Extracellular ciliary axonemes associated with the surface of smooth muscle cells of ctenophores

SIGNHILD TAMM* and SIDNEY L. TAMM*

Station Zoologique, Villefranche-sur-Mer, 06230, France and Boston University Marine Program, Marine Biological Laboratory, Woods Hole, Massachusetts 02543, USA

* Present address: Boston University Marine Program, Marine Biological Laboratory, Woods Hole, MA 02543, USA

Summary

We describe the first example of bare ciliary axonemes existing outside eukaryotic cells. The axonemes run in longitudinal invaginations of the surface membrane of giant smooth muscle cells in ctenophores. No motility of the surface-associated axonemes has been detected in living muscles. The axonemes are truly extracellular and in direct contact with the extracellular matrix (mesoglea), as shown by the ultrastructural tracer horseradish peroxidase. The axonemes appear partially degraded and disorganized, and individual doublet microtubules are difficult to distinguish. Nevertheless, immunofluorescence microscopy shows that the axonemes retain antigenic sites reacting with mouse monoclonal anti-beta-tubulin. The origin of the extracellular axonemes is unknown: no attached basal bodies (extracellular or intracellular) have been found. The muscle-associated axonemes may play a unique role in smooth muscle function and/or development, and may be related to the evolution of muscle cells in soft-bodied invertebrates that exploit cilia for a wide variety of functions.

Key words: extracellular axonemes, smooth muscle, ctenophores.

Introduction

Eukaryotic cilia and flagella consist of a 9+2 microtubular axoneme enclosed by an extension of the cell membrane. Whether functioning in motility or in sensory receptors, all cilia and flagella described so far are intracellular organelles. Prokaryotic flagella, on the other hand, are bare protein filaments that project externally from rotary motors embedded in the bacterial cell membrane.

In this report we describe the first example of bare microtubular axonemes existing outside eukaryotic cells. The extracellular axonemes run in longitudinal infoldings of the plasma membrane on the surface of giant smooth muscle cells in ctenophores. This unique association between a nonmuscle motility system and a muscle one may be related to the evolution of muscle cells in simple animals that exploit cilia for a wide variety of functions.

Materials and methods

Beroë cucumis was collected around Woods Hole or shipped from Friday Harbor Laboratory, WA, by Dr Claudia Mills. *B. gracilis* was collected at the Biologische Anstalt Helogland, Helgoland, West Germany. *B. mitrata* and *B. ovata* were collected at Station Zoologique, Villefranche-sur-Mer, France. Several *B. mitrata* were sent from Friday Harbor by Dr Claudia Mills. *Mnemiopsis leidyi* was collected at Woods Hole. *Ocyropsis crystallina* was collected in the Gulf Stream aboard the R.V. Oceanus. Animals were kept in large containers of freshly flowing sea water, and used soon after collection.

Living muscles

Small pieces of *Beroë* body wall mesoglea were placed in a few drops of appropriate solution (see below) on vaseline-edged microscope slides. The tissue was gently minced with fine forceps to separate and disperse the embedded muscle fibers. Enzymatic digestion (Hernandez-Nicaise *et al.* 1982) was *not* used to dissociate muscle cells.

Muscle preparations were bathed in (1) normal sea water, (2) 7 % $MgCl_2$: sea water (1: 1) or 7 % $MgCl_2$: Ca-free artificial sea water (1: 1; relaxing solution), or (3) 50 mM-KCl–50 mM-$CaCl_2$ sea water (contraction solution).

Sections of *Mnemiopsis* comb rows were cut out and mounted in sea water on microscope slides for observations of comb row retractor muscles *in situ*.

Muscle fibers were photographed with Zeiss phase-contrast and DIC optics (40×/0.75 NA objective) using an Olympus 35 mm camera (OM-2N) and Olympus electronic flash with Kodak Tech Pan (2415) film.

Immunofluorescence microscopy

Small pieces of *B. ovata* body wall were fixed in 3.7 % formaldehyde, 0.1 % Triton X-100, 0.15 M-NaCl, 0.2 M-sodium cacodylate, pH 7.5, for 30 min at room temperature. Tissue was

washed in 0.15 M-NaCl, 0.025 M-sodium phosphate buffer, pH 7.0 (PBS), for 1 h, then divided into three groups. Groups 1 and 2 were incubated in mouse monoclonal anti-beta-tubulin IgG (Amersham, Arlington Hts, IL) diluted 1:250 in PBS, 0.1% bovine serum albumin, 0.1% sodium azide for 1 h. Group 3 was incubated in PBS, 0.2% bovine serum albumin (BSA) without primary antibody during this time. All groups were washed in PBS for 1 h, and then treated with biotinylated goat anti-mouse IgG+IgM (H+L) (Janssen, Biotech, Belgium) diluted 1:250 in PBS, 0.1% BSA, 0.1% sodium azide for 1 h. All three groups were washed in PBS for 1 h. Groups 1 and 3 were treated with rhodamine–streptavidin (Immunotech Co., Marseilles) diluted 1:50 in PBS, 0.1% BSA, 0.1% sodium azide for 1 h. Group 2 was treated in PBS, 0.2% BSA without rhodamine–streptavidin during this time.

Tissue pieces were mounted in PBS, 50% glycerol, 5% *n*-propyl gallate on a microscope slide and examined with Zeiss epifluorescence optics (TRITC filter set) using a 63×/1.4 NA planapochromat objective. Photographs were taken on Kodak Tri-X film pushed to ASA 1600 with Perfection XR-1 developer.

Electron microscopy

Pieces of *Beroë* body wall and *Mnemiopsis* comb rows were fixed in 2.5% glutaraldehyde, 1% paraformaldehyde, 1% osmium tetroxide, 0.075 M-NaCl (0.15 M for Mediterranean specimens), 0.01 M-$CaCl_2$, 0.2 M-sodium cacodylate, pH 7.8, for 1 h at 0°C, washed in 0.3 M-NaCl, 0.2 M-sodium cacodylate, pH 7.8, at 0°C, postfixed in 1% osmium tetroxide, 0.38 M-NaCl, 0.1 M-sodium cacodylate, pH 7.8, for 15 min at 0°C, washed in distilled water at 0°C, and placed in 1% uranyl acetate overnight at 4°C. Tissue was dehydrated in acetone, embedded in Araldite, thin-sectioned with a Diatome diamond knife on a Reichert ultramicrotome, stained with lead and uranyl salts, and examined with a Zeiss 10C electron microscope at 80 kV.

Ocyropsis comb rows were fixed aboard ship in 2.5% glutaraldehyde, 0.14 M-NaCl, 0.2 M-sodium cacodylate, pH 7.5, for 2 h at room temperature, washed in 0.3 M-NaCl, 0.2 M-sodium cacodylate for 1 h, postfixed in 1% osmium tetroxide, 0.38 M-NaCl, 0.1 M-sodium cacodylate for 1 h, washed in distilled water, treated with 0.5% uranyl acetate for 2 h, then dehydrated and embedded in Araldite.

An osmium ferricyanide–tannic acid fixation method (McDonald, 1984) was also used for some tissue. Body wall pieces of *B. cucumis* were fixed in 2.5% glutaraldehyde, 1% paraformaldehyde, 0.075 M-NaCl, 0.01 M-$CaCl_2$, 0.2 M-sodium cacodylate, pH 7.5, for 1 h at room temperature. Tissue was washed in 0.3 M-NaCl, 0.2 M-sodium cacodylate, pH 7.5 (buffer wash), for 2 h at room temperature, then postfixed in 0.5% osmium tetroxide, 0.8% potassium ferricyanide, 0.375 M-NaCl, 0.1 M-sodium cacodylate, pH 7.5, for 30 min at 0°C. Following buffer washes, tissue was treated in 0.2% tannic acid in buffer wash for 30 min at room temperature. Tissue was washed in buffer, then distilled water, and placed in 2% uranyl acetate overnight at 4°C. Tissue was dehydrated in ethanol and processed for electron microscopy as above.

Extracellular tracer

Pieces of *B. cucumis* body wall were incubated in 1 mg ml^{-1} horseradish peroxidase (Sigma type II) in sea water for 5 h at 4°C. Tissue was fixed in 2.5% glutaraldehyde, 1% paraformaldehyde, 0.075 M-NaCl, 0.01 M-$CaCl_2$, 0.2 M-sodium cacodylate, pH 7.5, for 1 h at room temperature, washed in 0.3 M-NaCl, 0.2 M-sodium cacodylate, pH 7.6, for 3 h, and incubated in 0.5 mg ml^{-1} diaminobenzidine tetrahydrochloride (DAB, Sigma D-5637) in 0.1 M-Tris–HCl, pH 7.5, for 2 h in the dark. Tissue was placed in DAB/Tris containing 0.01% H_2O_2 for 2 h in the dark, washed in distilled water, and postfixed in 1% osmium tetroxide, 0.38 M-NaCl, 0.1 M-sodium cacodylate for 1 h at room temperature. Following distilled water washes, tissue was left in 1% uranyl acetate overnight at 4°C. Tissue was dehydrated in ethanol, embedded in Araldite, and thin-sectioned for electron microscopy as above.

Results

Background

Ctenophores are among the simplest animals with individual muscle cells. With the exception of striated muscles in the tentilla of the cydippid ctenophore, *Euplokamus* (Mackie *et al.* 1988), all muscles in ctenophores are smooth (non-striated). The giant, multinucleated smooth muscle cells of the ctenophore *Beroë* are the largest smooth muscle cells known, reaching 6 cm in length and up to 50 μm in diameter (Hernandez-Nicaise *et al.* 1980, 1982, 1984; Hernandez-Nicaise and Amsellem, 1980).

Beroë muscles are organized into three main systems: (1) longitudinal fibers that run through the mesoglea parallel to the meridional comb rows; (2) radial fibers that cross the mesogleal body wall and branch extensively at both ends on the pharyngeal and outer epithelial layer; and (3) circular fibers that run around the pharyngeal cavity under the epithelium (Hernandez-Nicaise and Amsellem, 1980; Hernandez-Nicaise *et al.* 1980).

Mnemiopsis and *Ocyropsis*, lobate ctenophores distantly related to *Beroë*, possess sagittal bundles of long smooth muscle fibers running along the pharyngeal wall (Hernandez-Nicaise *et al.* 1984; Anderson, 1984). In addition, lobate ctenophores have transverse bundles of comb row retractor muscles lying in the mesoglea midway between adjacent comb plates.

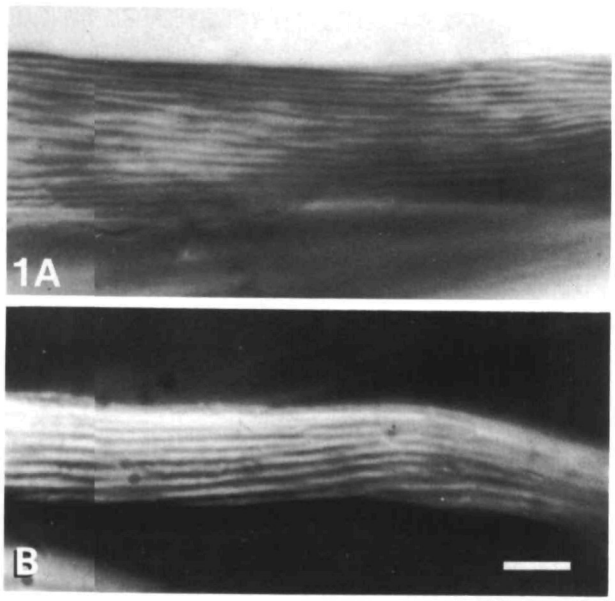

Fig. 1. Longitudinal striations on the surface of living muscle cells. A. Radial muscle in body wall of *B. cucumis* (relaxation solution). B. Comb row retractor muscle of *Mnemiopsis*. Bar, 10 μm.

Light microscopy of living muscles

The transparency of the mesoglea allows DIC and phase-contrast microscopy of living muscle fibers in whole-mount preparations. In all species of *Beroë* examined, the surface of longitudinal and radial muscles is scored by numerous closely spaced striations that run parallel to the long axis of the fiber (Fig. 1A). The surfaces of *Mnemiopsis* muscles are similarly marked by longitudinal striations

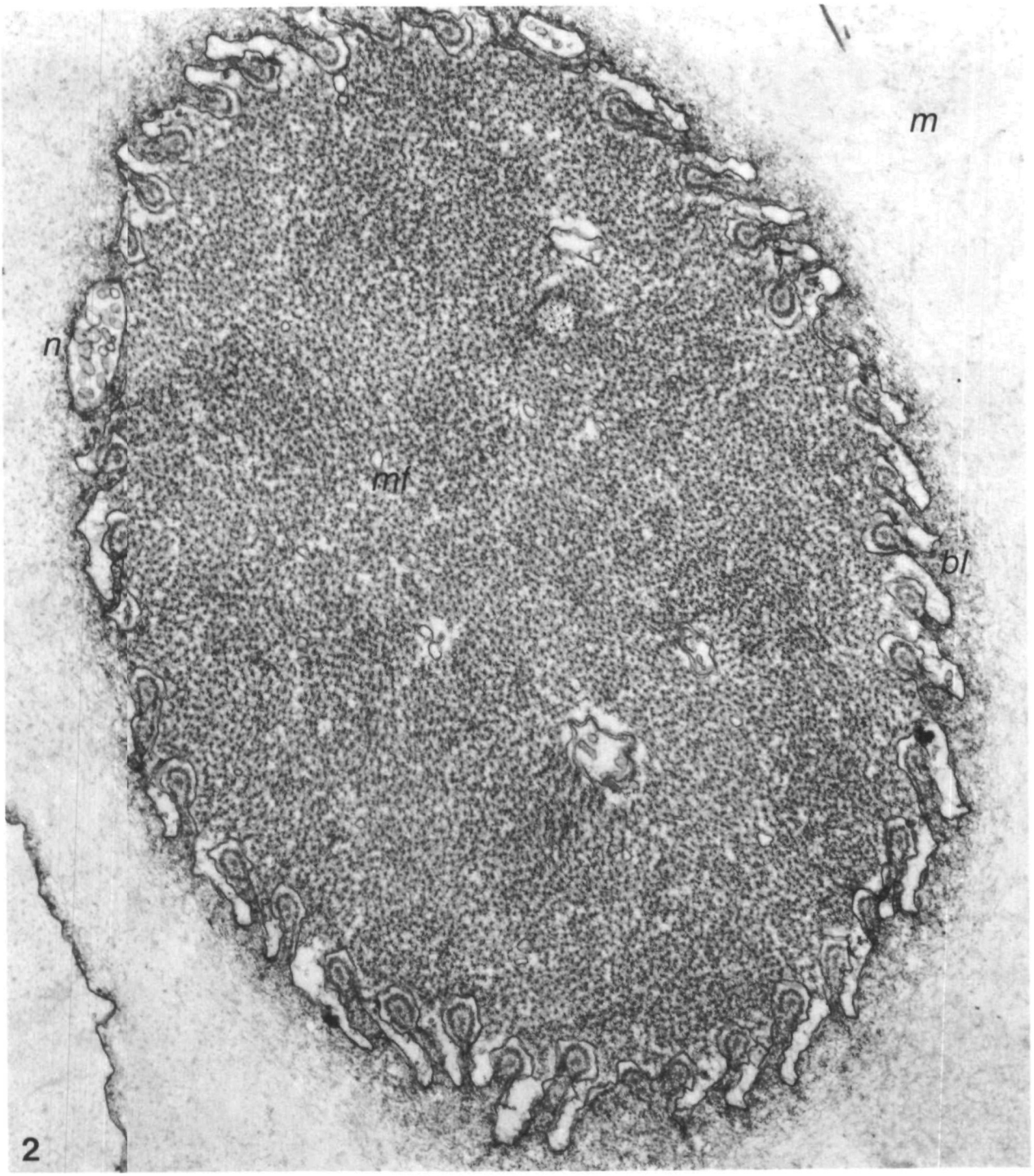

Fig. 2. Transverse section of a smooth muscle cell in the mesoglea body wall (m) of *B. mitrata*. Longitudinally running axonemes (shown in cross-section) lie in gutter-like infoldings of the sarcolemma. The axonemes are in direct contact with the mesoglea through the narrow necks of the membrane invaginations. The axis of the central-pair microtubules in many axonemes is oriented parallel to the muscle surface. Axonemal walls are split open with a gap facing the opening of the sarcolemmal furrows. A surface coat (basal lamina, bl) surrounds the muscle fiber. Myofilaments (mf) are shown in cross-section. Neurite (n) contacts muscle cell at upper left; axonemes and sarcolemmal furrows are absent in this region. ×16 900.

(Fig. 1B). These longitudinal stripes on the surface of ctenophore muscle cells were first observed by Will (1844) and later described by Eimer (1873) and Chun (1880), but have not been reported in recent studies (Hernandez-Nicaise *et al.* 1980, 1984; Hernandez-Nicaise and Amsellem, 1980; Anderson, 1984).

As we will show, the striations represent extracellular axonemes running in grooves or furrows of the sarcolemma. Discontinuities or terminations of the stripes have not been observed by light microscopy; the axonemes may therefore extend the entire length of the muscle fiber (with the exception noted below).

No motility of the surface striations (i.e. undulations or bending) has been seen in living muscles, regardless of the ionic composition of the medium (i.e. sea water, relaxation solution or contraction solution).

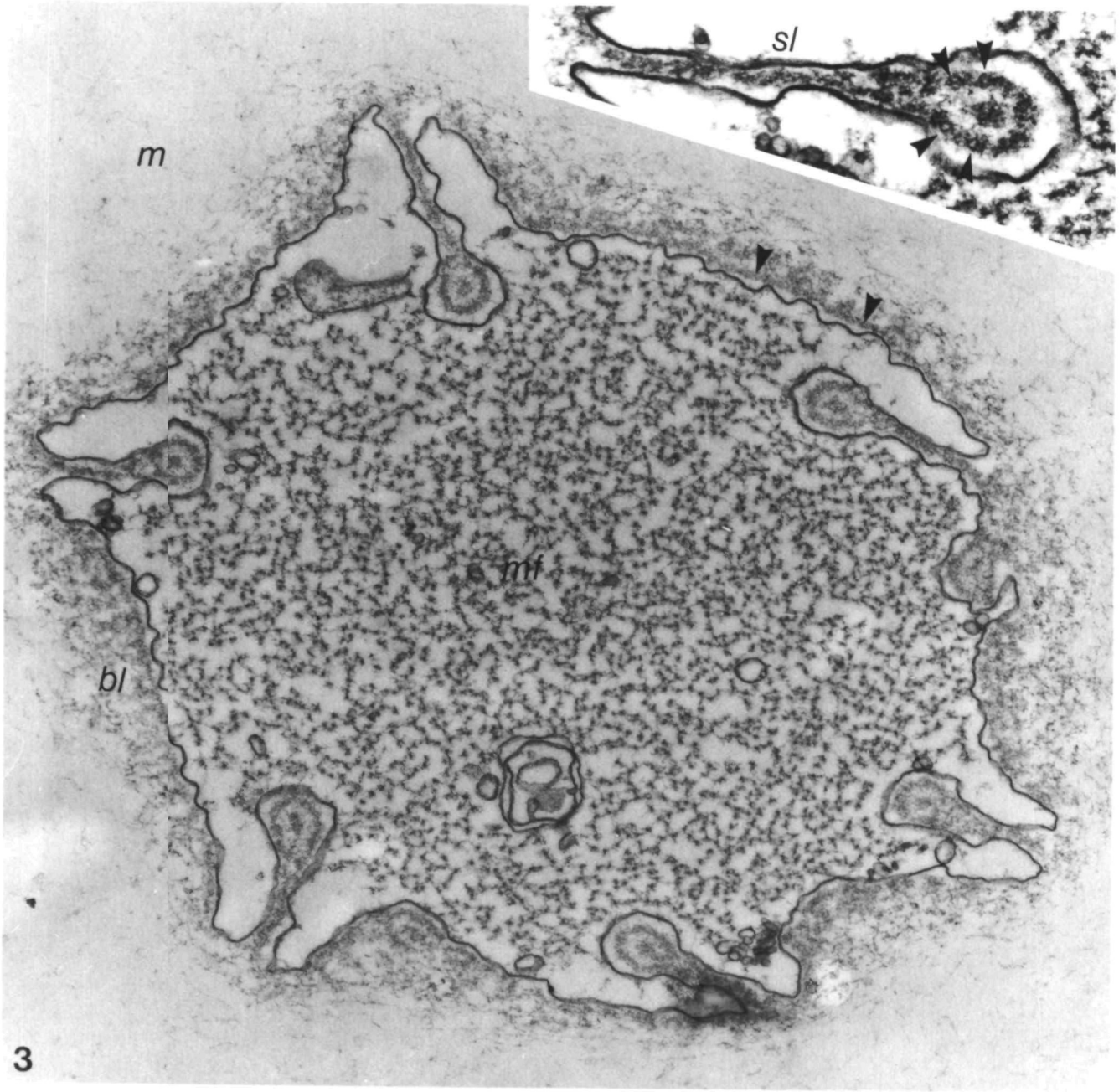

Fig. 3. Transverse section through a small-diameter branch of a radial smooth muscle cell of *B. mitrata*. The sarcolemmal (sl) infoldings with their bare axonemes are particularly obvious (inset, higher magnification of another muscle). The gutter-like sarcolemmal invaginations are separated from the axonemes by a clear space. The axonemes are directly continuous with the extracellular matrix (mesoglea, m) through the narrow openings of the membrane infoldings. The axonemal wall appears granular and fuzzy, but microtubules are sometimes distinguishable (arrowheads, inset). The axonemal cylinder is split open on the outer side, and dense material extends from this gap into the narrowed membrane infolding, continuing around the outside of the sarcolemma as a thin dense layer (arrowheads, main figure). A broader, more flocculent surface coat (basal lamina, bl) surrounds the muscle fiber. Myofilaments (mf) are cut transversely. ×31 000. Inset, ×57 000.

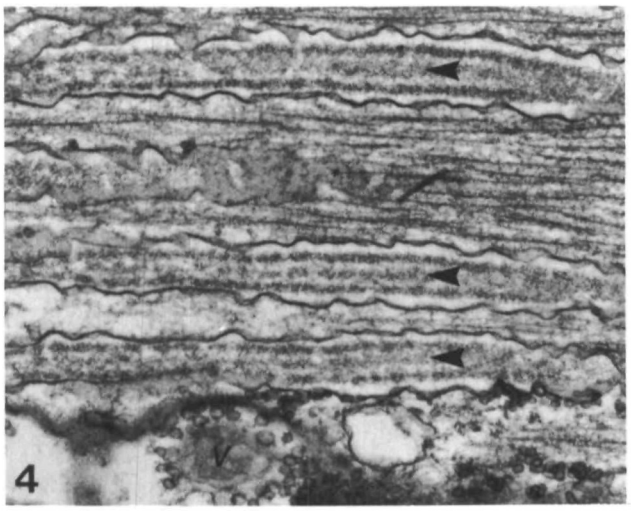

Fig. 4. Longitudinal section through the surface of a mesogleal muscle fiber of *B. mitrata*. Parallel axonemes run in longitudinal grooves of the sarcolemma, alternating with surrounding areas of myofilaments. The axonemal central-pair microtubules (arrowheads) and doublets are fuzzy and granular. A neurite with synaptic vesicles (v) forms a neuromuscular function at the bottom of figure. ×23 700.

Electron microscopy

Thin sections through longitudinal and radial muscle fibers of *Beroë* show bare axonemes running longitudinally in grooves or infoldings of the sarcolemma (Figs 2,3,5–7). The axonemes are regularly spaced around the circumference of the muscle cell. This parallel array of axonemes in membrane furrows gives rise to the longitudinally striped appearance of the muscle surface as seen by light microscopy (Fig. 1). Axonemes are not found on circular (subepithelial) muscle fibers of *Beroë* (see also Tamm and Tamm, 1987). Recent investigators did not report the presence of muscle-associated axonemes, and interpreted the longitudinal infoldings of the sarcolemma as tubular sacs (Hernandez-Nicaise *et al.* 1980; Hernandez-Nicaise and Amsellem, 1980) analogous to the caveolae of vertebrate smooth muscle (Gabella, 1971).

The axonemes have a typical diameter of 0.2–0.25 μm, but their appearance depends on the species. The most distinct axonemes found so far are on muscles of *B. mitrata* (Figs 2–4). Even in this species, however, the outer microtubular wall is amorphous and granular, making individual doublets difficult to distinguish. Tannic acid fixation improves the clarity of the doublet microtubules (Fig. 6), but dynein arms have not yet been identified. The central-pair microtubules, when recognizable, are often oriented parallel to the surface of the muscle cell (Fig. 2). The axonemal cylinder is typically split open, with a gap on the side facing the neck of the sarcolemmal furrow (Figs 2,3,5A).

In longitudinal thin sections the axonemes run straight along the surface of the muscle fiber, and the granular appearance of the microtubules is evident (Figs 4,5B). Except at neuromuscular junctions (below), no terminations of the axonemes have been observed, nor have attached basal bodies been found.

The absence of a surrounding membrane and the indistinct appearance of the extracellular axonemes is not a fixation artifact, since comb plate cilia and macrocilia in the same thin sections are well preserved.

In *B. mitrata* the deep sarcolemmal furrows increase the surface area of the muscle cells by 5- to 10-fold. In this species the membrane infoldings are usually angled to the muscle surface (Figs 2,3). The infoldings are particularly noticeable in small diameter branches of radial muscles (Fig. 3). The sarcolemmal invaginations widen at the bottom and surround most of the circumference of the axonemes like a gutter. The axonemes are separated from the sarcolemma by a clear space that is bridged by wispy strands (Fig. 3). The membrane gutters containing the axonemes are embedded in, and closely surrounded by, myofilaments at the periphery of the sarcoplasm. No cytoplasmic microtubules have been detected within the muscle cells.

At the gap in the axonemal wall the sarcolemmal infoldings narrow and are filled with dense material that resembles a continuation of the axonemal wall (Figs 2,3). This material extends upward through the neck of the membrane invaginations, and continues out around the surface of the muscle fiber. A broader outer surface coat (basal lamina) lies above this layer and faces the surrounding mesoglea (Figs 2,3,5A).

In *B. ovata* (Fig. 5) the muscle-associated axonemes are not as distinct as in *B. mitrata*. The shaft of the axonemes is often filled with amorphous material, partially obscuring the central-pair microtubules (Fig. 5B).

In radial muscles of *B. ovata*, particularly those fixed in a contracted state, adjacent axonemes are separated by bulbous evaginations of the sarcolemma devoid of myofilaments, giving cross-sections a daisy-like appearance. These sarcolemmal evaginations are rarely present in longitudinal muscles of *B. ovata*.

In muscles of *B. cucumis* the sarcolemmal furrows are shallower, and the axonemal cylinders are split open with the open side facing the mesoglea (Fig. 6).

In *B. gracilis* the axonemal walls are further unrolled and lie in wide shallow depressions of the sarcolemma (Fig. 7).

Mnemiopsis comb row retractor muscles resemble *B. cucumis* muscles, in that the axonemes are partially unrolled and lie in shallow furrows of the sarcolemma (not shown).

Ocyropsis comb row retractor muscles possess numerous closely spaced axonemes running in U-shaped grooves separated by thin folds of the sarcolemma (not shown).

Neuromuscular junctions

Smooth muscle cells are innervated along their length by processes of the subepithelial nerve net. At neuromuscular junctions the axonemes and sarcolemmal infoldings are locally obliterated at the broadened region of contact between the ending of the neurite and the muscle cell (Fig. 8). Synapses have the characteristic 'presynaptic triad' appearance (single layer of synaptic vesicles, endo-

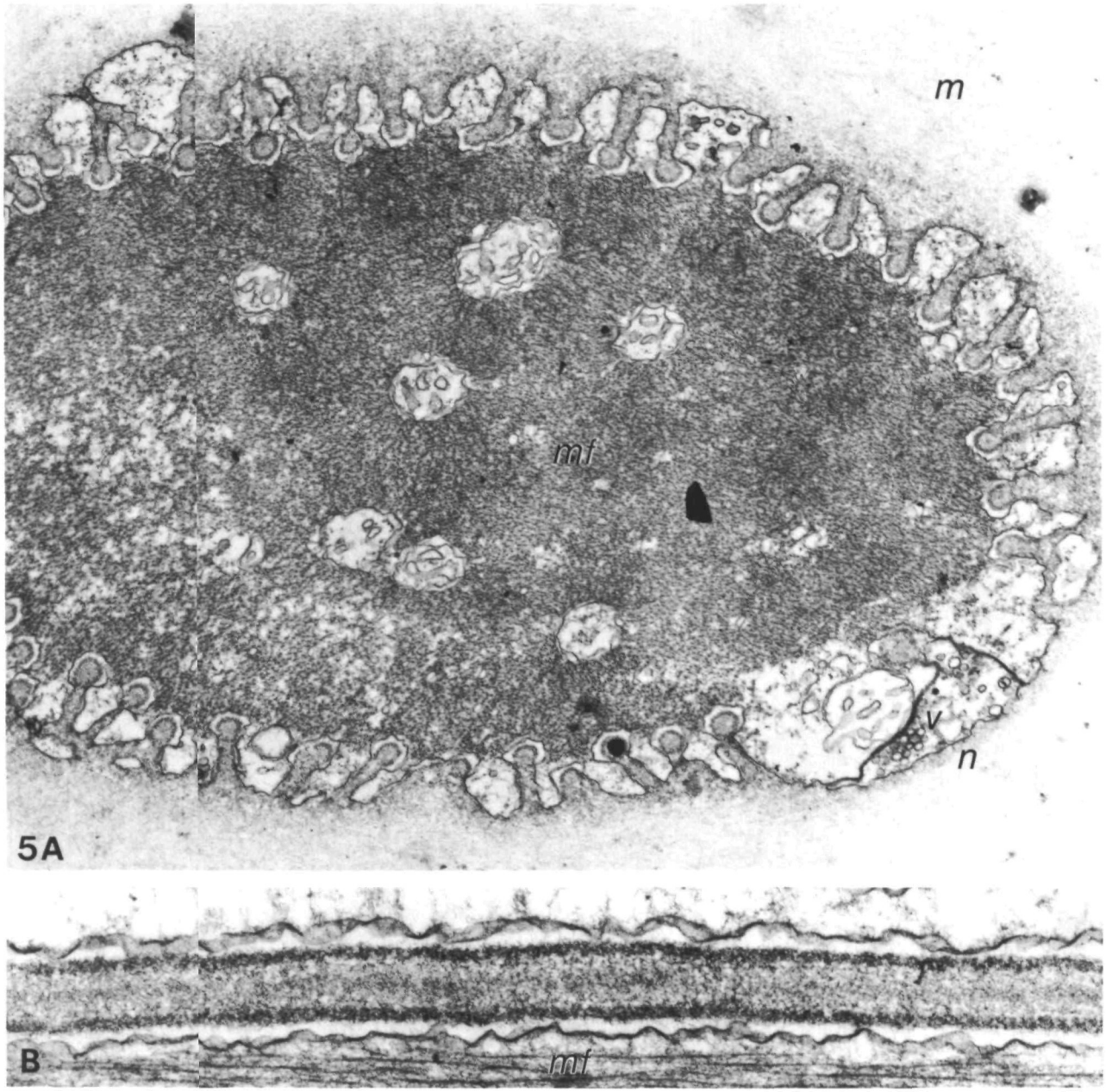

Fig. 5. Radial smooth muscle cells of *B. ovata*. A. Transverse section showing numerous bare axonemes running in longitudinal invaginations of the sarcolemma. The lumens of the axonemes contain dense material, and the central-pair microtubules are not clear. Sarcolemmal furrows and axonemes are locally obliterated at a neuromuscular junction (n, neurite; v, synaptic vesicles). ×12 100. B. Longitudinal section through the surface of a muscle fiber showing an axoneme in a sarcolemmal groove. Doublet microtubules appear granular and fuzzy, and the lumen of the axonemes is filled with dense material. mf, myofilaments. ×34 000.

plasmic reticulum sac, and closely opposed mitochondrion; Fig. 8) described for ctenophore synapses (Hernandez-Nicaise, 1973). Typically, large mitochondria of the muscle cell are closely apposed to the postsynaptic side of the junction; this feature of ctenophore neuromuscular junctions has not been reported previously (cf. Hernandez-Nicaise *et al.* 1980).

Extracellular tracer

An ultrastructural cytochemical marker technique utilizing horseradish peroxidase (Graham and Karnovsky, 1966; Karnovsky, 1967) was used to trace connections between the axonemes and extracellular matrix (mesoglea) in *Beroë*. Horseradish peroxidase fills the sarcolemmal infoldings and permeates the axonemes, as evidenced by the presence of electron-opaque reaction product at these sites (Fig. 9).

Therefore, the muscle-associated axonemes are in direct contact with the mesoglea, and are truly extracellular.

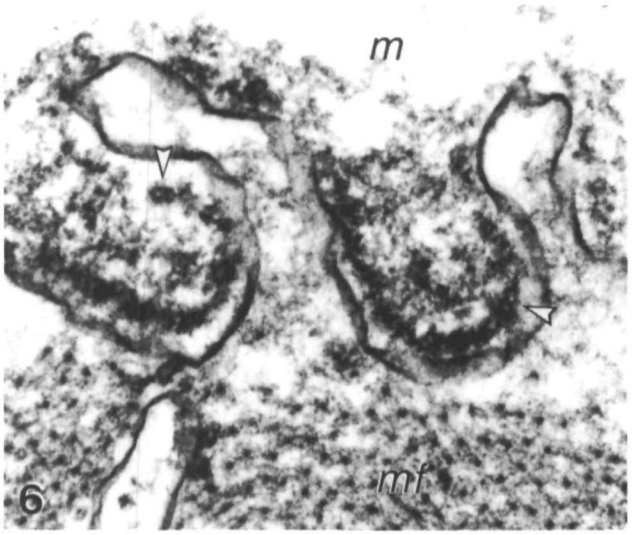

Fig. 6. Transverse section through axonemes in sarcolemmal grooves of a *B. cucumis* muscle fixed with tannic acid. Several doublet microtubules are evident (arrowheads). In this species the opening in the axonemal wall is wider, and the axonemes are partially unrolled. mf, myofilaments; m, mesoglea. ×64 600.

Anti-tubulin staining

Fluorescence microscopy of anti-tubulin rhodamine–streptavidin-treated tissue of *B. ovata* shows bright staining of the longitudinal striations on the muscle surface (Fig. 10B,D). Macrocilia, consisting of hundreds of 9+2 axonemes (Horridge, 1965; Tamm and Tamm, 1984, 1985), are also brightly fluorescent (Fig. 10F), showing the specificity of the staining for microtubules.

Controls in which either anti-tubulin or rhodamine–streptavidin was omitted did not show staining of longitudinal striations on muscle fibers, or of macrocilia (not shown).

These findings show that, despite the indistinct appearance of the extracellular axonemes, they contain tubulin.

Discussion

This is the first report of ciliary or flagellar axonemes existing outside cells. It is also the first known example of an association between axonemes (extracellular or intracellular) and muscle cells, or, more broadly, between a nonmuscle motility system and a muscle one.

In the following discussion we interpret these findings and consider the functional significance of this unique association.

Occurrence of muscle-associated axonemes

The cytology and ultrastructure of smooth muscles in *Beroë* and *Mnemiopsis* have been studied extensively by Hernandez-Nicaise and her colleagues (Hernandez-Nicaise *et al.* 1980, 1982, 1984; Hernandez-Nicaise and Amsellem, 1980). The presence of longitudinal striations on the surface of ctenophore muscle cells, although described by 19th century German cytologists (Will, 1844; Eimer, 1873; Chun, 1880), was not noticed by recent workers; nor have axonemes in longitudinal infoldings of the sarcolemma been reported. However, these features are evident in published electron micrographs of muscle fibers of *B. ovata* (Hernandez-Nicaise and Amsellem, 1980, Fig. 4; Hernandez-Nicaise *et al.* 1980, Fig. 3) and *Mnemiopsis leydii* (Hernandez-Nicaise *et al.* 1984, Fig. 8).

On the basis of our own observations of smooth muscles in various species of *Beroë* and in the lobates *Mnemiopsis* and *Ocyropsis*, as well as previously published micrographs, we believe that axonemes are a common surface feature of mesogleal muscles in ctenophores.

Extracellular location of axonemes

The muscle-associated axonemes truly lack membranes of their own and are in direct contact with the mesoglea, as is shown by the ultrastructural cytochemical tracer, horseradish peroxidase (Karnovsky, 1967; Graham and Karnovsky, 1966). Electron-opaque reaction product fills the sarcolemmal infoldings and permeates the axonemes (Fig. 9). It is unlikely that the bare and indistinct appearance of the axonemes is a fixation artifact, since the membranes and microtubules of comb plate cilia and macrocilia in the same sections are well preserved. In addition, the same electron-microscopic methods used here preserve the microtubular architecture of demembranated axonemes of ctenophore cilia (Tamm and Tamm, 1984, 1989).

The disorganized appearance of the axonemes and granular nature of the microtubules may reflect partial degradation due to their location in the extracellular matrix. Nevertheless, our immunofluorescence results show that antigenic sites reacting with mouse monoclonal anti-beta-tubulin are present in the axonemes. The splitting open and unrolling of the axonemal wall is reminiscent of structural changes undergone by sea-urchin sperm flagellar axonemes during the early stages of trypsin digestion (Summers and Gibbons, 1973). In this study, the nexin links between the outer doublets and the radial spoke connections to the central sheath were most sensitive to proteolysis.

Origins

The origin of the extracellular axonemes is unknown. We have not observed beginnings or ends of axonemes, except where they terminate locally at neuromuscular junctions. Nor have we found attached basal bodies. It is unlikely that the axonemes maintain connections to intracellular basal bodies (centrioles), since this would require ~0.2 μm diameter holes somewhere in a plasma membrane. Instead, we think that the axonemes are detached remnants of cilia of unknown origin that have secondarily lost their membranes in association with the outer surface of the muscle cells.

Functional significance

The absence of observable movements of axonemes on living muscle cells, together with their physical separ-

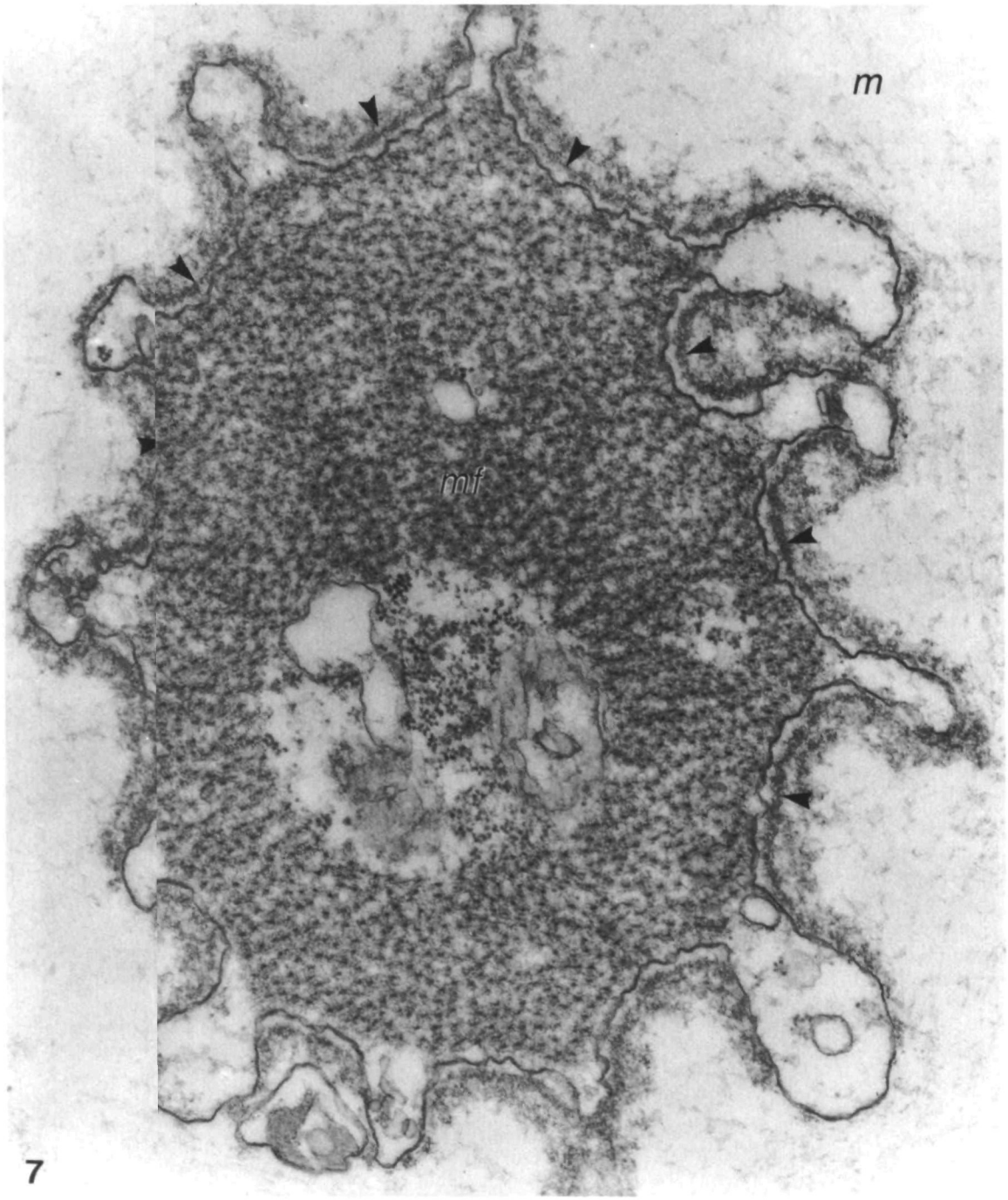

Fig. 7. Transverse section through a mesogleal muscle of *B. gracilis*. Axonemal walls are unrolled and appear as a thin layer of dense material lining the shallow wide grooves of the sarcolemma (arrowheads). mf, myofilaments; m, mesoglea. ×38 800.

ation from possible energy sources, indicates that they do not play a direct motile role in muscle function.

One way of investigating the function of the axonemes is to remove them and determine the effects, if any, on muscle function. This has been done unintentionally by previous investigators, who used enzymatic digestion to isolate viable muscle cells from the body wall of *Beroë* and *Mnemiopsis* (Hernandez-Nicaise *et al.* 1982, 1984; Anderson, 1984). Their aim was to obtain smooth muscle preparations suitable for microelectrode studies. Examination of their published light micrographs, and transmission and scanning electron micrographs reveals that enzymatically isolated mucle fibers have an essentially smooth surface (relaxed fibers) without axonemes (Hernandez-Nicaise *et al.* 1984, Figs 3,11,12; Hernandez-Nicaise *et al.* 1982, Fig. 1; Anderson, 1984, Fig. 3). Enzymatic dissociation evidently digests the axonemes, as might be expected from their extracellular location, and also eliminates the sarcolemmal furrows in which the axonemes originally resided. These enzyme-induced

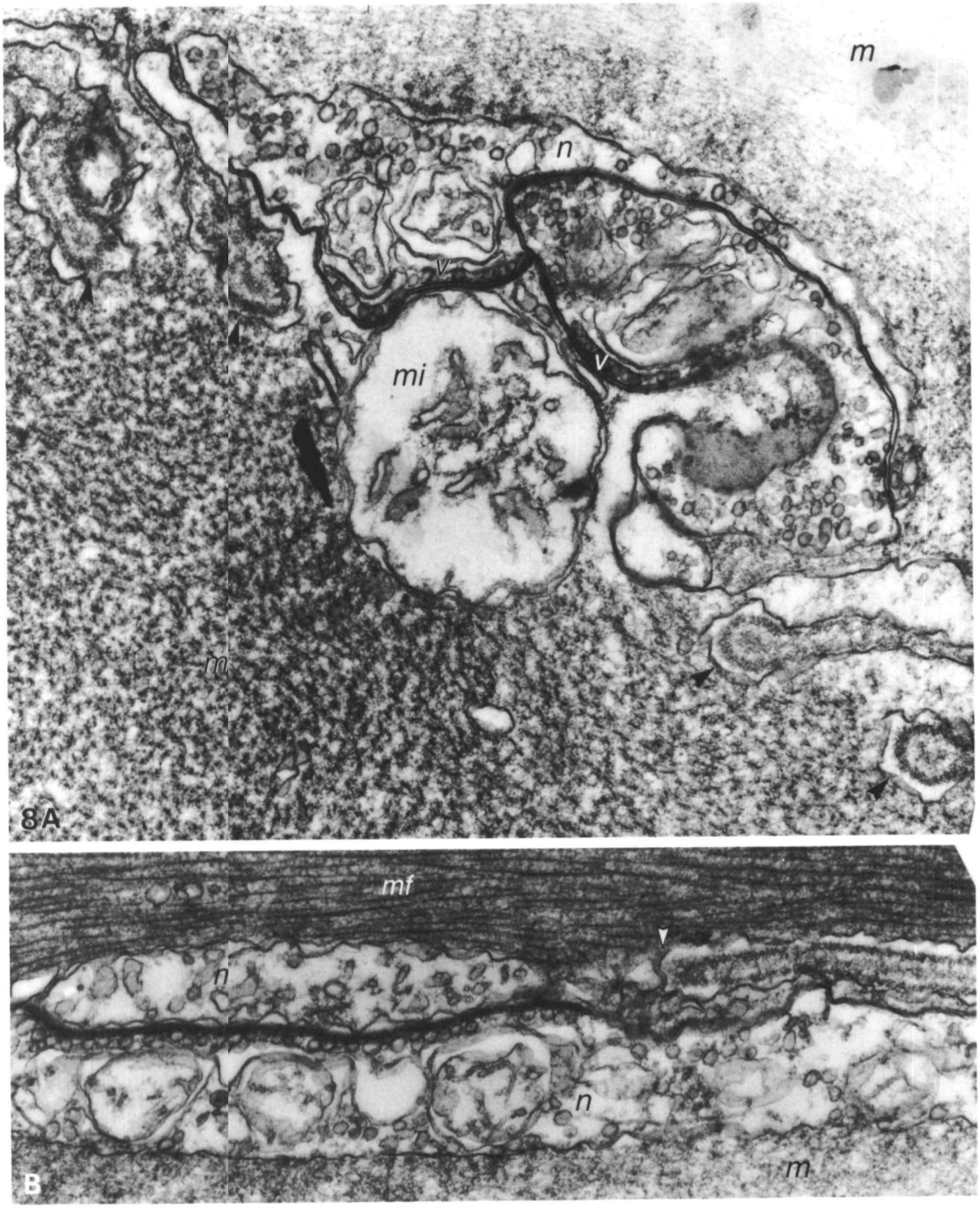

Fig. 8. Neuromuscular junctions in *B. mitrata*. A. Transverse section of muscle showing synaptic region of neurite (n). The 'presynaptic triad' consists of mitochondria, smooth endoplasmic reticulum, and a layer of synaptic vesicles (v). Large muscle mitochondria (mi) lie close to the postsynaptic side of the junction. The sarcolemmal infoldings with their axonemes (arrowheads) terminate at the broadened junctional region. ×30 500. B. Longitudinal section of synapse. Note termination of an axoneme and its sarcolemmal groove (arrowhead) to right of synaptic cleft. The neurite (n) contains mitochondria, smooth endoplasmic reticulum sacs, and a single layer of synaptic vesicles (v). Large mitochondria (mi) are present on the postsynaptic side. mf, myofilaments; m, mesoglea. ×26 000.

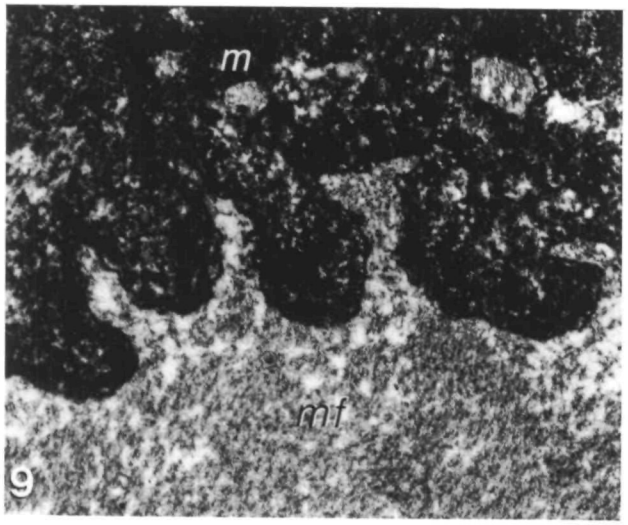

Fig. 9. Horseradish peroxidase-treated body wall of *B. cucumis* showing edge of muscle in transverse section. Electron-dense reaction product fills mesoglea (m) and sarcolemmal grooves, permeating and obscuring the axonemes. mf, myofilaments. ×21 100.

structural alterations of isolated muscle cells were not recognized in previous studies (Hernandez-Nicaise *et al.* 1982, 1984; Anderson, 1984).

Electrical properties of enzymatically isolated muscle cells have been compared with those of *in situ* cells, where technically possible. Resting potentials and action potentials of isolated muscle cells from *Beroë* and *Mnemiopsis* are similar to those recorded from cells *in situ* (Anderson, 1984; Bilbaut *et al.* 1988*a*,*b*). However, the input impedances of isolated cells of *Mnemiopsis* were substantially higher and more variable than those of *in situ* cells (Anderson, 1984). This difference may be due to the altered surface topography of isolated cells.

Isolated muscle fibers contract if stimulated electrically, chemically or mechanically, and slowly relax (Hernandez-Nicaise *et al.* 1981, 1982; Bilbaut *et al.* 1988*a*,*b*; Anderson, 1984). Contraction requires external calcium (Bilbaut *et al.* 1988*b*).

So far, then, no obvious functional changes can be attributed to the absence of axonemes on isolated cells. However, a complete functional comparison of contractile properties, tensile strength and extensibility of isolated *versus in situ* muscle cells has not been made. In addition, the effects on muscle function and/or development of eliminating axonemes from muscles *in situ*, without enzyme treatment, have not been tested.

For these reasons, the following possible roles of the axonemes are being considered for future experiments.

First, the axonemes may be remnants of inwardly grown embryonic cilia, that served to guide elongating muscle fibers along specific paths through the mesoglea during development. A similar role has been proposed for cytoplasmic microtubules in myoblasts during myotube formation in higher animals (Warren, 1974). We are currently investigating this possibility by studying muscle development in larval *Beroë*.

Alternatively, the cage of parallel axonemes around the muscle fiber may play a mechanical or skeletal role in these soft-bodied animals. Ctenophore mesoglea, unlike that of the Porifera and the Cnidaria, lacks periodically cross-banded collagen fibrils. Instead, fine filaments, presumed to be collagen, are randomly dispersed throughout the extracellular matrix (Franc *et al.* 1976). In the absence of a more reinforced matrix gel, the axonemes may serve as longitudinal tensile elements to limit muscle extension during movements of the body wall, thereby controlling stretch-activated contractions of these large smooth muscle fibers. The axonemes may also act as struts to limit shortening and/or prevent coiling of muscles during contraction, as happens in isolated fibers without axonemes (Hernandez-Nicaise *et al.* 1982; Anderson, 1984). In addition, if the axonemes resisted dimensional changes of muscle fibers of constant volume, then extension or contraction would stiffen the fiber and provide support for the mesogleal body wall (Kier and Smith, 1985). Such an increase in rigidity might help to prevent collapse of the body wall during inhalation of prey into the stomach cavity of *Beroë* (Wolcott, 1981; Tamm, 1982). This suggests that the giant smooth muscle cells with their axonemal cages may act as individual 'muscular hydrostats' (Kier and Smith, 1985) to support and control muscular movements of the body wall of ctenophores. An argument against this possibility is our ultrastructural observation that the axonemes are not totally tubular, and thus may not be suitable skeletal elements.

Finally, the surface axonemes may play an active role in smooth muscle function. In smooth muscle, as in skeletal and cardiac muscle, contraction is activated by an increase in cytosolic calcium concentration (Somlyo, 1985; Rüegg, 1986). However, the source(s) of activator calcium remain unclear: the relative contributions of transmembrane calcium influx *versus* calcium release from intracellular stores (sarcoplasmic reticulum or plasma membrane sites) for tonic and phasic responses of smooth muscles have been debated (Somlyo, 1985; Bond *et al.* 1984; Triggle *et al.* 1989; Van Breemen and Saida, 1989). In the absence of a T-tubule system, a considerable portion of the sarcoplasmic reticulum in vertebrate smooth muscle is located peripherally, where it forms special junctions with the adjacent sarcolemma (Bond, 1984; Somlyo and Franzini-Armstrong, 1985).

An extensive system of peripheral sarcoplasmic reticulum with junctions onto the sarcolemma has not been observed in *Beroë ovata* muscles (Hernandez-Nicaise *et al.* 1980; Hernandez-Nicaise and Amsellem, 1982), although Ca-binding sites on the plasma membrane have been reported (Nicaise *et al.* 1982). However, the most obvious peripheral feature of ctenophore smooth muscle membranes is the longitudinal infoldings of the sarcolemma (Fig. 3). The axonemes lie in gutters at the bottoms of the membrane furrows, which themselves are embedded in peripheral myofilaments.

Since many motile axonemes contain calmodulin or other Ca-binding proteins (Otter, 1989), we envision that the nonmotile muscle axonemes of ctenophore muscles may bind Ca and serve as an external Ca store and/or sink

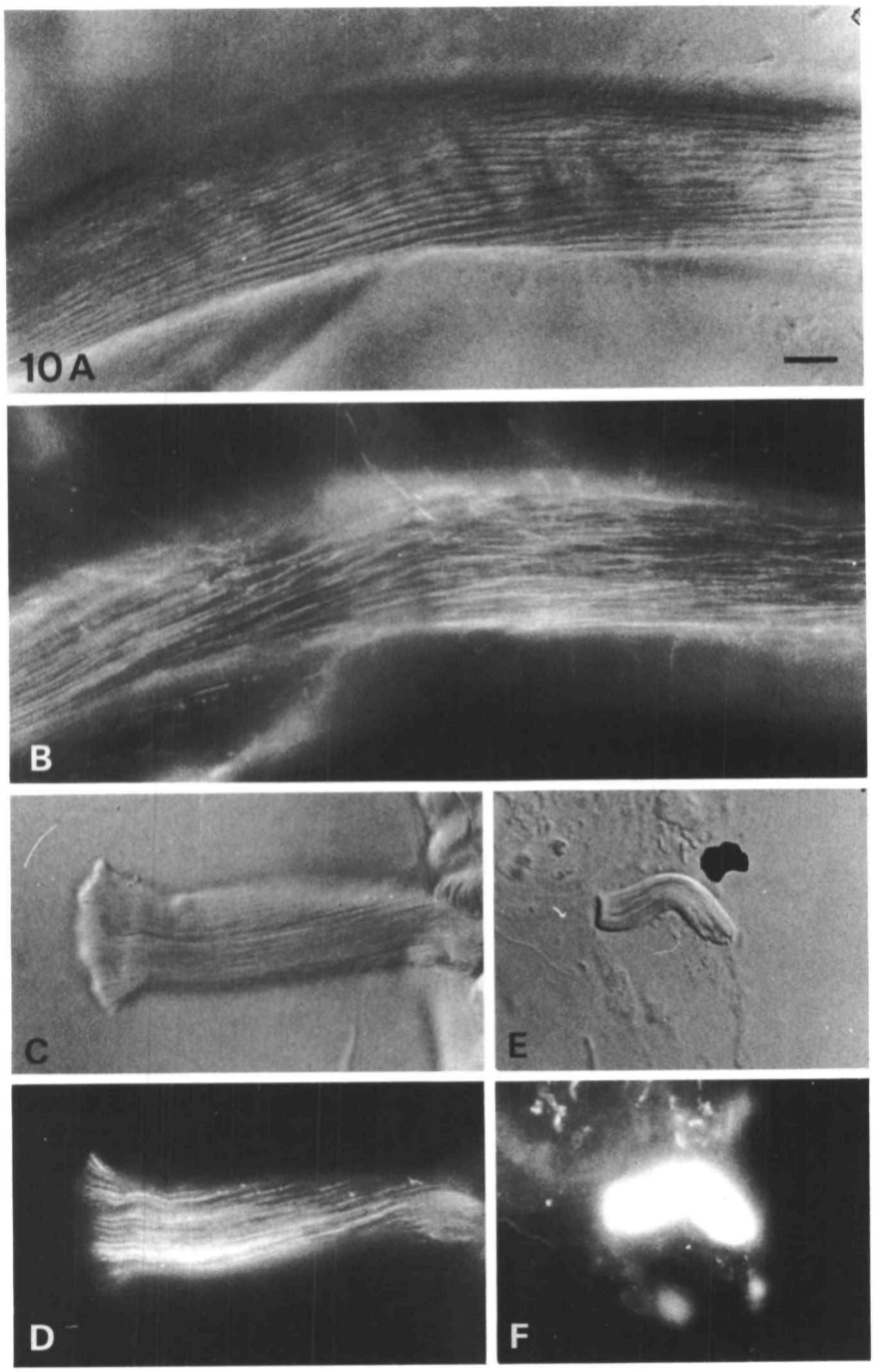

Fig. 10. Pairs of Nomarski (A,C,E) and fluorescence (B,D,F) photographs of anti-beta-tubulin-stained muscles (A–D) and macrocilium (E,F) of *B. ovata*. Longitudinal surface striations of muscles (A,C) correspond to brightly fluorescent anti-tubulin lines (B,D). Bright lines projecting from the muscle surface (B) may be detached broken axonemes. C,D. Cut slightly twisted end of a muscle cell. Fluorescent anti-tubulin lines resemble strands at the end of a cut rope. E,F. Detached macrocilium with flat basal end (to left) and pointed tip fluoresces brightly. Bar, 10 μm.

for excitation-contraction coupling. Isolated *Beroë* muscle cells require external Ca for contraction (Bilbaut *et al.* 1988*a*,*b*), perhaps because the axonemes have been lost.

In conclusion, ctenophores, possessing the largest smooth muscles and the longest cilia in nature, have put their actions together here. The muscle-associated axonemes may play a unique role in muscle function. This extraordinary collaboration may be related to the evolution of muscle cells in soft-bodied animals that exploit cilia for a wide variety of functions (Tamm, 1982).

We thank Dr Christian Sardet and the scientists of the Station Zoologique, Villefranche-sur-Mer, France, for their generous hospitality and assistance during this work. We first noticed the extracellular axonemes in *B. mitrata* kindly sent to us by Dr Claudia Mills, Friday Harbor, WA. Dorothy Hahn once again skillfully and patiently processed these words. This research was performed during sabbatical leave from Boston University, and was supported by NIH GM 27903.

References

ANDERSON, P. A. V. (1984). The electrophysiology of single smooth muscle cells isolated from the ctenophore *Mnemiopsis*. *J. comp. Physiol. B*, **154**, 257–268.

BILBAUT, A., HERNANDEZ-NICAISE, M.-L., LEECH, C. A. AND MEECH, R. W. (1988*a*). Membrane currents that govern smooth muscle contraction in a ctenophore. *Nature, Lond.* **331**, 533–535.

BILBAUT, A., MEECH, R. W. AND HERNANDEZ-NICAISE, M.-L. (1988*b*). Isolated giant smooth muscle fibres in *Beroë ovata*: Ionic dependence of action potentials reveals two distinct types of fibre. *J. exp. Biol.* **135**, 343–362.

BOND, M., KITAZAWA, T., SOMLYO, A. P. AND SOMLYO, A. V. (1984). Release and recycling of calcium by the sarcoplasmic reticulum in guinea pig portal vein smooth muscle. *J. Physiol.* **355**, 677–695.

CHUN, C. (1880). Der Ctenophoren des Golfes von Neapel, und der engrenzenden Meeres-Abschnitte. *Flora and Fauna des Golfes von Neapel*, vol. 1, pp. 1–311. Engelmann, Leipzig.

EIMER, T. (1873). *Zoologische studien auf Capri. I. Ueber* Beroë ovatus. *Ein beitrag zur anatomie der rippenguallen.* Engelmann, Leipzig.

FRANC, S., FRANC, J. M. AND GARRONE, R. (1976). Fine structure and cellular origin of collagenous matrices in primitive animals: Porifera, Cnidaria and Ctenophora. *Front. Matrix. Biol.*, vol. 3, pp. 143–156. Karger, Basel.

GABELLA, G. (1971). Caveolae intracellulares and sarcoplasmic reticulum in smooth muscle. *J. Cell Sci.* **8**, 601–609.

GRAHAM, R. C. AND KARNOVSKY, M. J. (1966). The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**, 291–302.

HERNANDEZ-NICAISE, M.-L. (1973). The nervous system of ctenophores. III. Ultrastructure of synapses. *J. Neurocytol.* **2**, 249–263.

HERNANDEZ-NICAISE, M.-L. AND AMSELLEM, J. (1980). Ultrastructure of the giant smooth muscle fiber of the ctenophore *Beroë ovata*. *J. Ultrastruct. Res.* **72**, 151–168.

HERNANDEZ-NICAISE, M. L., BILBAUT, A., MALAVAL, L. AND NICAISE, G. (1982). Isolation of functional giant smooth muscle cells from an invertebrate: structural features of relaxed and contracted fibers. *Proc. natn. Acad. Sci. U.S.A.* **79**, 1884–1888.

HERNANDEZ-NICAISE, M.-L., MACKIE, G. O. AND MEECH, R. W. (1980). Giant smooth muscle cells of *Beroë*. Ultrastructure, innervation, and electrical properties. *J. gen. Physiol.* **75**, 79–105.

HERNANDEZ-NICAISE, M.-L., NICAISE, G. AND ANDERSON, P. A. V. (1981). Isolation of giant smooth muscle cells from the ctenophore *Mnemiopsis*. *Am. Zool.* **21**, 1012.

HERNANDEZ-NICAISE, M.-L., NICAISE, G. & MALAVAL, L. (1984). Giant smooth muscle fibers of the ctenophore *Mnemiopsis leydii*: ultrastructural study of *in situ* and isolated cells. *Biol. Bull. mar. Biol. Labs Woods Hole* **167**, 210–228.

HORRIDGE, G. A. (1965). Macrocilia with numerous shafts from the lips of the ctenophore *Beroë*. *Proc. R. Soc. Lond. B*, **162**, 351–364.

KARNOVSKY, M. J. (1967). The ultrastructural basis of capillary permeability studied with peroxidase as a tracer. *J. Cell Biol.* **35**, 213–236.

KIER, W. M. AND SMITH, K. K. (1985). Tongues, tentacles and trunks: the biomechanics of movements in muscular hydrostats. *Zool. J. Linn. Soc.* **83**, 307–324.

MACKIE, G. O., MILLS, C. E. AND SINGLA, C. L. (1988). Structure and function of the prehensile tentilla of *Euplokamus* (Ctenophora, Cydippida). *Zoomorphology* **107**, 319–337.

MCDONALD, K. (1984). Osmium ferricyanide fixation improves microfilament preservation and membrane visualization in a variety of animal cell types. *J. Ultrastruct. Res.* **86**, 107–118.

NICAISE, G., HERNANDEZ-NICAISE, M.-L. AND MALAVAL, L. (1982). Electron microscopy and X-ray microanalysis of calcium-binding sites on the plasma membrane of *Beroë* giant smooth muscle fibre. *J. Cell Sci.* **55**, 353–364.

OTTER, T. (1989). Calmodulin and the control of flagellar movement. In *Cell Movement*, vol. 1: *The Dynein ATPases*, pp. 281–298 (ed. Warner, F. D., Satir, P. and Gibbons, I. R.) A. R. Liss, New York.

RÜEGG, J. C. (1986). *Calcium in Muscle Activation*. Springer-Verlag, New York.

SOMLYO, A. P. (1985). Excitation-contraction coupling and the ultrastructure of smooth muscle. *Circul. Res.* **57**, 497–507.

SOMLYO, A. V. AND FRANZINI-ARMSTRONG, C. (1984). New views of smooth muscle structure using freezing, deep-etching and rotary shadowing. *Experientia* **41**, 841–856.

SUMMERS, K. E. AND GIBBONS, I. R. (1973). Effects of trypsin digestion on flagellar structures and their relationship to motility. *J. Cell Biol.* **58**, 618–629.

TAMM, S. L. (1982). Ctenophores. In *Electrical Conduction and Behaviour in 'Simple' Invertebrates* (ed. G. A. B. Shelton), Oxford University Press, England, pp. 266–358.

TAMM, S. L. AND TAMM, S. (1984). Alternate patterns of doublet microtubule sliding in ATP-disintegrated macrocilia of the ctenophore *Beroë*. *J. Cell Biol.* **99**, 1364–1371.

TAMM, S. L. AND TAMM, S. (1985). Visualization of changes in ciliary tip configuration caused by sliding displacement of microtubules in macrocilia of the ctenophore *Beroë*. *J. Cell Sci.* **79**, 161–179.

TAMM, S. L. AND TAMM, S. (1987). Massive actin bundle couples macrocilia to muscles in the ctenophore *Beroë*. *Cell Motil. Cytoskel.* **7**, 116–128.

TAMM, S. L. AND TAMM, S. (1989). Calcium sensitivity extends the length of ATP-reactivated ciliary axonemes. *Proc. natn. Acad. Sci. U.S.A.* **86**, 6987–6991.

TRIGGLE, D. J., ZHENG, W., HAWTHORN, M., KWAN, Y. W., WEI, X.-Y., JOSLYN, A., FERRANTE, J. AND TRIGGLE, A. M. (1989). Calcium channels in smooth muscle. Properties and regulation. *Ann. N.Y. Acad. Sci.* **560**, 215–229.

VAN BREEMEN, C. & SAIDA, K. (1989). Cellular mechanisms regulating $[Ca^{2+}]_i$ smooth muscle. *A. Rev. Physiol.* **51**, 315–329.

WARREN, R. H. (1974). Microtubular organization in elongating myogenic cells. *J. Cell Biol.* **63**, 550–566.

WILL, J. G. F. (1844). *Horae Tergestinae. Beschreibung und Anatomie der im Herbste 1843 bei Trieste beobachteten Akalephen.* Voss, Leipzig.

WOLCOTT, T. G. (1981). Inhaling without ribs: the problem of suction in soft-bodied invertebrates. *Biol. Bull. mar. Biol. Labs Woods Hole* **160**, 189–197.

(Received 7 August 1989 – Accepted 18 August 1989)