.

APPENDIX

Sources of ATPase in *Physarum*

The expected contributors to ATP consumption in *Physarum* in the context of the present model are listed below. Numerical estimates (in $\mu M.s^{-1}$) for average values over one oscillation cycle are given in

brackets. The parameter values used are listed below or appear in Table 1.

•The calcium pump to vacuoles: (0.44),

$$R_1 = 5k_V(a)n_c \quad (a = [\mathrm{ATP}]), \tag{9}$$

assuming five molecules of ATP are hydrolyzed for each translocated Ca^{2+} ion (Kato, 1979).

•Phosphorylation of the P-light chain of myosin: (1.25)

$$R_2 = N_M[k_P(1 - \phi - \theta_a)(1 - q_{2a}) + (\phi - \theta_b)(1 - q_{2b})]$$
$$= N_M k_D(\theta_a + \theta_b). \tag{10}$$

•Phosphorylation of the myosin kinase: (0.48)

$$R_3 = N_M k_Q(n_c)(1 - \phi). \tag{11}$$

•The actomyosin ATP-ase: (0.50)

$$R_4 = N_M \frac{fg}{f + g}(\theta_a + \theta_b), \tag{12}$$

where f and g are the binding and dissociation rates for a myosin head with/from actin in Huxley's model, and for simplicity, regulation is assumed to be slow compared with the contraction cycle. However, the estimates $f = 0.4$, $g = 0.1\ s^{-1}$ (Hai and Murphy, 1988) for smooth muscle suggest that the rates of regulation and cycling are similar.

•Production of cAMP: ($\sim$0.1?) The rate is hard to estimate; the rate constant is perhaps $>1\ s^{-1}$ but the concentration of adenylate cyclase is not known and possibly $<0.1\ \mu M$, suggesting this contribution is negligible.

•Assembly and disassembly of actin filaments. We believe that this contribution can also be neglected, in spite of the very high concentration, $\sim$1 mM (Hartshorne, 1987), of actin filaments expected. According to Pollard and Cooper (1986), actin polymerization is reversible but can be biased in the forward direction by actin monomers carrying ATP and in the backward direction by ADP-monomers. F-actin, i.e., polymerized actin, is the ATPase with a rate constant of 0.02–0.06 s^{-1}. However, ADP must dissociate from actin monomers before they can pick up ATP and continue a cycle of assembly/disassembly, and this step has a very slow rate indeed, $\sim$0.001 s. Under steady-state conditions the ATP-ase rate is therefore expected to be 0.001 × (ADP monomer concentration). The monomer concentration should be raised by calcium acting on fragmin to sever filaments.

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