

OSCILLATORY CONTRACTION ACTIVITY IN *PHYSARUM*

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SUMMARY

The plasmodia of *Physarum polycephalum* show different oscillatory phenomena (time period approximately 1.3 min) in their contraction behaviour and their protoplasmic flow. The force generating system for these phenomena is cytoplasmic actomyosin. The biochemical nature and location(s) of the oscillator(s), i.e. the clock governing these phenomena are unknown.

The following locations are discussed as possible sites of the oscillator: (1) cytoplasmic actomyosin, (2) the energy supply system, (3) inner Ca^{2+} stores, and (4) the plasmalemma, which must be involved at least in modulating the force generated by the contractile machinery during a chemotactic response.

The following oscillatory phenomena were used to assess the effects of externally and internally applied substances (e.g. calcium antagonistic drugs, caffeine, D_2O) on oscillating force output:

(1) persistence of longitudinal contractile activity of veins (for external application of test substances),

(2) persistence of radial activity of veins (for internal application of the test substances),

(3) *de novo* generation of contractile activity in protoplasmic drops (external application).

The data seem to exclude rhythmical Ca^{2+} , Na^+ or K^+ transport across the plasmalemma as a triggering function for the oscillation. Contractile activity seems to represent a spontaneous, endogeneous oscillation which can be modulated via the plasmalemma during chemotaxis.

INTRODUCTION

The plasmodial phase of the acellular slime mould *Physarum polycephalum* represents a multinuclear mass of protoplasm. Migrating plasmodia (Fig. 1a) are differentiated into a front region, i.e. a compact sheet of protoplasm, and in the rear region into protoplasmic 'veins' or 'strands'. Within the veins protoplasm is transported with velocities up to 1.3 mm/s (Kamiya, 1959). Much of the interest in studying the physiology of *Physarum* lies in the fact that the plasmodia are used to analyse the function of cytoplasmic actomyosin (Komnick *et al.* 1973; Wohlfarth-Bottermann, 1975b).

The protoplasmic veins (Fig. 1b) are differentiated into an ectoplasmic tube

Physarum polycephalum plasmodia

Total size : 1–1000 mm

Vein diameter : 0.1–1 mm

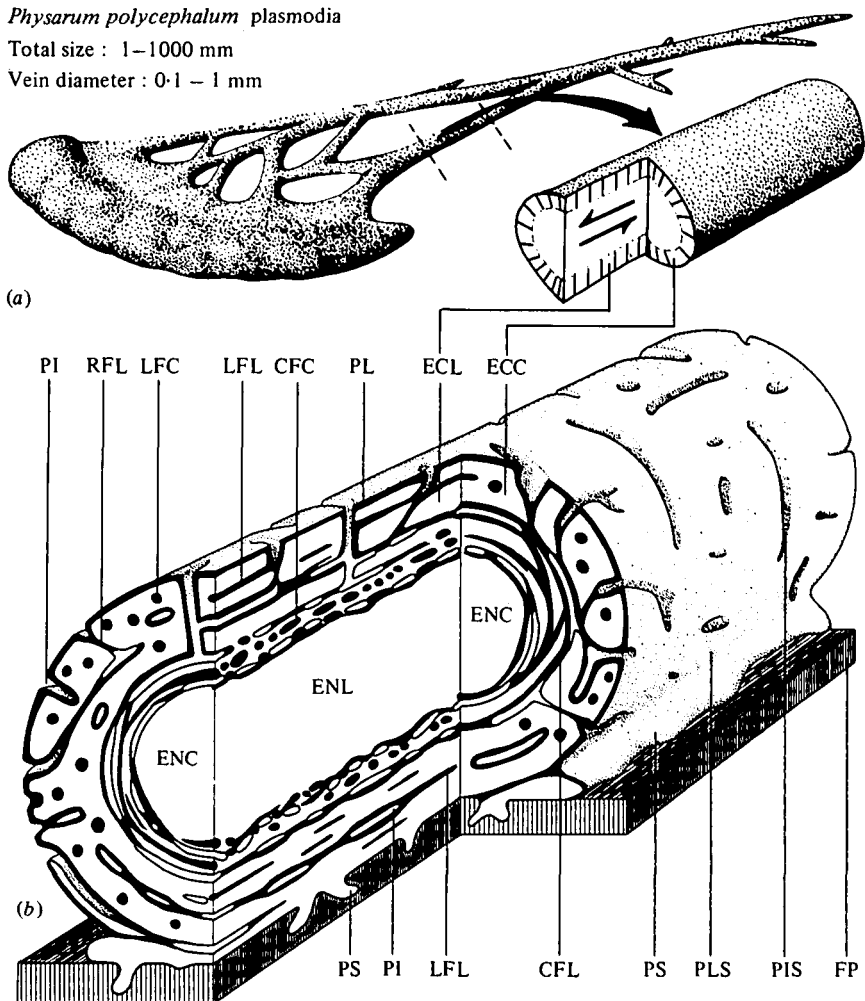


Fig. 1 (a). Diagram of a locomoting *Physarum* plasmodium. Left side: front region; right side: protoplasmic veins in the rear part. The inset (arrow) symbolizes the internal vein structure: hatching = ectoplasmic tube; white = endoplasmic core; \rightleftharpoons changing directions of endoplasmic transport during shuttle streaming.

(b) The detailed structure of a vein. Actomyosin fibrils are restricted to the ectoplasmic region. CFC, Circular fibrils in cross-section; CFL, circular fibrils in longitudinal section; ECC, ectoplasm in cross-section; ECL, ectoplasm in longitudinal section; ENC, endoplasm in cross-section; ENL, endoplasm in longitudinal section; FP, filter paper; LFC, longitudinal fibrils in cross-section; LFL, longitudinal fibrils in longitudinal section; PI, plasmalemma invagination; PIS, plasmalemma invagination in surface view; PL, plasmalemma; PLS, plasmalemma surface; PS, pseudopodium; RFL, radial fibrils in longitudinal section (Fleischer & Wohlfarth-Bottermann, 1975).

(possessing a complicated system of plasmalemma invaginations with intimately connected cytoplasmic actomyosin structures in the form of sheets and fibrils) and an endoplasmic core (representing the more fluid transported protoplasm (Wohlfarth-Bottermann, 1974; 1975c). The rhythmical ebb and flow of endoplasm has a period within the minute range (shuttle streaming), i.e. protoplasmic streaming oscillates ■

respect to its direction (Kamiya, 1959; Sachsenmaier & Hansen, 1973; Hülsmann & Wohlfarth-Bottermann, 1978*b*). A 'long period oscillation' (8–10 h) related to synchronous mitoses (Sachsenmaier & Hansen, 1973) will not be discussed in this contribution.

Morphological basis and contraction phenomena

When considering the question of the nature (Hess & Boiteux, 1971) of the physiological oscillator(s) which drives the periodic streaming phenomena, we have to consider the motile mechanisms responsible for endoplasmic flow. The endoplasm flows passively due to hydrostatic pressure differences developed in different areas of the veins (Kamiya, 1959). These pressure differences are brought about by rhythmic contractions of the ectoplasmic tube, i.e. by oscillatory contractions of the cytoplasmic actomyosin within the ectoplasm (Wohlfarth-Bottermann, 1962, 1965; Hatano & Oosawa, 1966; Fleischer & Wohlfarth-Bottermann, 1975; Wohlfarth-Bottermann & Fleischer, 1976). Recordings of contractile phenomena can be made by cinematography and more recently also by tensiometric and other techniques (Kamiya, 1970; Wohlfarth-Bottermann, 1975*a*; Samans, Götz von Olenhusen & Wohlfarth-Bottermann, 1978; Grebecki & Cieslawska, 1978). Tensiometry is a convenient technique in addition to providing a direct measurement of contractile force in millipond (1 kp = 9.80665 Newtons) of single veins. With this equipment it is possible to register automatically

(1) longitudinal contractile activity of isolated veins (under isometric and isotonic conditions) (Kamiya, 1970),

(2) radial contractile behaviour of veins *in situ* (Wohlfarth-Bottermann, 1975*a*),

(3) longitudinal contractions of veins *in situ* (Hülsmann & Wohlfarth-Bottermann, 1978*a*),

(4) protoplasmic flow rates (in mg/min) of single veins by using the tensiometric electrobalance for weighing inflowing and outflowing endoplasm (Hülsmann & Wohlfarth-Bottermann, 1978*b*).

What are the time periods of the different oscillating phenomena? The mean average time period for longitudinal contractions of isolated veins under isometric conditions lies in the range of 2.1 min, whereas the corresponding value for radial contractions is 1.3 min (Wohlfarth-Bottermann, 1977). The latter value corresponds with the mean average value of the shuttle streaming period (Hülsmann & Wohlfarth-Bottermann, 1978*b*).

These observations raise the question of whether there are different contraction frequencies in one vein (i.e. 2.1 min (longitudinal) and 1.3 min (radial and streaming)). If the tensiometric analysis of radial activities and longitudinal activities of veins are performed *in situ* and both measurements are done simultaneously in one and the same vein by using a twin tension transducer and a special arrangement of the vein (Hülsmann & Wohlfarth-Bottermann, 1978*a, b*), it can be shown that there is only one common time period of 1.3 min and that both contractile activities have a close phase identity.

When radial, longitudinal and streaming phenomena were recorded simultaneously (Fig. 2*a*), all three curves (Fig. 2*b*) reveal identical frequencies and close phase

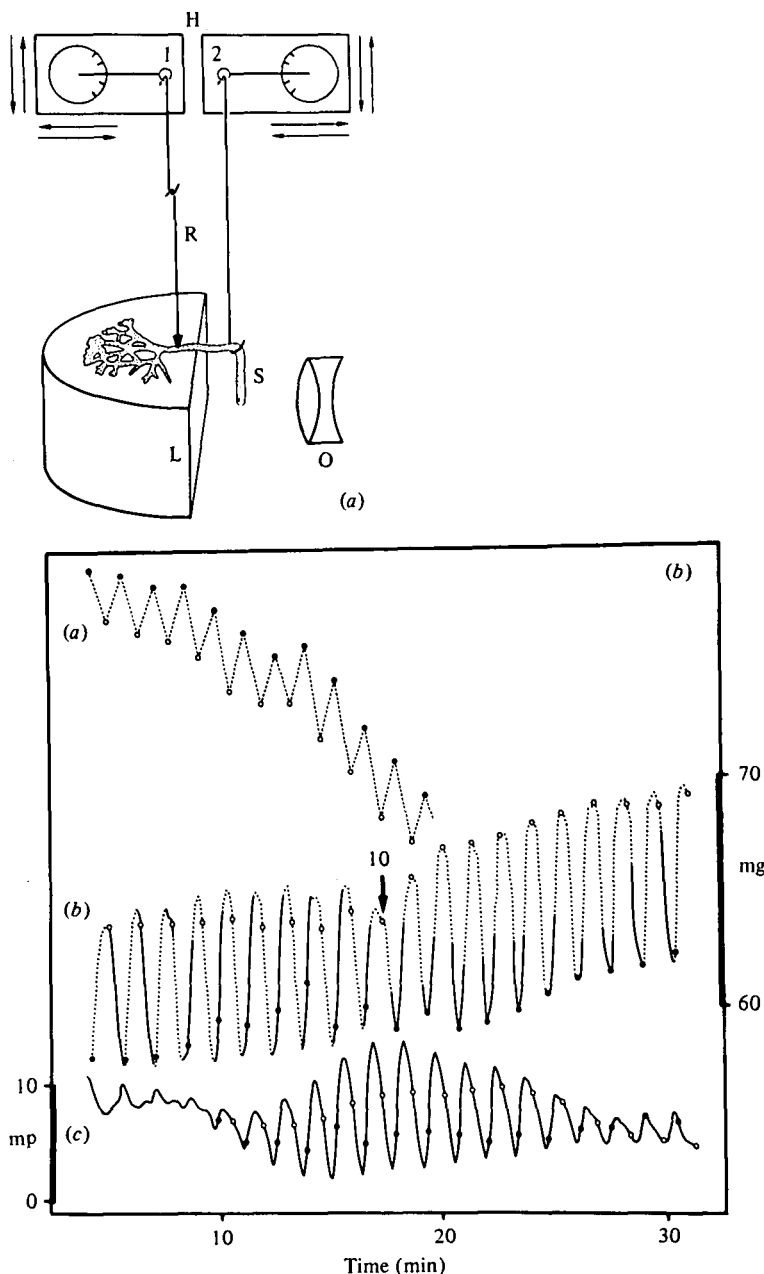


Fig. 2 (a) Twin tension transducer for the simultaneous measurement of radial contractile activity (R), protoplasmic flow rates (shuttle streaming) and isotonic longitudinal contractions (O) on a semi-isolated strand of *Physarum*. H, Tensiometric electrobalances, adjustable in horizontal and vertical directions (arrows). 1, Measuring head for radial activities. 2, Head for measurements of weight changes. R, Rod transferring the radial activity under isometric conditions to the measuring head. S, Strand hanging on a hook and remaining partly in its original position on filter paper. O, Objective of monitoring system.

(b) Experimental arrangement as in (a). Simultaneous registration of (a) isotonic longitudinal contraction rhythms in the suspended strand region (S) registered by TV monitoring (o); (b) weight changes (streaming phenomena) of the suspended region (S) and (c) of radial contraction cycles at the vein part which remained *in situ*. The circles and dots represent the time points of maximal elongation (relaxation, ○) and maximal shortening (contraction, ●) of the suspended part S. The phases of radial relaxation, i.e. of vein dilation (curve C) are marked by dotted lines in the streaming curve (b) (Hülsmann & Wohlfarth-Bottermann, 1978b).

relationships. In summary, the time period of approximately 1.3 min seems to represent the genuine and characteristic frequency for these oscillatory phenomena in *Physarum*.

According to Yoshimoto & Kamiya (1978*a-d*) 'the strand has no active contractile rhythm in both radial and longitudinal directions as long as it is part of the network or ramified region of the advancing plasmodium'. However, the presence of rhythmicity has been demonstrated by applying infrared (Samans *et al.* 1978) as well as cinematographic (Grebecki & Cieslawska, 1978) recording techniques which, in contrast to tensiometry, register radial oscillations without touching the veins. Hülsmann & Wohlfarth-Bottermann (1978*b*) have provided further evidence and arguments against the claims of Yoshimoto & Kamiya (1978*d*) cited above.

Because the sinusoidal form of oscillation curves often suggest the existence of interference phenomena by two waves with slightly different frequencies (Kamiya, 1959, 1970), it has also to be considered that different oscillators may be operating. However, the observed interference phenomena can also result from small local phase shifts of contraction phenomena working with identical frequencies but having a spatially different arrangement (Hülsmann & Wohlfarth-Bottermann, 1978*a,b*). Principally, it is undecided, whether

(i) there are different physiological oscillators interfering with each other, or whether

(ii) there is only one oscillator, and spatially different effector systems interfere with each other.

Contraction-relaxation cycles of strand segments become synchronized under tensiometric conditions (Yoshimoto & Kamiya, 1958*a*). Grebecki & Cieslawska (1978) concluded that 'the entire system of major veins forming the network contracts synchronously'. This statement, however, is not supported by data obtained from infrared techniques (Z. Hejnowicz & K. E. Wohlfarth-Bottermann, unpublished).

The nature and location of the oscillator

What is the physiological nature and location of the oscillator and is there some sort of 'pacemaker' or 'trigger' which governs the contractions? It seems reasonable to discuss the following possibilities concerning the nature and location of the unknown clock:

(1) oscillatory behaviour based on the force generating system itself (i.e. the *cytoplasmic actin and/or myosin*),

(2) oscillation phenomena of the enzymes of the *energy supply* system (glycolysis and/or aerobic respiration),

(3) rhythmical translocation of calcium across the internal membrane systems which regulate the free calcium concentration (10^{-6} to 10^{-7} M) and thereby control contractile activity of cytoplasmic actomyosin,

(4) rhythmical ion movement across the plasmalemma which may act as a trigger for the contractile machinery, either directly or indirectly by inducing calcium release from the inner stores.

One approach is to try and exclude some of these possible mechanisms in order to narrow down the alternatives for future investigations.

The existence of a regulating mechanism synchronizing contraction rhythms

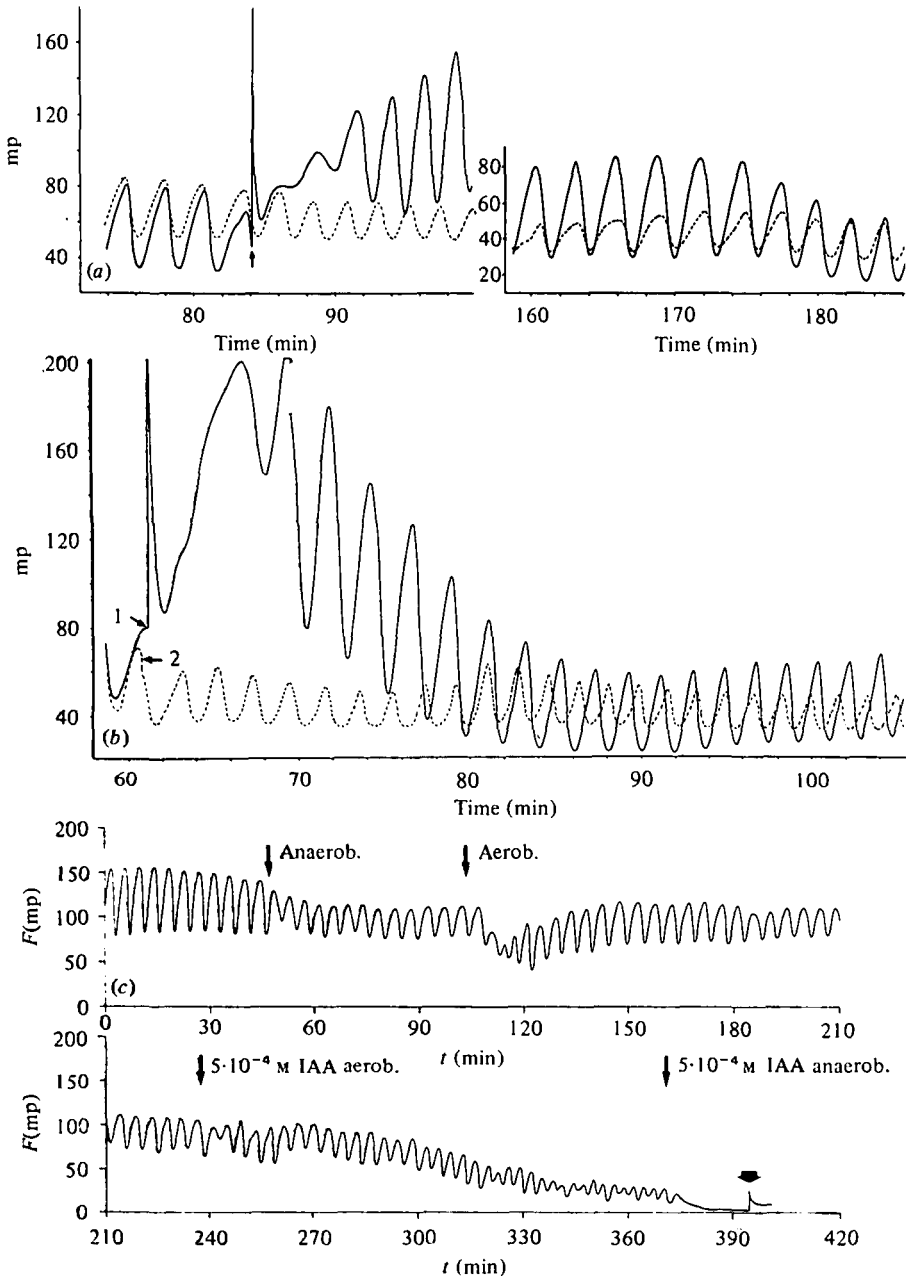


Fig. 3 (a) Simultaneous and independent registration of isometric longitudinal contractile activities of two ends of *one* vein, mounted in a twin tension transducer in the form of an inverse trapeze. One end of the vein (continuous line) was stretched 50% to the original length of this vein part (arrow). The stretch procedure resulted in an increased force output and in a phase shift. The curves on the right show that oscillations of the two vein parts (both stretched and unstretched) were resynchronized later on if the connecting part of both vein ends was not interrupted (Krüger & Wohlfarth-Bottermann, 1978).

(b) In a corresponding experiment the part connecting the two ends was separated (at arrow 2) just before the stretch procedure (at arrow 1). The stretch-induced phase shift was not resynchronized (Krüger & Wohlfarth-Bottermann, 1978).

(c) Longitudinal activity of a vein under isometric conditions of measurement. The arrows indicate the subsequent anaerobiosis, reoxygenation, the application of 5×10^{-4} M iodoacetate (IAA) and a combination of oxygen deprivation and glycolysis inhibition. Note the diminished force output under anaerobic conditions as well as after glycolysis inhibition. A complete cessation of force output occurred after applying a combination of both conditions, i.e. a total blockade of the energy supply (Büttner, unpubl., see Wohlfarth-Bottermann, 1978).

throughout a large plasmodium was clearly established by Takeuchi & Yoneda (1977), Yoshimoto & Kamiya (1978*a*), Krüger & Wohlfarth-Bottermann (1978). Yoshimoto & Kamiya (1978*c*) conclude from their results that the regulating factor is transmitted by the flowing endoplasm. However, since rhythmicity of the ectoplasmic tube continues when endoplasm is replaced by artificial media (Ueda *et al.* 1978), an exclusively 'endoplasmic seat' seems unlikely.

Let us consider some alternative locations in somewhat more detail.

Cytoplasmic actomyosin

The example of the oscillatory behaviour of insect flight muscle (Rüegg, 1973) shows that in principle actomyosin can oscillate *sui generis*. Force output of *Physarum* veins is increased by stretching the strands (Kamiya, 1970; Wohlfarth-Bottermann, 1975*a*) and strong stretches induce a phase shift of the oscillation (Krüger & Wohlfarth-Bottermann, 1978) (Fig. 3*a, b*). These two facts seem to favour the idea that a mechanical component (tension) is involved in the control mechanism. Yoshimoto & Kamiya (1978*b*) deny the possibility of shifting the phase mechanically and point to the possibility that the clock mechanism 'advances, even when contraction *per se* is inhibited'.

In spite of many experiments, we have been unable to restore oscillatory activity in a glycerinated or detergent-treated strand (i.e. in a 'cell free' system) in a significant and reproducible manner. Thus, the question of whether the oscillator is influenced by tension is still undecided. Oscillatory behaviour of the actomyosin itself remains to be established, but it should be mentioned that the contraction-relaxation cycle needs a periodic actin transformation (Isenberg & Wohlfarth-Bottermann, 1976). If this actin cycle is inhibited, e.g. by the injection of Phalloidin (Fig. 4*d*), which shifts the equilibrium irreversibly towards the F-form of actin all oscillations cease as soon as the drug reaches the contractile elements (Götz von Olenhusen & Wohlfarth-Bottermann, 1979*a*).

The regulatory mechanisms which control this actin transformation are thus potential candidates for the oscillator. Conceivably, changes in tension may influence not only the actin equilibrium, but also calcium homeostasis by triggering mechanosensitive calcium channels (e.g. in the plasmalemma or in the membranes enclosing internal calcium stores).

Glycolysis and aerobic respiration

It is known that the glycolytic system of yeast cells can oscillate (Hess & Boiteux, 1971; Richter, Betz & Giersch, 1975). Sachsenmaier & Hansen (1973) have speculated that the rhythmical contractions of *Physarum* might be driven by such a glycolytic oscillator. Daniel (1970) has observed that the reduced pyridin nucleotide level in *Physarum* seems to oscillate in phase with contractile activity. However, it is difficult to monitor such biochemical changes using conventional fluorometric techniques because such 'optical oscillations' may result mainly from rhythmic alterations of vein geometry (Sachsenmaier & Hansen, 1973). Another approach to this problem is to study the response of longitudinal contractions to anaerobic conditions and to inhibitors of glycolysis (Fig. 3*c*). In contrast to earlier observations about the effects of these conditions upon protoplasmic streaming (Kamiya, 1959; Sachsenmaier &

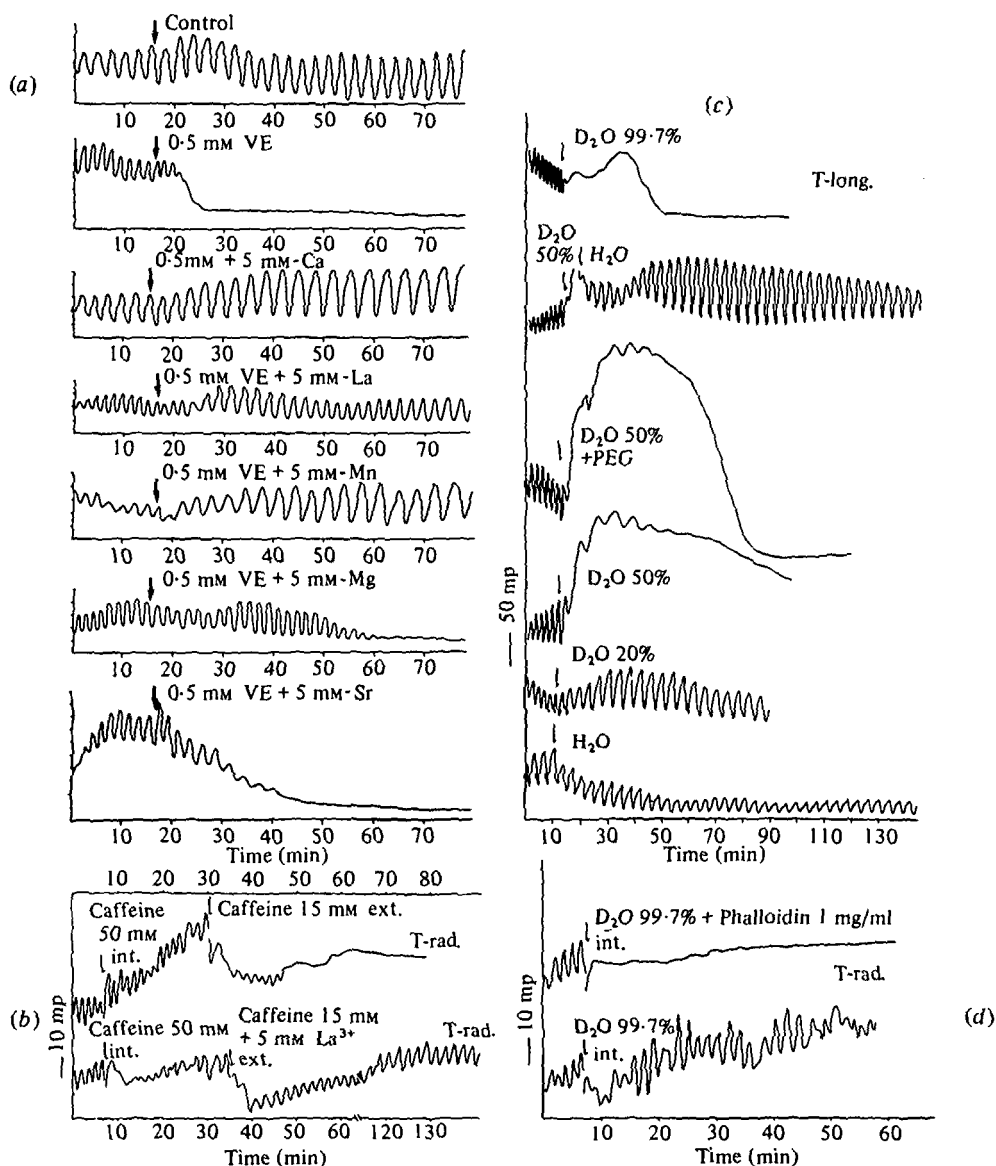


Fig. 4 (a) Effect of 0.5 mM verapamil (VE) on longitudinal contractile activity and the inhibition of the effect of this Ca^{2+} antagonistic drug by 5 mM Ca^{2+} , La^{3+} or Mn^{2+} . Mg^{2+} and Sr^{2+} are not able to inhibit the drug effect. One unit of the ordinate = 10 mp (Wohlfarth-Bottermann & Götz von Olenhusen, 1977). (b) Internal (int.) and external (ext.) effects of caffeine and the inhibition of the relaxing effect of this drug during its external application by 5 mM La^{3+} (Götz von Olenhusen & Wohlfarth-Bottermann, 1979b). (c) Effects of different concentrations of D_2O on longitudinal contraction activities of veins (PEG = addition of 10% polyethylene glycol to 50% D_2O) (Götz von Olenhusen & Wohlfarth-Bottermann, 1979b). (d) The internal application of 99.7% D_2O is ineffective in inhibiting contractile activity (compare with the external application depicted in Fig. 4c). The addition of Phalloidin 1 mg/ml to the heavy water immediately stops rhythmicity and proves that the D_2O reached the contractile substrate (Götz von Olenhusen & Wohlfarth-Bottermann, 1979b).

Hansen, 1973), there was a decline in the longitudinal force output recorded tensiometrically during oxygen deprivation. After reaeration of the outer medium, contractile amplitude recovered. Application of 5×10^{-5} M iodoacetate (IAA) also reduced contractile amplitude. Complete inhibition of oscillatory activity occurred only after the simultaneous application of IAA and the removal of oxygen, i.e. a complete energy blockade (Fig. 3c). The energy source seems to be variable, dependent upon the availability of oxygen. In the experiments described in Fig. 3(c), variations in frequency were not significant, irrespective of whether aerobic or anaerobic cell respiration was available. This seems not to be in favour of an assumption that there is a metabolic oscillator which functions to create regular oscillations via the level of ATP.

Internal Ca^{2+} stores

The cytoplasmic actomyosin of *Physarum* is sensitive to changes in the level of free calcium (Hatano, 1970). Contractile activity can be controlled by injecting varying calcium concentrations (Ueda, Götz von Olenhusen & Wohlfarth-Bottermann, 1978). Histochemical and biochemical techniques have revealed the existence of internal stores which can accumulate calcium against concentration gradients by means of energy-requiring calcium pumps (Braatz & Kornick, 1973; Kato & Tonomura, 1977). However, there is no unequivocal evidence to suggest that a rhythmical release and accumulation of calcium by these stores is the basis of the oscillator. Ridgeway & Durham (1976) used the photoprotein aequorin in an attempt to detect fluctuations in intracellular calcium. Unfortunately, the oscillations in light output which they described may be explained by rhythmic radial volume changes of the veins and may not necessarily reflect rhythmical changes in the level of calcium.

The use of streaming direction by Ridgeway & Durham (1976) as a criterion for contractile phases is of limited value, since streaming and contraction activity are not always in identical phases. However, the thesis that there is a rhythmical translocation of calcium across the membranes of the inner calcium stores is nevertheless a very reasonable hypothesis which must be followed up in future experiments.

Unfortunately, the preparation of membrane fractions from *Physarum* plasmodia in our experience is much more difficult as compared with other cells because the outer slime layer causes different cell components to stick together after homogenization. This hinders their separation during differential centrifugation. A clear-cut demonstration of rhythmical activities of inner Ca^{2+} stores and their functional analysis has to be attempted on purified ultracentrifugal fractions (Kato & Tonomura, 1977).

Plasmalemma

Similar automatic contraction events as in *Physarum* are the slow rhythms (minute rhythms) of some smooth muscle (Golenhofen, see Bülbiring & Shuba, 1976) and myogenic activity of heart muscle. These are supposed to be triggered by a periodic influx of Ca^{2+} across the plasmalemma which may then trigger a further release of calcium from inner Ca^{2+} stores through a mechanism of calcium-dependent calcium release (Fabiato & Fabiato, 1975; Endo, 1977; Lüttgau, 1977). Therefore the existence of a similar trigger mechanism must be taken into account for *Physarum*, and it is

important to know whether rhythmic changes in ionic permeabilities or electrogenic pump activity across the plasmalemma may play a role as a pacemaker mechanism.

Electrophysiological recordings on *Physarum* reveal that the membrane potential fluctuates by 54 mV (Meyer & Stockem, 1979). The period of the bioelectrical phenomena responsible for this oscillatory behaviour has a time period in the range of streaming- and contractile-activity (Kishimoto, 1958*a,b*; Kamiya, 1959; Rhea, 1966). This observation suggests that changes in ionic permeabilities and/or electrogenic ion pumps may trigger contractile activity. The plasmalemma should have an important function at least in the modulation of contractile activity (Hato *et al.* 1976; Ueda *et al.* 1975, 1976; Ludlow & Durham, 1977), because external chemotactic signals must cross this structure before they lead to appropriate alterations of the oscillating motor. Unfortunately, however, electrophysiological measurements of membrane potentials are difficult to perform because *Physarum* has a strong tendency to separate off microelectrodes by forming new plasmalemma around them (Rhea, 1966). Furthermore, ion flux measurements (Ludlow & Durham, 1977) cannot give reliable information because there is an extended extracellular space within the veins in the form of highly branched pockets of deeply penetrating plasmalemma invaginations (Fig. 1*b*), PI). Thus, flux measurements and electrophysiological methods are of restricted applicability and possible physiological events occurring in the plasmalemma have to be deduced indirectly from tensiometric recordings of responses to externally and internally applied test solutions. *Physarum* offers several advantages for this type of experimental approach. It is possible to study:

(1) The effects of *external* application on *persistence* of longitudinal contractile activity of veins under isometric conditions of measurement (Wohlfarth-Bottermann, 1975*a*) (Fig. 4*a, c*).

(2) The effects of *injected* test solutions on *persistence* of radial contractile activity of veins *in situ* (in addition, internal and external solutions can be applied simultaneously) (Fig. 4*b, d*) (Ueda *et al.* 1978).

(3) The effects of *externally* applied test solutions on *de novo* generated contractile activity of protoplasmic drops (Fig. 6) (Wohlfarth-Bottermann & Götz von Olenhusen, 1977; Götz von Olenhusen & Wohlfarth-Bottermann, 1979*b*).

These modes of application as well as the different preparations (veins or drops at different age stages) have various suitabilities for answering certain questions. Advantages and shortcomings cannot be discussed here in detail (see Götz von Olenhusen & Wohlfarth-Bottermann, 1979*b*).

The importance of calcium for the contractile rhythm has been investigated in several different ways. Studies with radioactive calcium seem to show a rapid and continuous $^{45}\text{Ca}^{2+}$ uptake into migrating plasmodia. This influx may represent either an active uptake process or a passive inflow down the large concentration gradient which exists between the external (10^{-3} M) and internal compartment (10^{-7} M). This large influx of calcium is balanced by a corresponding efflux which may be associated with slime secretion. The uptake of $^{45}\text{Ca}^{2+}$ into migrating plasmodia is strongly reduced by 5 mM- La^{3+} without obviously decreasing the viability of the plasmodium over a period of many hours (Dierkes, unpublished).

Reducing external calcium to 10^{-5} to 10^{-8} M in the external medium likewise had little effect in that the automaticity of longitudinal contractions persists for 1 to 2 h.

After this time a declining longitudinal contraction force may be traced back to an increasing loss of internal Ca^{2+} , which can be prevented by the addition of 5 mM- La^{3+} to the 10^{-8} M- Ca^{2+} EGTA buffer (Wohlfarth-Bottermann & Götz von Olenhusen, 1977).

Verapamil (0.5 mM), a calcium antagonistic drug, abolishes longitudinal force generation within a few minutes after external application (Fig. 4*a*). However, since the action of this drug is inhibited by 5 mM- Ca^{2+} , La^{3+} or Mn^{2+} (not by Mg^{2+} or Sr^{2+}) it is difficult to believe that this drug acts to inhibit calcium movement across the plasmalemma as it does in smooth muscle: the outer application of 5 mM- La^{3+} probably is not compatible with a persisting rhythmical Ca^{2+} transport across the plasmalemma. Dörrscheid-Käfer (1977) points to a possible action of the Ca^{2+} antagonist upon inner stores of the skeletal muscle.

After the introduction of a new technique for replacing of endoplasm by artificial media, it was possible to compare the influence of drugs when applied to either the inside or to the outside of the ectoplasmic tube (Ueda *et al.* 1978). It became apparent that theophylline and caffeine (Fig. 4*b*) have a strong effect when applied externally, but not when injected internally. The strong relaxing effect of 15 mM external caffeine was inhibited when 5 mM- La^{3+} was added to the drug solution. This external sensitivity to caffeine (Hatano & Nakajima, 1961; Hatano, 1970) on the outside and the insensitivity of strands after injection of the drug to the inside may indicate that the drug influences contractile activity via the plasmalemma (blockade of phosphodiesterase activity?).

A corresponding differential sensitivity was found when heavy water (D_2O) was applied either externally or internally (Fig. 4*c,d*). Longitudinal contractile activity stopped immediately when 50% D_2O was added to the bathing solution, but the injection of 99.7% D_2O did not lead to a cessation of contractile rhythmicity. The addition of Phalloidin to the heavy-water proved that the injected medium actually reached the contractile elements (compare Götz von Olenhusen & Wohlfarth-Bottermann, 1978, 1979*a*). The addition of 10% polyethyleneglycol (PEG) to the external D_2O did not slow down the fast action of the heavy water. These observations on the effects of both caffeine and D_2O on veins initially suggest that both substances act primarily on some processes on the plasmalemma which may be important for the continuation of contractile automaticity. One has, however, to consider (i) that caffeine and D_2O do rapidly penetrate into the cell, and (ii) the effective concentration of substances within the ectoplasmic tube may be low after injection in comparison to external application. Therefore, D_2O and caffeine could still act within the cell, e.g. by altering calcium movement across internal stores (Syson & Huddart, 1976; Kaminer, 1977).

When discussing the effects of certain chemicals applied externally upon veins, we have to consider 6 principal compartments: (1) the extracellular medium, (2) the slime layer, (3) the cell membrane, (4) plasmalemma invaginations with slime content, (5) the cytoplasm, (6) different internal Ca^{2+} stores.

The use of another preparation, i.e. protoplasmic drops (Wohlfarth-Bottermann, 1963; Götz von Olenhusen, Jücker & Wohlfarth-Bottermann, 1979; Götz von Olenhusen & Wohlfarth-Bottermann, 1979*b*), has four essential advantages in comparison to the use of veins:

(a) Instead of the *persistence* of contractile activities, the *de novo* generation of rhythmicity is used as a criterion for the effects of externally applied substances.

(b) The slime layer of protoplasmic drops is much thinner than that of veins, i.e. there is faster and more intimate contact of test solutions with the external plasmalemma.

(c) The extracellular space within the plasmalemma invagination system is much smaller in normal *protoplasmic drops* as compared with the extended invagination system in veins. Therefore bathing solutions are more likely to reach the innermost pockets of the invaginations.

(d) In *caffeine-treated drops* (Fig. 5), plasmalemma invaginations are absent thus removing all possible doubt that solutions can reach all parts of the plasmalemma. In spite of their inability to form plasmalemma invaginations, the caffeine-treated drops (as normal drops) undergo a fibrillogenesis of cytoplasmic actomyosin (drop age 5–15 min) and subsequently a *de novo* generation of contractile activity (drop age 20 min).

Fig. 6(a) shows that contractile activity of normal protoplasmic drops (control in Fig. 5) can be generated *de novo* if the outer concentration of Ca^{2+} ions is as low as 10^{-8} M, and even in the presence of the calcium antagonistic drug D 600 (0.5 mM). Caffeine (5 mM) does not hinder the initiation of activity (Fig. 6b), but 5% D_2O inhibits the newly generated activity (Fig. 6c). The results characterized in Fig. 6(a–c) were obtained in normal drops. In order to be completely sure that the total area of the plasmalemma is reached by the experimental solutions, we tested the influence of different chemicals on caffeine-treated drops, i.e. on drops without plasmalemma invaginations (see Fig. 5).

The relative amount of interior membrane in normal drops compared to caffeine-treated drops at 10 and 20 min was estimated by a morphometric analysis of the semi-thin sections shown in Fig. 5. The interior membrane area (invaginations and vacuoles) amounts to 265% (10 min) and 365% (20 min) of the exterior plasmalemma area of normal drops, whereas the corresponding values in the caffeine-treated drops are only 18 and 65% respectively (representing mainly vacuoles).

The large amount of interior plasmalemma and vacuoles in normal drops may indicate physiologically important structures delimiting at least partly an extracellular space in the drops' interior which perhaps is not readily accessible to externally applied test solutions. Thus, the caffeine-induced plasmodial stage which lacks plasmalemma invaginations is completely accessible to experimentally applied solutions. Fig. 6(d) demonstrates that such drops equilibrated with 5 mM caffeine generate contractile activity even in the presence of 5 mM La^{3+} and 50 mM outer K^+ . Even 100 mM- K^+ does not hinder completely the *de novo* generation of an oscillation in caffeine-treated drops. Oscillatory phenomena cannot be registered in the combined presence of 5 mM caffeine and 70 mM- K^+ . Likewise no oscillation appears when high K^+ is replaced by high Na^+ (Fig. 6d).

Evaluating these data one can conclude that oscillation seems to be widely independent of a contraction triggering plasmalemmal Ca^{2+} pump (Ludlow & Durham, 1977). There are no data favouring the view that a rhythmical Ca^{2+} transport across the plasmalemma triggers the oscillation.

The same can be concluded with respect to the involvement of a hypothetical

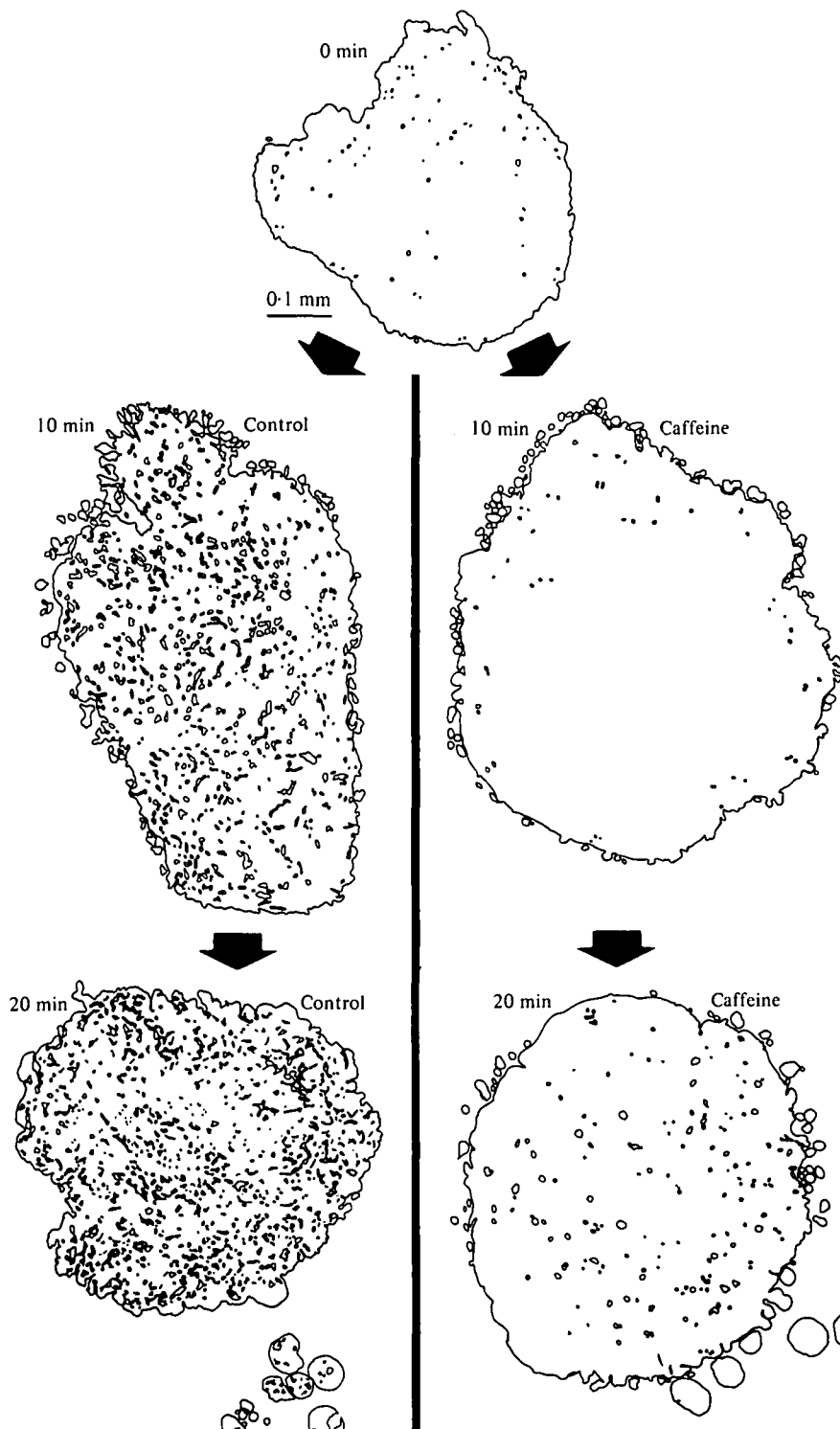


Fig. 5. Drawings of semithin sections of normal protoplasmic drops (control) and of 5 mM caffeine-treated drops demonstrating the differences in interior membrane areas (plasmalemma invaginations and vacuoles). The cytoplasmic actomyosin fibrils are not depicted. Magnification $90\times$ (Götz von Olenhusen, Jücker & Wohlfarth-Bottermann, 1979).

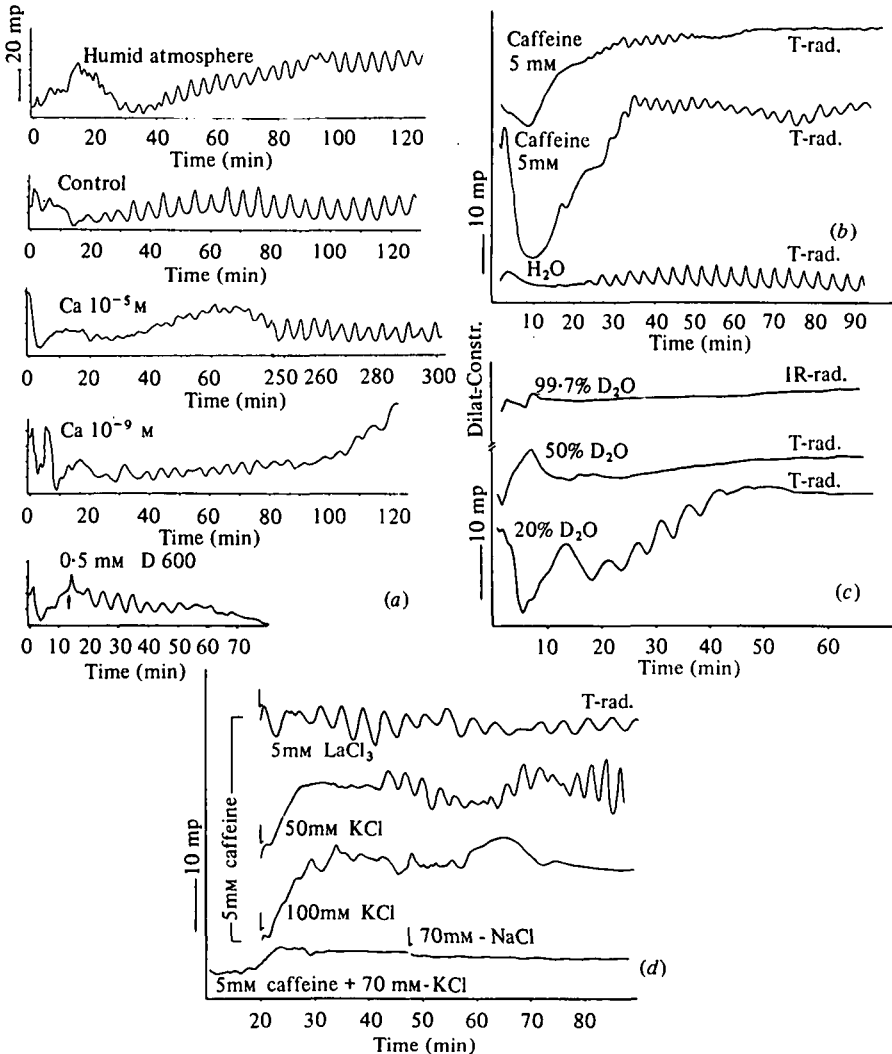


Fig. 6 (a) *De novo* generation of contractile activity of normal protoplasmic drops in spite of very low levels of external Ca^{2+} or the presence of the calcium antagonistic drug D 600 (0.5 mM) (Wohlfarth-Bottermann & Götz von Olenhusen, 1977). (b) *De novo* generation of contractile activity of normal protoplasmic drops in spite of the presence of 5 mM caffeine within the outer medium (Götz von Olenhusen & Wohlfarth-Bottermann, 1979b). (c) Influence of different D_2O concentrations on the *de novo* generation of contractile activity in normal protoplasmic drops (Götz von Olenhusen & Wohlfarth-Bottermann, 1979b). (d) *De novo* generation of contractile activity of protoplasmic drops in spite of the presence of 5 mM- La^{3+} and 50 or 100 mM- K^+ respectively after a period of treatment with 5 mM caffeine (drops without plasmalemma invaginations). The lowest curve demonstrates that the initiation of contractile activity is inhibited, if 5 mM caffeine and 70 mM- K^+ are applied continuously and that no oscillation can be registered even when this solution is replaced by 70 mM- Na^+ (Götz von Olenhusen & Wohlfarth-Bottermann, 1979b).

Na^+ - K^+ pump. The ineffectiveness of ouabain on persistence and *de novo* generation of activity and on ATPase activity of *Physarum* homogenates (Achenbach & Achenbach, 1979) rules out any role for the Na^+ - K^+ pump in triggering oscillations. Caffeine obviously prevents the formation of the plasmalemma invagination!

perhaps by interfering with Ca^{2+} -sensitive membrane sites which may influence the anchoring of actin molecules to the plasmalemma and/or blocking plasmalemmal phosphodiesterase activities. Only speculations are possible at the present time about the molecular action of heavy water (conformational change of membrane proteins and interaction with structured water which surrounds membranes). In muscle, D_2O uncouples excitation from contraction by affecting inner calcium stores (Kaminer, 1977).

The possible existence of a proton pump should be mentioned. However, according to pilot experiments, 10 mM KSCN and 10^{-6} M valinomycin did not inhibit the initiation of contractile activity in invagination-free drops (Götz von Olenhusen & Wohlfarth-Bottermann, 1979(b) and unpubl. results). Experiments depicted in Fig. 5(d) also argue against an oscillation triggering role for some hypothetical proton pump ($\text{H}^+-\text{HCO}_3^-$ or H^+-K^+) (Daniel & Eustace, 1972; Matveeva *et al.* 1978).

In summary, we can probably exclude calcium and Na^+-K^+ ion pumps as trigger mechanism on the plasmalemma. This does not preclude the existence of some other unknown mechanism located in the plasmalemma or within the peripheral cell cortex. The role of external Ca^{2+} as a force modulator in the sequence of chemotactic responses remains to be analysed. The bioelectrical phenomena observed to date (Meyer & Stockem, 1979) do not prove rhythmic plasmalemma ion pumps as a trigger, since the phenomena may either reflect intracellular ion fluxes or represent a consequence of contraction activity (Kishimoto, 1958a,b): this activity continues also when streaming activity is restrained (Wohlfarth-Bottermann, 1975a) and thus can also be present under conditions when there is no spontaneous streaming. The strict dependence of oscillatory behaviour upon bioelectric trigger phenomena in the plasmalemma could only be shown by demonstrating that intrinsic *contractile* activity ceased when these electrical signals in the plasmalemma were blocked experimentally.

To further analyse the role of the surface membrane it would be very important to determine whether oscillations would occur in strands whose plasmalemma is completely destroyed by detergents. Teplov *et al.* (1978) recently described that oscillation could be revived in Tween-treated strands. When trying to reproduce these interesting results in our laboratory, we confirmed that the Tween-treated veins behaved as described by Teplov *et al.* (1978). However, when we put the Tween-treated 'models' on to nutrient agar, at the end of the experiment, small parts of the tensiometrically measured veins developed into a new microplasmodium 12 h later. This means that the revival of oscillations supposed by Teplov *et al.* (1978) was perhaps not performed with a real 'cell free' system, but that the revival of contractile activity may be traced back to small areas of the veins possibly remaining unaffected by the detergent. In any case, in our laboratory (Achenbach, unpublished), it was not possible to revive oscillations in those veins which afterwards did *not* grow to new microplasmodia, i.e. in veins which were in fact 'dead' and thus in fact a cell free system.

The caffeine-treated protoplasmic drop, which lack plasmalemma invaginations thus allowing free access to external solutions, may prove a useful experimental model for future measurements of ion fluxes and electrical phenomena. It may be easier to identify the precise role of the plasmalemma in generating oscillatory activity using this simpler plasmodial stage. A recent observation in our laboratory (F. Achenbach, unpublished) that invaginations reappear after removal of caffeine underlines the

vitality of this stage and presents the opportunity of studying the contribution of these invaginations to oscillatory activity.

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