THE PATTERNED ORGANIZATION OF THICK AND THIN MICROFILAMENTS IN THE CONTRACTING PSEUDOPOD OF *DIFFLUGIA*

B. S. ECKERT* AND S. M. McGEE-RUSSELL

Department of Biological Sciences, State University of New York at Albany, 1400 Washington Avenue, Albany, New York 12222, U.S.A.

SUMMARY

The lobopodial pseudopods of the shelled amoeba *Diffugia* undergo a rapid active contraction and length shortening which results in movement of the organism, or rapid pseudopod retraction. In polarized light, the pseudopod shows high birefringence before and during this normal contraction, suggesting a high degree of linear organization, and a complex pattern of changes. Immediately conventional fixation begins, pseudopods retract rapidly, and show changes in birefringence. When longitudinal sections are viewed in the electron microscope, such fixed, contracting pseudopods are seen to contain thick (14–16 nm) and thin (5–8 nm) microfilaments. Montages demonstrate that these microfilaments are found in close parallel association with each other, and lie parallel and peripheral to the longitudinal axis of the pseudopod, which is the axis of contraction. This distribution suggests that the patterned microfilaments could be involved in the shortening process and that they could account for the birefringence seen in the contracting pseudopod. Rapid alignment, or assembly, is also suggested.

INTRODUCTION

The motile process of *Diffugia* is an interesting variation upon the motile processes of other, better known, naked amoebae (Allen, 1961, 1970). Its sandy shell is a considerable mass that the amoeba must move by means of pseudopods (Figs. 1, 2), and presumably requires the adaptive variation in behaviour.

The movement of *Diffugia* was described in detail by Mast (1931). Movement of *Diffugia* results, usually, from the activity of a single pseudopod, or a small group of two or three, which extends through the opening in the shell. This pseudopod advances without attachment and may swing slowly from side to side. Finally it attaches to the substrate by an attachment area near the tip. It then contracts longitudinally, so pulling the shell forward towards the anterior end of the pseudopod. By the time this contraction occurs, usually, another pseudopod has extended, which then goes through the same process. The contraction is often accompanied by the rapid appearance of numerous hyaline blisters on the surface of the pseudopod. Wohlman & Allen (1968) suggested that these are a result of syneresis. They showed that high birefringence in the pseudopod is associated with contraction, while extension involves little or no detectable birefringence. This suggests an increase in the proportion of linear organization accompanying contraction which the authors (p. 108) stated preceded.

* Present address: Department of Biological Structure, University of Miami School of Medicine, Box 875, Biscayne Annex, Miami, Florida 33152, U.S.A.
pseudopod retraction: 'Upon retraction, birefringence typically fades...'. Observations of our material did not entirely accord with this statement, which we believe represents an oversimplification of the events which occur. We have therefore, with the permission of the authors, re-examined the original data of the films, using frame-by-frame analysis. This analysis, together with our own data, confirms a complex pattern of changes in the contracting pseudopod.

Hence pseudopod withdrawal in Diffugia differs from that in naked amoebae such as Chaos. In these other amoebae, the pseudopod is withdrawn as a result of cytoplasmic streaming; endoplasm flowing out causes a pseudopod to be reduced progressively (Allen, 1968). In Diffugia the pseudopod decreases in length as a result of a very rapid active contraction, similar, apparently, to the contraction which moves the shell, as a process independent of, and uncoupled from, the direction of streaming (Wohlman & Allen, 1968).

Microfilaments have been demonstrated in several amoeboid organisms. Nachmias (1964, 1968) found thick and thin microfilaments in Chaos chaos, and suggested that they play a role in cytoplasmic movement. The work of Pollard & Ito (1970) demonstrated the presence of thick and thin microfilaments in motile cytoplasmic extracts of Amoeba proteus. Pollard, Shelton, Weiheing & Korn (1970) and Pollard & Korn (1971) showed that the thin (8-nm) filaments of Acanthamoeba and Amoeba proteus are actin-like. This has also been established for Chaos carolinensis by Comly (1972) by heavy meromyosin (HMM) binding.

We undertook first, to examine the ultrastructure of the specialized lobopod of Diffugia, to establish, in the first instance, whether thick and thin microfilaments could be detected in thin sections, possibly in organized array; and secondly to re-examine the reported changes in birefringence associated with active deformation of the pseudopod.

MATERIALS AND METHODS

Cultures, source and maintenance

Diffugia lobostoma samples were obtained from Carolina Biological Supply Company. They were maintained in large Petri dishes containing Marshall's medium and Spirogyra as their food supply.

Light microscopy

Observations with the light microscope were made using Zeiss Photomicroscopes I and II equipped with Nomarski differential interference optics and polarized light optics. Animals were observed under an agar overlay for extended periods of time, and monitored during the fixation response, using a microperfusion chamber (McGee-Russell & Allen, 1971). The internal camera and automatic exposure devices of the photomicroscopes were used for 35-mm photomicrography on Kodak Panatomic X film which was developed in Digna. An Arriflex 16-mm camera was used for microcinematography with Kodak Plus X film, also developed in Digna.

Electron microscopy

Specimens were fixed with 6.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, for 40 min at room temperature. They were postfixed in 2% osmium tetroxide in 0.1 M...
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Cacodylate buffer for 30 min at room temperature. The primary fixative was administered either under observation in a microperfusion chamber, or by placing the organisms in a very small drop of medium in a Petri dish and then flooding rapidly with a large excess of fixative. The specimens were dehydrated through an ethanol series (20, 30, 50, 80, 90, 100 % 2 changes of 20 s each), passed through propylene oxide (2 changes of 5 min each), 1:1 propylene oxide: Epon (1 change, overnight) and embedded in Epon. Silver and gold sections were cut on a Reichert OMU-2, stained with 2 % aqueous uranyl acetate, and lead citrate (Reynolds, 1963) and observed in an AEI EM6B electron microscope at 60 kV with 50- or 100-μm objective apertures.

By careful sectioning, serial longitudinal sections of pseudopods could be obtained when the embedded organism was in the configuration shown in Fig. 1. From such a suitable series, a photo-montage could be prepared for analysis of almost the entire length of the pseudopod. An example is shown in Fig. 3.

Observations

Light microscopy

Shell locomotion appears to be the result of successive pseudopod extensions and contractions. When the pseudopod is firmly attached to the substrate, its shortening causes the shell and enclosed cell body to be pulled toward the attachment point. This movement of the sandy shell can only be accomplished through a force applied to it by the pseudopod. The active work involved in the application of such a force to move the shell implies that an energy-driven contraction occurs. If the pseudopod is unattached and such a contraction occurs the pseudopod rapidly shortens and retracts toward the shell. Hyaline blisters appear during the latter half of the contraction process in both attached and unattached pseudopods.

Fixation response

When fixative is perfused into a microperfusion chamber or flooded quickly across an animal, pseudopods, both attached and unattached, retract immediately (Fig. 5). The retraction is quite rapid, and may be completed in 1 s. Fixation, therefore, must be equally rapid or pseudopods may be entirely withdrawn. Some variation occurs among individuals; some may take several seconds to withdraw and these are most suitable for recording by cinematography. Some pseudopods appear more sensitive than others and show a clear-cut 'damage' response, with excessive internal bubbling and distortion of shape. Pseudopods of this type were not analysed.

Rapid flooding of numerous active animals gave the greatest yield of fixed, partially extended, pseudopods of life-like form. The immediacy of response to fixative is such that all the animals used in this study must be considered to be in the process of retracting. Fixed pseudopods, prepared in this way, observed after embedding, retain much of the birefringence seen in the living state.

Observations in polarised light

In our observations, the birefringence detectable in the living state shows a more complex cycle of development than was briefly suggested in the previous publication from this group by Wohlman & Allen (1968). We agree that the pseudopods are not birefringent, or at most, are very weakly so, during extension. We also agree that strong
positive birefringence develops upon pseudopod attachment. However we cannot agree that the statements 'As the pseudopod retracts, the birefringent fibrillar array disappears...' (Wohlman & Allen, 1968, p. 105) and 'Upon retraction, birefringence typically fades...' (p. 108) fully describe what appears to be a complex pattern of changes in birefringence associated with pseudopod shortening and shell translocation. In our work, and in our re-examination of the original films, at least 3 processes have been observed: (1) The shell and pseudopod may remain stationary while birefringence develops, fades and then reappears. Here presumably either a contractile system is developed which is not activated, or an actively working system does not result in a resolution of forces by movement. (2) A pseudopod may detach and shorten towards the shell with a continuing and changing level of birefringence detectable within it, until retraction is 'completed', and the pseudopod disappears from sight beneath the shell (Fig. 6). These observations do not contradict, but extend the observations of Wohlman & Allen, since at no time in this process is the level of birefringence as high as it is at attachment before shortening starts; yet incontrovertibly, frame-by-frame analysis confirms (Fig. 6) strong and changing birefringence persistent throughout prominent length changes in a withdrawing pseudopod. (3) The shell and cell body may be moved towards the attachment point, with a fairly high level of birefringence maintained throughout movement. Definite fading of this birefringence occurs significantly at the very end of the presumed contraction cycle.

Electron microscopy

As shown in Fig. 3, the pseudopod can be seen to be divisible into 2 zones. The outer zone is a denser one, 0-3–0-5 μm wide. It contains few vesicles or large granules; most of the microfilamentous complement is present here. The central zone is less dense and is 2–5 μm wide. It contains a majority of the vesicles and large granules present in the pseudopod. Very few microfilaments are found in this layer. The large granules contain a pseudocrystalline material of unknown composition (Fig. 4). The contents of the large vesicles stain very lightly, appear granular or slightly reticular and are of unknown composition. These vesicles may be a result of the syneresis related to pseudopod contraction.

The microfilaments which are seen in the outer layer are often organized into bands (Fig. 7). These lie parallel to the longitudinal axis of the pseudopod, which is the axis of contraction. They are composed of 2 types of microfilaments, thick and thin. (See Eckert & McGee-Russell, 1972.) Both types of filaments are found within a single band. Bands containing only one type of filament have not been found. This constitutes a significant difference from the previous observations of Wohlman & Allen (1968). The thick filaments measure 14–16 nm in diameter and up to 0-5 μm in length. Their appearance does not in any way suggest that they are an aggregation of thin filaments but rather they appear to be a distinct type of their own. The thin filaments measure 5–8 nm in diameter and appear to be longer than the thick filaments.

The thick and thin microfilaments are usually found in close parallel association with each other, a thick and a thin filament often lying close together with a spacing as small as 10 nm. This patterning is shown in Fig. 8 (arrows).
DISCUSSION

Pseudopod withdrawal in *Difflugia* is a more rapid process than in naked amoebae such as *Chaos*. A considerable force is required for shell translocation and this movement seems to require both pseudopod attachment and shortening. We suggest, therefore, that this retraction, causing shell movement, is an active contraction process, resembling on the microscale in many aspects, the contractile function of the columella muscle of gastropod molluscs. The appearance and redistribution of birefringence before and during pseudopod retraction, plus the rapidity of response to fixatives, suggest that unattached pseudopod retraction is also accomplished by an active contractile system. The level and the patterning of the birefringence suggest a high degree of organization of microfilaments in the pseudopod, which would be required for an active directional contraction.

The ultrastructural evidence presented here is the first to indicate a possible *dual* system of thick and thin filaments of, perhaps, actin- and myosin-like character, in *Difflugia*, with a suggestion of 3-dimensionally patterned array, paralleling a known axis of physiological activity. Fixation has stimulated and preserved a state of contraction, and an organized system of thick and thin microfilaments is revealed with dimensions similar to those found in naked amoebae by others. Close parallel association suggests interaction between them, similar to the interaction suggested by Pollard & Ito (1970) for filaments in *Amoeba proteus* extracts.

If we assume that the birefringence depends upon the development of the microfilamentous system, then clearly the latter is rapidly assembled or rapidly aligned. At present it is impossible to determine whether the system is in a non-aligned condition, or a non-assembled condition in the isotropic extending pseudopod, either *in vivo* or after fixation, unless the fixation response can be eliminated.

The strong birefringence in pseudopods unaccompanied by movement of shell or pseudopod is noteworthy, since it may indicate the capacity of the contractile system to maintain tension of an overburden, comparable to isometric contraction in muscle. Lack of resolution of the forces may lead to cyclic disassociation and re-organization of the contractile system, until sufficient elements and/or sufficient order are generated by continued recruitment, to match and overcome the load of the shell (process 1, described above).

The apparently prominent degree of organization of the microfilamentous system makes *Difflugia* an excellent organism for the further study of active contraction in amoeboid organisms. We conclude, as a working hypothesis, that the microfilamentous system, possibly through rapid filament assembly and interaction, is capable of producing the contraction required for shell translocation or pseudopod retraction.

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REFERENCES


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Fig. 1. Orientation of *Difflugia* for longitudinal pseudopod sectioning.

Fig. 2. Living *Difflugia* with pseudopods (arrow) extended. Zeiss Nomarski differential interference optics.
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Plane of section

1

Shell composed of stones

5 \mu m

100 \mu m

50 \mu m

Fixed pseudopod
Fig. 3. Photo-montage of longitudinal section of a pseudopod. The outer zone (arrow a) and the inner zone (arrow b) are clearly visible. A few thick filaments may be seen in the outer zone.

Fig. 4. Oblique section of pseudopod at higher magnification than in Fig. 3. Note large granules (g) and vesicles (v). Mitochondria (m) are also visible.
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Fig. 5. Single frame prints from cine film showing retraction of pseudopod (arrow) in response to fixative. The fixative arrives at about the time of the first frame and by the third frame, fixation is complete. This particular individual was slower in responding than most, so that the pseudopod is fixed before it entirely retracts. A, frame 1, 0 s; B, frame 44, 2.75 s; C, frame 80, 5 s.

Fig. 6. Single frame prints from film taken in polarized light by Dr Alan Wohlman and Dr Robert D. Allen. The arrow points to the birefringence which persists during pseudopod retraction. Printed by kind permission of the authors. A, frame 1, 0 s; B, frame 60, 3.75 s; C, frame 78, 4.98 s.
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Fig. 7. Electron micrograph of pseudopod in longitudinal section showing a bundle of microfilaments in the outer zone. Note thick (th) and thin (tn) filaments.

Fig. 8. Higher magnification of a different region from that in Fig. 7. Arrows indicate regions of close-parallel association of thick and thin microfilaments.
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