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The cytoskeleton of the fiber cells of *Trichoplax adhaerens* (Placozoa)

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Summary. The cytoskeleton of *Trichoplax adhaerens* fiber cells was studied after chemical fixation, freeze-substitution, lysis of attached cells with nonionic detergents and by immunofluorescence. Cytoskeletal elements present in the cell bodies and reaching into the extensions include microtubules, intermediate filaments, 6–7 nm and 2–3 nm microfilaments. The latter seem to interconnect other cytoskeletal elements. Actin-like microfilaments are found both as networks and parallel strands. Immunofluorescence with anti-actin shows the presence of actin in the cell body, underneath the plasmalemma and within the extensions. Both the results of immunofluorescence and the identification of 6–7 nm actin-like microfilaments support the concept of contractility of the fiber cells as the cause of the rapid shape changes of *Trichoplax*. Anti-tubulin fluorescence corresponds to the location of microtubules in the extensions as well as the cell bodies of the fiber cells. The extensions are withdrawn upon depolymerization of the microtubules by colchicine.

A. Introduction

The fiber cells of *Trichoplax* form a mesenchyme-like tissue in the space between the dorsal and the ventral epithelium. Their long and aborizing extensions (Figs. 1 and 2 in Ruthmann 1977) make specialized contacts (Grell and Benwitz 1974) with each other and the cell bodies of other fiber cells forming a three-dimensional network which may be instrumental in the coordination of movements. These include a slow gliding over the substrate by the ventral cilia and relatively rapid changes of the outer shape of the organism ascribed to the contractility of the fiber cells (Grell and Benwitz 1971). However, microfilaments as the molecular basis of contractility have not been found as yet. In a short note Klauser and Ruppert (1981) suggested, therefore, that all non-flagellar movements (which also include a buckling up during feeding) may be due to a terminal web in the cells of the ventral epithelium. Since failure to find microfilaments in the fiber cells may also be due to the poor preservation observed after the conventional double fixation in glutaraldehyde and osmic acid, we sought for alternative procedures to demonstrate both microfilaments and microtubules. In addition, we used specific antibodies to localize polymerized actin and tubulin by fluorescence microscopy.

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B. Material and methods

Trichoplax, strain E 5, was originally obtained from Prof. Grell (Tübingen) and cultured as described previously (Grell and Benwitz 1971).

For freeze-substitution, the organisms were washed in calcium-free seawater, fragmented with a 10 µl constriction pipette, transferred to thin Thermanox plates and immersed for 10 s in propane at −180° C. The shock-frozen samples were collected in liquid nitrogen and then transferred to the substitution mixture (1% OsO_4 in 100% ethanol, colled to −70° C by dry ice) for 4 days. After slow warming to room temperature, the osmic acid was washed out with absolute ethanol.

After washing in calcium-free seawater, tissue fragments obtained as described above were fixed for 30 min at room temperature in 2.5% glutaraldehyde in PIPES buffer (pH 7.4) containing 1% low molecular weight tannic acid (Mallinckrodt) and 10^{-5} M phalloidine to stabilize microfilaments. After two rinses in buffer, the samples were post-fixed in 1% OsO_4 in distilled water.

All samples were embedded in Epon. Thin sections were double stained with 4% uranyl acetate in 25% ethanol (15 min) and in lead citrate (15 min).

To study lysed cells by whole-mount electron microscopy, the substrate has to be made hydrophilic to permit the attachment of the fiber cells. Formvar-coated nickel grids were stabilized by evaporated carbon and gassed with pentylamine. The surface remains hydrophilic enough for 2–6 days to permit attachment. After mechanical dissociation of the tissue the cells were pipetted onto the grids and kept for about 30 min in a moist chamber to allow time for attachment.

Cells were lysed from 1–6 min using either 0.1% saponin or 0.1% Trixon X-100 as a detergent. Besides detergent, the lysis medium contained 0.05 *M* PIPES buffer (pH 7.4), 0.02 *M* EGTA as a calcium-specific chelator, 0.02 *M* KCl, 0.02 *M* $MgCl_2$ and 30% (w/v) polyethylene glycol-200 (Merck). After extraction, the cytoskeletons were fixed in 2.5% glutaraldehyde, dissolved in 0.05 *M* PIPES buffer (pH 7.4) containing 30% polyethylene glycol-200. Dehydration was carried out stepwise in ethanol with 0.5% uranyl acetate in the 70% ethanol step to obtain contrast. In some

experiments, 10^{-5} *M* colchicine was added to the lysis medium containing 0.1% saponine.

For indirect immunofluorescence, isolated cells were pipetted onto coverslips and fixed after attachment for 5 min in 2.5% glutaraldehyde in 0.15 *M* cacodylate buffer (pH 7.4) to which dimethylsulphoxide (DMSO) had been added to a final concentration of 10%. After dehydration, a drop of glycerol buffer (50% glycerol, 1% DMSO, 5 m*M* $MgCl_2$, 1 m*M* EGTA in 5 m*M* Sörensen phosphate buffer) was added to the coverslips to prevent drying. Samples treated in this manner could be stored in a moist chamber in the refrigerator for several days. Before antibody treatment, the cells were rinsed for 15 min in 1% borohydride in 50% ethanol which was subsequently washed out with phosphate buffer. Affinity column-purified rabbit antibodies against native porcine brain tubulin and chicken smooth muscle actin, kindly provided by Prof. Hauser (Bochum), were applied in a moist chamber at a concentration of 10 µg/ml. After washing in phosphate buffer, FITC-labelled goat IgG against rabbit (obtained from Miles) was used as the second antibody. The incubation time with the antibodies was 30 min at 37° C. After several rinses, the cells were immersed in the glycerol buffer containing 10% paraphenylenediamine to avoid bleaching and photographed with a Zeiss photomicroscope equipped with phase contrast and epifluorescence optics, using high-speed emulsions (400 ASA).

C. Results

1. Cytoskeletal structures in ultrathin sections

Figure 1 shows a survey of some characterstic features of fiber cells as seen after freeze-substitution. The prominent mitochondrial complex (m) next to the nucleus (n) consists of dense mitochondria closely associated with less dense microbodies of unknown function as first described by Grell and Benwitz (1971). Next to it is the single Golgi complex (g). The large concrement vacuole (k), possibly a lysosomal compartment, is also found in epithelial cells. Endoplasmic reticulum, recognizable only by the rows of attached ribosomes, is found within the cell body but does not reach into the extensions of the cell. It contains (not shown) presumably endosymbiotic bacteria within widened cisternae (Grell and Benwitz 1971). In contrast to the cells of both epithelia, the fiber cells are tetraploid. They multiply by mitosis (Ruthmann 1977).

Microfilaments are difficult to discern in sections after freeze-substitution because of the high background density of the cytoplasm. The inset in Fig. 1 shows a bundle of filaments in an extension of a fiber cell which can be followed into the peripheral cytoplasm of the cell body. The thicker ones are in the range of intermediate filaments (10 nm) while the thin ones measure 6 nm and less.

Some aspects of the distribution of microtubules can be discerned in Fig. 2. While some microtubules radiate from the centrioles close to the Golgi apparatus into the cytoplasm, it is obvious that these organelles are not the only or even the major microtubule organizing centers in the cell. This holds especially for the microtubules, often in bundles, which are in the cell extensions and for microtubules close to the cell margin.

Microtubules and microfilaments can also be demonstrated in tissue fragments fixed with glutaraldehyde containing phalloidin and tannic acid and postfixed in osmium tetroxide. Both are abundant in fiber cell extensions (Fig. 4). Microfilaments in the 6 nm range are also often found close to the cell periphery where the extensions originate (Fig. 3). In these locations they frequently form dense webs (compare also Fig. 6) rather than parallel bundles.

2. Lysed cells

A more complete survey of the cytoskeletal structures can be obtained by the study of fiber cells attached to the supporting film and lysed in a suitable detergent. Brief treatment (1 min) with Triton-X 100 solubilizes the plasmalemma, most internal membrane systems and many cytoplasmic proteins (Schliwa et al. 1981 b) while an equally short application of 0.1% saponin is said to cause only small holes in the cell membrane (Mesland and Spiele 1984). The degree of extraction can further be controlled by the addition of polyethylene glycol.

After brief extraction with detergent, the fiber cell remains attached and preserves its general shape. Figure 5 shows a cell after 1 min saponin treatment. It should be stressed that the shape of these cells which are attached all over one surface to the supporting film differs from that in the organism. The cell body and the extensions are flattened to the substrate and the extensions, which have formed anew after isolation, are unbranched and often curved. Frequently, they show a concave side which is supported by a bundle of microtubules and a convex side resembling a broad lamellipodium. Cytoplasmic remnants and cytoskeletal elements form a more or less dense meshwork (Figs. 6–8). Figure 6 shows a microtubule bundle reaching into a fiber cell extension and a network of 6–7 nm filaments at its base. Single microtubules and small bundles are found in various directions in the peripheral cytoplasm (Fig. 7) while the central part of the cell is too thick and dense to study cytoskeletal structures. Microfilaments of about 6–7 nm oriented in different directions contribute to the network-like appearance of the cytoplasm. In addition, filaments of an intermediate size (10–14 nm) and much thinner fibers of 2–3 nm can be made out (Fig. 7, inset). Longer extraction (6 min) reveals no other cytoskeletal features but the background of adhering material is reduced and the spongy network appears loosened (Fig. 8). Bundled microtubules which reach into the extensions can be followed deep into the cell interior.

Triton-X 100 leads to a more extensive solubilization but the overall shape of the cell is still well preserved (Fig. 11), although there is some damage at the periphery. Some extensions appear shrunken and occasionally extensions have torn away. In spite of these shortcomings, some aspects of the distribution of cytoskeletal structures can

Fig. 1. Fiber cell, freeze-substitution. *n* nucleus, *m* mitochondrial complex, *g* Golgi apparatus, *k* concrement vacuole. Scale: 0.5 µm. *Inset:* Enlargement of the fiber cell extension; *arrows* intermediate filament. Note microfilaments in the 6 nm-range below the intermediate filament. Scale: 0.2 µm

Fig. 2. Freeze-substitution. *g* Golgi apparatus, *c* centrioles. *arrows* microtubules, some radiating from the centriole region. Scale: 0.5 µm

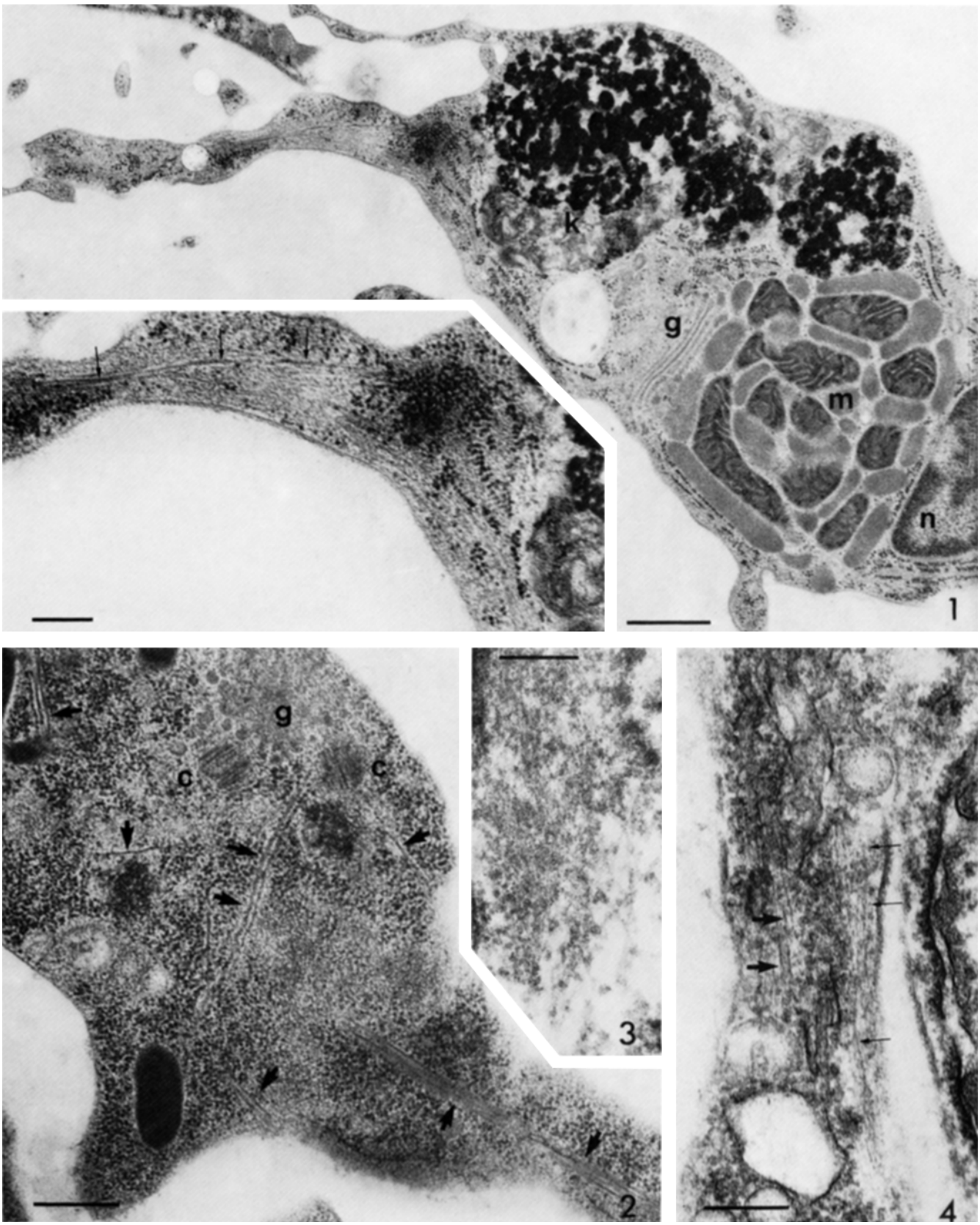


Fig. 3. Criss-crossing microfilaments beneath the cell membrane of a fiber cell extension. Tannic acid, glutaraldehyde, phalloidine; postfixation OsO_4. Scale: 0.2 μm

Fig. 4. Microtubules (*large arrows*) and microfilaments (*small arrows*) in a fiber cell extension. Tannic acid, glutaraldehyde, phalloidine; postfixation OsO_4. Scale: 0.2 μm

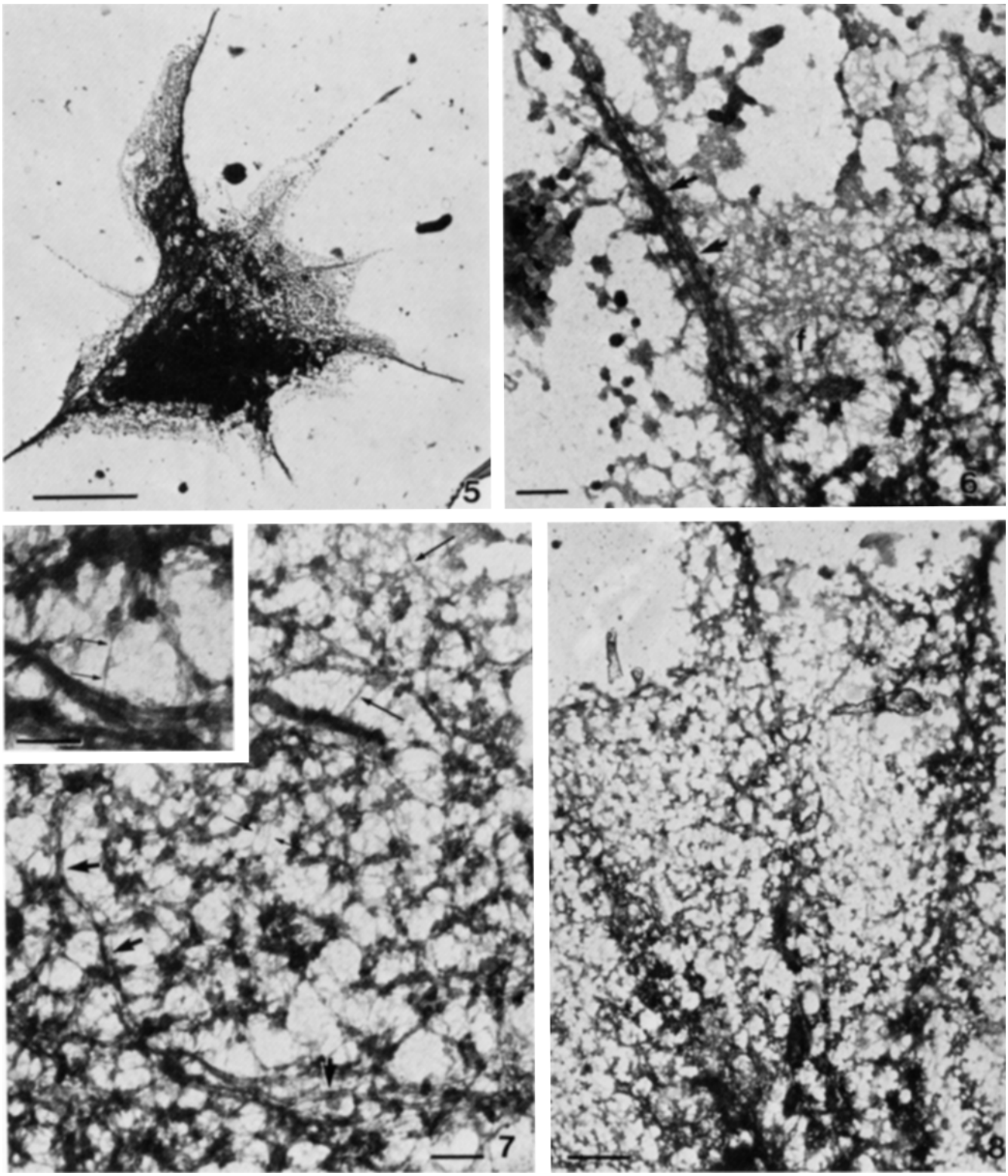


Fig. 5. Survey of a fiber cell extracted for 1 min with 0.1% saponin. The extensions, supported by microtubules, given the appearance of a network while the central regions of the cell contain much unextracted material. Scale: 5 μm

Fig. 6. Enlargement of Fig. 5. Note bundled microtubules (*arrows*) and criss-crossing microfilaments (*f*) in a fiber cell extension. Scale: 0.2 μm

Fig. 7. Peripheral cytoplasm of the cell shown in Fig. 5. Note the complex network of microbutules (*heavy arrows*), intermediate filaments (*long arrows*) and microfilaments (*short arrows*). Scale: 0.2 μm. *Inset:* Enlarged part of Fig. 7, showing 3 nm-filament (*arrows*). Scale: 0.1 μm

Fig. 8. Survey of the peripheral region of a fiber cell extracted for 6 min with 0.1% saponin. Bundles of dense material containing microtubules radiate to the periphery. Scale: 0.1 μm

be discerned. Long tracts of fibrillar structures seem to converge from the cell periphery and the interior unextracted part to the remaining extensions. Within the extension, microtubules, intermediate filaments and microfilaments show a preferentially longitudinal orientation within a meshwork of an undefined nature (Fig. 9). The peripheral cytoplasm (Fig. 10) shows long stretches of intermediate filaments as well as microfilaments of 6–7 nm and about 3 nm thickness. Further extraction of cytoplasmic remnants is achieved if Triton-X100 is dissolved in 10% (instead of 30%) polyethylene glycol. An example is shown in Fig. 12 where the microfilaments of a fiber cell extension stand out particularly clearly. The thicker filaments are oriented lengthwise within the extension and seem to be cross-connected by the 3 nm filaments.

Application of colchicine (10–5 *M*, 5 min) in the saponin lysis mixture leads to the withdrawal of all extensions. The cells remain attached but they round off and become too dense for an analysis of their cytoskeletal components.

3. Immunofluorescence

The presence of both actin and tubulin in fiber cells is demonstrated with specific antibodies labelled with a fluorochrome. Figures 14 and 15 illustrate the staining pattern obtained with anti-actin. A weak but distinct fluorescence is seen in all fiber cell extensions. The tips of the extensions may show a more intense reaction, especially when they are enlarged to a more or less disc-shaped adhesion plaque (comp. Figs. 13 and 14). There is a diffuse fluorescence throughout the cytoplasm. At the cell periphery, there is a thin rim of fluorescence, indicating an association of actin microfilaments with the cell membrane.

The pattern of anti-tubulin fluorescence is entirely different (Fig. 16). There is only a weak reaction in the cytoplasmic interior in accordance with the relatively sparse microtubules found there. The rim of the cell and the extensions show a more pronounced fluorescence suggesting the presence of bundled microtubules. In addition, the short microspikes found at the periphery of this cell contain microtubules as does the edge of a broad lamellipodium seen at the lower right of the figure. Such lamellipodia are often found in isolated fiber cells attached to a substrate.

D. Discussion

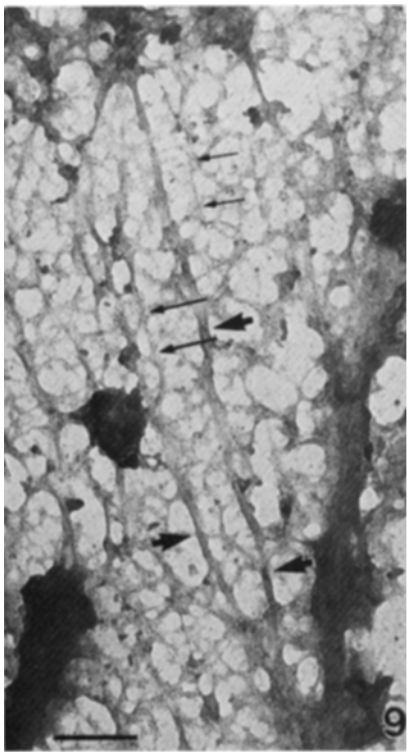
When *Trichoplax* is observed under a low-power dissecting microscope two types of movement are readily discerned. One is a slow gliding over the surface at speeds of 0.5 to 2 mm/min and is undoubtedly due to the action of the ventral cilia against the substrate. The second type of movement consists of a relatively rapid change of the outer circumference superficially resembling amoeboid movement (Kuhl and Kuhl 1966; Grell 1973). Another type of shape change is the localized buckling-up of the ventral epithelium associated with extracellular digestion which can be ascribed to a bundle of presumably contractile microfilaments located distally at the level of the belt desmosomes (Ruthmann et al. in press). This is a slow process and can be looked upon as "temporary gastrulation" from a phylogenetic standpoint (Grell 1973).

The comparatively fast changes of the outer shape have been ascribed to contractility of the fiber cells whose branched extensions are in contact with each other and with both epithelia (Grell and Benwitz 1971). Under favorable circumstances, fiber cell contractions can be seen in the phase contrast microscope when looking through folds of the thin dorsal epithelium close to the margin of *Trichoplax*. Slight movement of the coverslip can lead to such folds which permit the observation of fiber cells without the light-scattering thick epithelium underneath. Altough the irregular twitching of single cells which is noted may, according to Grell and Benwitz, represent a 'physiological artefact' due to cover glass pressure, it does show the basic capacity of contraction. Under normal conditions, only the coordinated contraction of large numbers of fiber cells within a given area would be expected to lead to the pronounced shape changes of the organism. The mechanism of coordination remains unclarified. Specialized cell connections found in ultrathin sections by Grell and Benwitz (1974) are most likely to be involved. They have also been identified by scanning electron microscopy which shows, in addition, that the fiber cells are extensively interconnected by their branched extensions (Rassat and Ruthmann 1979).

In spite of these observations, microfilaments which might be responsible for contractility have not been demonstrated in the electron microscope as yet. This may, however, be due to poor preservation since *Trichoplax* is notoriously difficult to fix by conventional methods. In a previous study (Ruthmann et al. in press), a mixture of glutaraldehyde in PIPES buffer was shown to preserve epithelial cell structures including the terminal microfilament bundle rather well, although microtubules are depolymerized. In fiber cells, neither microtubules nor microfilaments of any kind could be demonstrated by the simultaneous or the sequential action of both fixatives. The apparent absence of microfilaments in fiber cells led Klauser and Ruppert (1981) to the suggestion that all contractile phenomena of *Trichoplax* might be due to the terminal web of microfilaments in the cells of the ventral epithelium.

As shown in the present paper, cytoskeletal elements can be demonstrated within the fiber cells by alternative methods. Freeze-substitution preserves both microtubules and microfilaments, although the cytoplasmic ground substance becomes too dense for a detailed study of the latter.

Tissue fragments obtained with the aid of a constriction pipette can be fixed by the routine glutaraldehyde and osmic acid-procedure, indicating that poor penetration due to the extensive covering of the animal with slime may be the primary cause of faulty fixation when whole *Trichoplax* are used. Double fixation of tissue fragments in the presence of phalloidin which protects F-actin against the destructive effects of osmic acid (Gicquaud et al. 1980) and tannic acid both for better preservation (Seagull and Heath 1979) and contrast has permitted the demonstration of microfilaments of the 6–7 nm range as well as microtubules in fiber cell extensions. Such microfilaments could also be shown both in the cell body and the extensions in cells viewed in toto after lysis. Both the morphological findings and the localization of actin by immunofluorescence suggest that fiber cell contractility has the same molecular basis as in other cell types. The presence of a large mitochondrial complex only in fiber cells would also seem to agree with specialization for energy-dependent contactility. The precise role of the microtubules in the cell body and the cell extensions and their relationship to the various filamentous structures remains to be established. Since colchicine leads to their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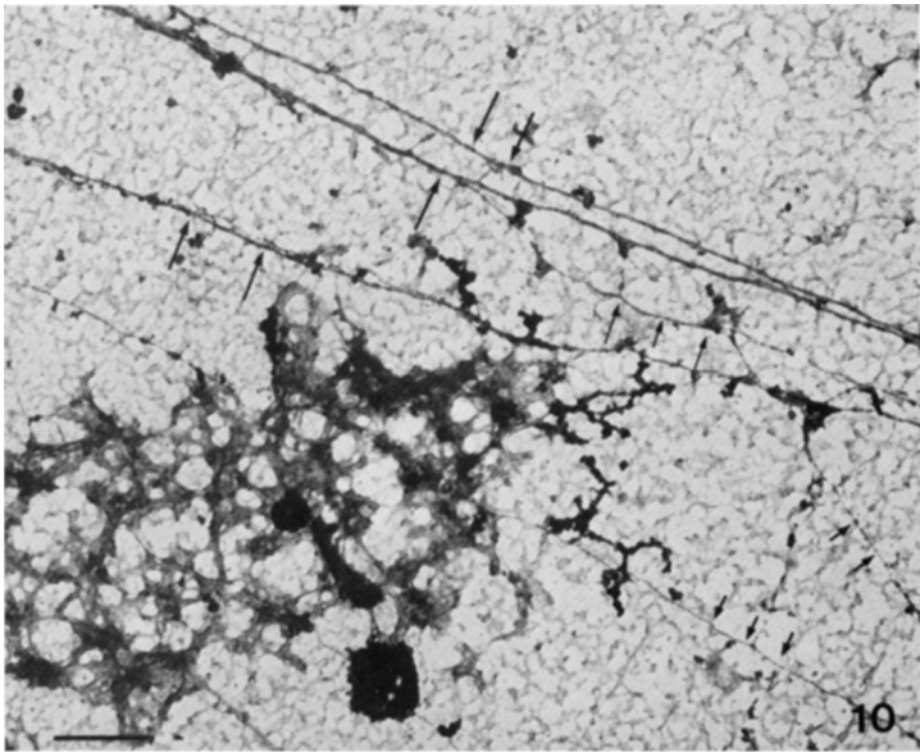
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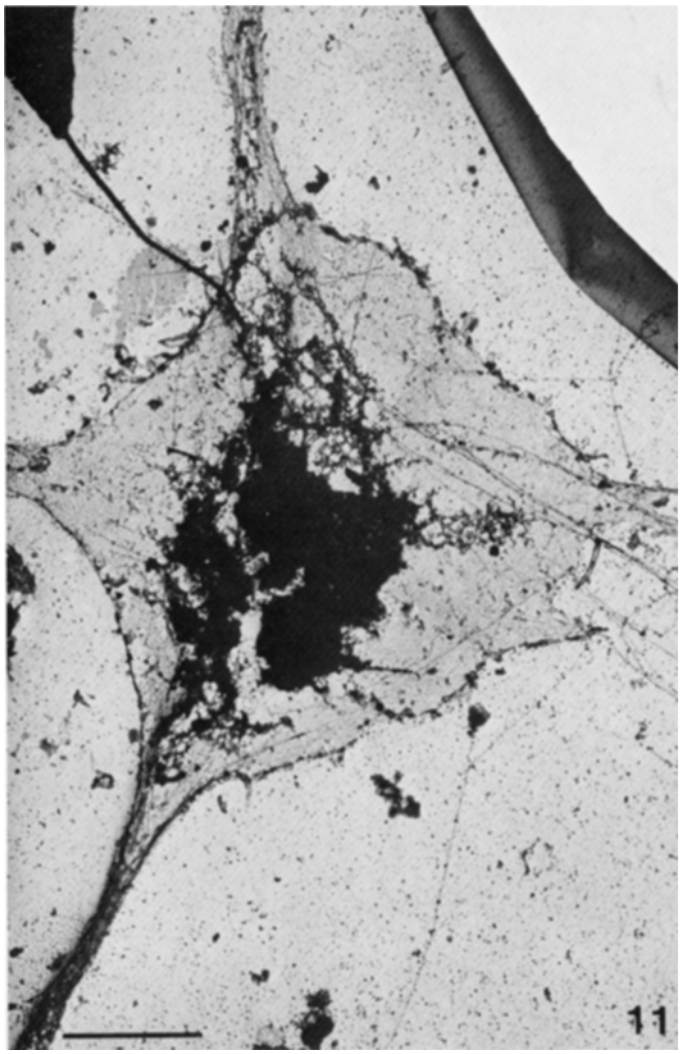
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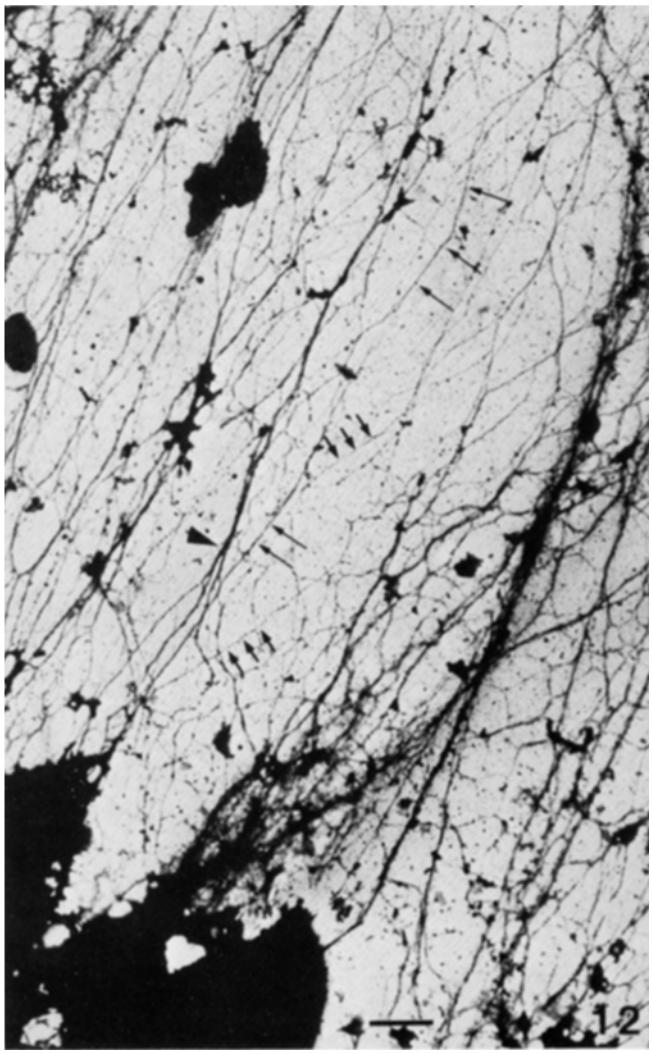
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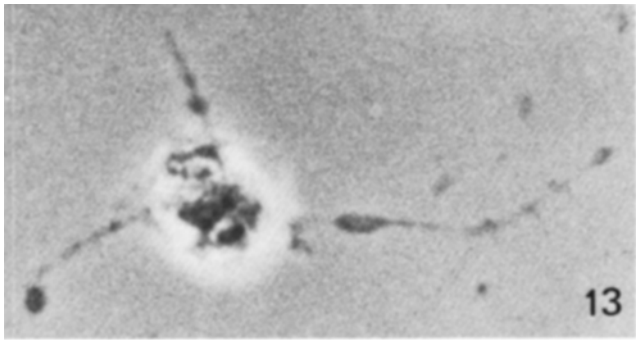

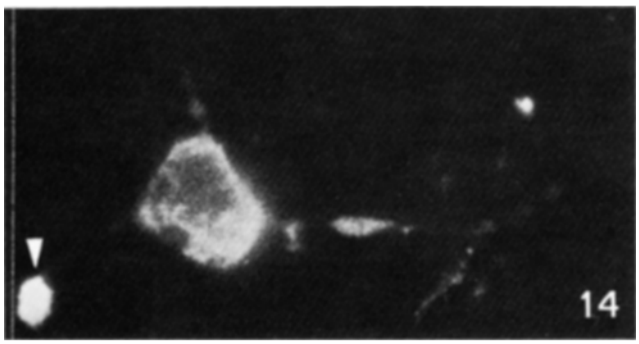

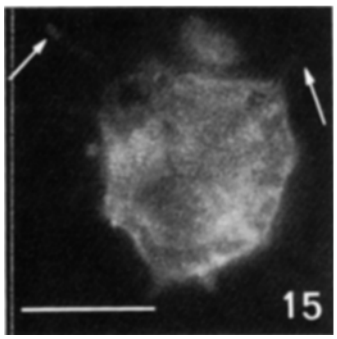

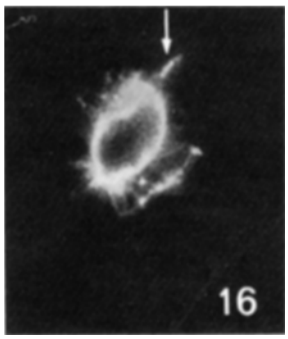

Figs. 13, 14. Fiber cell with long extensions, phase contrast and antiactin fluorescence. The *arrowhead* points to a knob-like adhesion plaque at the termination of an extension

Fig. 15. Antiactin fluorescence of a fiber cell with short extensions (*arrows*)

Fig. 16. Antitubulin fluorescence. Numerous short and one larger extension with microtubules. Note the intense fluorescence along the edge of a lamellipodium (*lower right*). Scale for all figures: 10 μm

withdrawal, the extensions seem to be stabilized by microtubules.

Extraction methods with nonionic detergents to study the cytoskeleton have become established and improved some years ago (Brown et al. 1976; Osborn and Weber 1977; Webster et al. 1978; Trotter et al. 1978; Heuser and Kirschner 1980; Schliwa et al. 1981a, b). The results obtained with tissue culture cells are in good accordance with our findings. Brief extraction with detergents such as Brij 58 leaves a cytoplasmic matrix of a spongy appearance consisting in part of a network of microfilaments and microtubules (Schliwa et al. 1981a) as in the saponin-treated cells of *Trichoplax*. In addition, there are lattice elements which are thinner in the middle than at the ends leading to an appearance like the microtrabecular system (Wolosewick and Porter 1979) whose real existence has, however, been doubted (Heuser and Kirschner 1980). The microtrabecular appearance is progressively lost if extraction times are extended or if Triton X-100 is used as a detergent. With the latter, the microfilaments stand out more clearly and can be followed over larger distances, but their number seems reduced. Similar observations have been made by Schliwa et al. (1981a) when comparing the effects of Brij 58 with Triton X-100. The latter compound led to a massive release of polypeptides from cells pre-extracted with Brij 58.

Besides microtubules, three types of filamentous structures were regularly found in cultured mammalian cells by Schliwa et al. (1981b). These include intermediate filaments, actin and 2–3 nm filaments of unknown biochemical nature. Actin and the 2–3 nm filaments seem to play a role as cytoskeletal cross-linkers. Although *Trichoplax* as the presumably most primitive metazoon is phylogenetically far removed from mammals, our own findings are in full accord with this, indicating that the filaments described are most likely of principal importance as structural and functional elements of animal cytoskeletons. In the absence of immunochemical data, the biochemical nature of the *Trichoplax* intermediate filaments remains obscure. Microfilaments of the 6–7 nm class may represent actin since the corresponding immunofluorescence is found both within the cell body and in the extensions. Actin may be involved both in contraction as well as cell adhesion. Its presence beneath the cell membrane may be indicative of a role in contraction since actin microfilaments have been shown to be indirectly linked to the cell membrane in other contractile systems (e.g. Mooseker and Tilney 1975; Condeelis 1979).

Fig. 9. Extension of a fiber cell extracted for 5 min with 0.1% Triton-X 100. Microtubules (*heavy arrows*), intermediate filaments (*long arrows*) and microfilaments (*short arrows*). Scale: 0.2 μm

Fig. 10. Peripheral cytoplasm, 5 min Triton-X 100. Note the long strands of intermediate filaments (*long arrows*), 6–7 nm microfilaments (*thin arrows*) and 3 nm filaments (*short arrows*). Scale: 0.5 μm

Fig. 11. Survey of a cell extracted for 5 min with Triton-X 100. Scale: 5 μm

Fig. 12. Part of a fiber cell extension, 5 min Triton-X 100, 10% polyethylene glycol. Compared with Figs. 9 and 10 (30% polyethylene glycol), the cytoplasmic ground substance appears completely extracted, leaving long strands of 6–7 nm filaments (*thin arrows*) and interconnecting 3 nm filaments (*short triple arrows*). Intermediate filaments cannot be definitely identified since smaller fibers seem to have collapsed to form thicker strands (*arrowhead*). Scale: 0.2 μm

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