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Microtubule nucleation and organization in teleost photoreceptors: microtubule recovery after elimination by cold

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Summary

Retinal photoreceptors have two separate populations of microtubules: axonemal microtubules of the modified cilium of the outer segment and cytoplasmic microtubules of the cell body. The axonemal microtubules originate from a basal body located at the distal tip of the photoreceptor inner segment and extend in a 9 + 0 configuration into the outer segment of rods and accessory outer segment of cones. The cytoplasmic microtubules of the cell body are axially aligned from the distal tip of the inner segment to proximal synapse, and are oriented with uniform polarity, their minus ends distal toward the outer segment and plus ends proximal toward the synapse (Troutt & Burnside, 1988). To investigate how this regular cytoplasmic microtubule array is generated, we have attempted to identify microtubule nucleation sites in the cones of the tropical teleost fish, Tilapia (*Sarotherodon mossambicus*) by examining the regrowth of cytoplasmic microtubules after cold disruption in whole retinas or in isolated cone fragments consisting of inner and outer segments (CIS-COS). Incremental stages of microtubule reassembly were examined both by electron microscopy of thin sections and by immunofluorescent localization of microtubules with an antitubulin antibody. Cold treatment completely abolished all cytoplasmic microtubules but did not disrupt axonemal microtubules. Within 2 min after rewarming, cytoplasmic microtubules reappeared in the most distal portion of the inner segment in a small aster-like array associated with the basal body, and subsequently appeared in more proximal parts of the cone. These observations suggest that a favoured microtubule nucleation site is associated with the basal body region of the cone outer segment, and thus that the basal body region could function as a microtubule organizing centre for the photoreceptor. These results are consistent with the findings of our previous investigation of cone microtubule polarity, which showed that the minus ends of the cytoplasmic microtubules of the cone are associated with the basal body region.

Introduction

The contributions of microtubules to cell motility and establishment of cell shape are critically dependent on their distribution throughout the cytoplasm. Thus, much effort is being expended in attempting to understand just how microtubule patterns are generated (Osborn & Weber, 1976; Brinkley *et al.*, 1981; Karsenti *et al.*, 1984; Kirschner & Mitchison, 1986; McNiven & Porter, 1988). Popular candidates for such organizing functions are microtubule-organizing centres (MTOCs). MTOCs have been identified in cells by their ability to nucleate microtubule assembly and maintain microtubules in organized arrays (see Brinkley, 1985). The two most commonly described MTOCs are: (1) perinuclear centrosomes (centrioles surrounded by dense amorphous material), and (2) basal bodies of cilia and flagella. In most cells so far examined, microtobules are oriented such that their fast-growing, 'plus' ends are directed away from perinuclear MTOCs (Heidemann & McIntosh, 1980; Euteneuer & McIntosh, 1981; McIntosh & Euteneuer, 1984; McNiven *et al.*, 1984; Soltys & Borisy, 1985). This microtubule polarity orientation appears to be conserved even when microtubules are not directly associated with an MTOC. For example, although the axonal microtubules of neurons do not appear to be contiguous with the centriolar region of the soma, their minus ends are oriented toward the cell body and their plus ends toward the synaptic ending (Heidemann & McIntosh, 1980; Burton & Paige, 1981; Filliatreau & DiGiamberardino, 1981; Heidemann *et al.*, 1981; Lyser, 1968).

In this study we have investigated the control of

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microtubule orientation in cones of teleost fish retinas. Microtubules have been shown to play a crucial role in elongation of the cone inner segment (Burnside, 1988). Teleost cones elongate and contract in a diurnal cycle which serves to reposition the photosensory outer segments for optimal function in bright or dim light (Burnside, 1988). All cytoplasmic microtubules in teleost photoreceptors, including those of the inner segment, are oriented with minus ends toward the outer segment and plus ends toward the synaptic ending (Troutt & Burnside, 1988). A single basal body gives rise during development to the connecting cilium of the outer segments, an axonemal derivative with a 9 + 0 core of doublet microtubules. In teleost cones the connecting cilium extends into an accessory outer segment rather than into the true outer segment as observed in rods (Yacob *et al.*, 1977; Kunz *et al.*, 1983; Nagle *et al.*, 1986; Troutt & Burnside, 1988).

The only structure in teleost photoreceptors that resembles a classical MTOC is the basal body of the connecting cilium. No perinuclear centrioles have been detected (Kinney & Fisher, 1978; Kunz *et al.*, 1983).

Since the minus ends of cytoplasmic microtubules are oriented toward and closely associated with the base of the connecting cilium, it is possible that the basal body (and/or associated material) might serve as an organizing centre for assembly of cytoplasmic microtubules in the cone cell body as well as for axonemal microtubules. To test this hypothesis, we monitored the reassembly of microtubules during recovery from a cold treatment that entirely eliminated cone cytoplasmic microtubules in the tropical freshwater cichlid Tilapia (*Sarotherodon mossambicus*). We report that reassembly of cone cytoplasmic microtubules appears first as an aster at the base of the connecting cilium; only later are microtubules found throughout the inner segment of the cell.

Materials and methods

Animals

Since cytoplasmic microtubules were cold stable in the temperate green sunfish used in our previous studies, we used the tropical freshwater fish, Tilapia (*Sarotherodon mossambicus*) for this study because its cone microtubules were found to be cold labile. Fish were maintained in a 28° C temperature-controlled outdoor pond on the campus of the University of California at Berkeley. Cone morphology and movements are very similar in Tilapia and sunfish.

Preparation of retinas

Fish were dark-adapted for 45–60 min before the experiment by placing them in a light-tight, aerated box. Under room illumination, fish were killed by spinal section; their eyes were removed and hemisected. Retinas were isolated by separating them from the choroid with a stream of calcium-free modified Earle's Ringer (containing 5 mM ethylene glycol bis-(β-aminoethyl ether) N,N,N',N'-tetracetic acid (EGTA), 1 mM $MgSO_4$, 24 mM sodium bicarbonate, 25 mM glucose, 3 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1 mM ascorbate, pH 7.4) and severing the optic nerve. Retinas were bisected along the choroid fissure. One half-retina was fixed immediately for electron microscopy as a T_0 (untreated control). Since cones contract during the light dissection, cone myoids were approximately 5 μm in length upon fixation.

Preparation of isolated cone inner segments plus outer segments

Fish were dark-adapted by placing them in an aerated, light-tight box for 2.5–3 h. Because cones elongate so extensively during this lengthy dark-adaption, the long delicate cone myoids break off during retinal detachment, and the distal part of the cone, the portion containing the inner and outer segment (CIS-COS), remains attached to the underlying retinal pigment epithelium. Fragments of retinal pigment epithelium with attached CIS-COS were washed from the eyecup by flushing with calcium-free modified Earle's Ringer. Retinal pigment epithelia from eight fish were pooled in pregassed (95% O_2 and 5% $C0_2$) modified Earle's Ringer, then transferred to a 12 ml conical tube containing 10 ml of Papain Digestion Medium (calcium-free modified Earle's Ringer, plus 8.6 units papain and 0.17 mg L-cysteine HCl monohydrate). After 2 min of digestion, the medium was removed and the retinal pigment epithelium was gently washed 3 times with 3 ml of washing solution (modified Earle's Ringer plus 0.1 mg ml^{-1} DNase, 0.006 mg ml^{-1} leupeptin, 1 mg ml^{-1} bovine serum albumin). After washing, 2 ml of calcium-free modified Earle's Ringer was added to the digested retinal pigment epithelium, and CIS-COS were dissociated from the retinal pigment epithelium by pipetting up and down through a 1 ml pipette five times. The CIS-COS, now suspended in calcium-free modified Earle's Ringer, were transferred to a clean tube. The dissociation procedure was repeated twice more with 1.5 ml of calcium-free Earle's Ringer added to the remaining retinal pigment epithelium, to collect a total of 5 ml of CIS-COS suspension. One aliquot was fixed immediately as a control (T_0, untreated condition).

Incubation at subzero temperature

Retinas in calcium-free modified Earle's Ringer containing 5 mM taurine and CIS-COS suspended in calcium-free modified Earle's Ringer were incubated at −3 to −4° C for 25–30 min, using a STIR KOOL cold plate (Model SK 12, Thermoelectrics Unlimited, Inc.). One half-retina and one aliquot of CIS-COS were fixed immediately after cold incubation to determine whether the microtubule array had been completely abolished. Cooling to temperatures above 0° C failed to disrupt all microtubules. Incubation at subzero temperatures for 30 min did not kill the cones; in an earlier study, we found that green sunfish cones exhibited normal retinomotor movements when cultured after rewarming (Troutt & Burnside, 1989).

Recovery of the microtubule array in cones

Cold-treated half-retinas and CIS-COS suspensions were further incubated at 28° C (the ambient temperature of pond water for Tilapia) and then fixed at intervals of 2, 4 and 7 min. Since microtubules had reassembled throughout the length of the inner segment by 7 min, longer times were not studied.

Immunofluorescence

CIS-COS suspensions were fixed by adding an equal volume of fix-lysis solution (final concentration: 1% glutaraldehyde, 3% paraformaldehyde and 0.1% Triton X-100 in PHEM buffer, which is composed of 50 mM piperazine-N,N'-bis [2-ethane-sulfonic acid]) Dipotassium salt (PIPES), 25 mM HEPES, 8 mM EGTA, and 2 mM $MgCl_2$, at pH 7.0. Glutaraldehyde concentrations between 0.01% and 1% were initially tested; 1% consistently gave the best microtubule preservation. Cells in suspension were allowed to settle for 5 min onto polylysine-coated cover slips, and then were rinsed with phosphate buffered saline (PBS) before treatment with 0.5 mg ml^{-1} sodium borohydride in PBS for 10 min to inactivate free aldehyde groups. The cover slips were rinsed 3 times with PBS and then incubated with a mouse hybridoma culture supernatant containing a monoclonal antibody against calf brain tubulin (kindly provided by Dr Ursula Euteneuer, University of California, Berkeley). The antibody recognizes the beta subunit of tubulin (Euteneuer, unpublished results).) Incubations were carried out for 50 min in a moist dark chamber at 33–37° C. Cover slips were rinsed 3 times with PBS, and then incubated with FITC-conjugated goat anti-mouse IgG (Cappel; the 1 mg ml^{-1} stock was diluted 1:30 in PBS) as in the primary antibody step. After 3 more washes with PBS, cover slips were mounted onto glass slides with a 9:1 mixture of glycerol and PBS containing 1 mg ml^{-1} p-phenylenediamine.

Immunoblots

Immunoblotting was performed to test the specificity of the antitubulin antibody. Retinas were isolated from Tilapia that had been dark-adapted for 60 min, and were homogenized in ice-cold buffer containing 50 mM Tris- HCl, pH 7.4, 1 mM EGTA, 5 mM $MgCl_2$ and protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine and 10 μg ml^{-1} of leupeptin, aprotinin and N-α-p tosyl-l-arginine methyl ester), at 0.5 ml per retina. Suspensions of photoreceptor fragments (inner segments and outer segments, is-os) were prepared from fish that were dark-adapted for 2 h by shaking isolated retinas in 1 ml of modified Earle's Ringer. Suspensions and homogenized retinas were filtered through Nitex (Tektco Inc., Elmsford, NY), diluted with an equal volume of 3% SDS (sodium dodecyl sulfate) sample buffer (Laemmli, 1970) and frozen without prior boiling. Protein concentration was determined by dye binding assay (BioRad, Richmond, CA) and photoreceptor counts were made using a haemocytometer and a × 16 objective. A tubulin standard purified from calf brain was provided by Mr Chris Staiger, University of California at Berkeley.

Samples were subjected to SDS gel electrophoresis (Laemmli, 1970) on 5–15% gradient polyacrylamide minigels of 0.5 mm thickness and then blotted onto nitrocellulose in buffer containing 20% methanol at 48 volts (270–310 mAmps) for 3 h according to Towbin and co-workers (1979). After visualizing total transferred proteins with Ponceau S stain (Sigma, St Louis, MO) and blocking the nitrocellulose with 5% 'Blotto' (Johnson *et al.*, 1984), filters were incubated overnight with mouse hybridoma culture supernatant containing the monoclonal antibody against tubulin. Antibody binding was visualized using horseradish peroxidase (HRP) conjugated goat anti-mouse antiserum (BioRad) and 4-chloro-1-napthol (Sigma) as colour developing reagent.

Electron microscopy

Retinas were prepared for EM using a modification of the procedure developed by McDonald (1984) for improving microtubule preservation over standard methods. Retinas were fixed with 2% glutaraldehyde in 50 mM cacodylate plus 5 mM $CaCl_2$, pH 7.4, for 30–60 min. Samples of 1–2 mm^2 were cut from the central region of each half-retina. Samples were washed in fix buffer, and then postfixed in 0.5% $0s0_4$ plus 0.8% potassium ferricyanide in the same buffer for 20 min. They were again washed in buffer, then in deionized distilled water, and stained with 1% aqueous uranyl acetate for 1.5 h in the dark. After ethanol dehydration, samples were embedded in Poly/Bed 812-Araldite resin. Thin-sectioning was performed on a Sorvall MT-2 ultramicrotome to observe cones in either cross or longitudinal section. Sections were stained with 1% uranyl acetate and with lead citrate, and examined on a JEOL IOO-S electron microscope.

Results

Cones from Tilapia retinas possess an arrangement of microtubules very similar to that previously described for green sunfish (Troutt & Burnside, 1988). A single basal body situated in the distal part of the inner segment at the base of the connecting cilium gives rise to nine outer doublet microtubules that diverge from one another as they project into the accessory outer segment (Fig. 1). The basal body of the cone connecting cilium is unusual in that it is conical in shape rather than cylindrical, the triplets (Fig. 2b) diverging toward the outer segment (Troutt & Burnside, 1988). Associated with the basal body are electron dense amorphous bodies ('fuzz') similar to centriolar bodies (Fig. 2). Throughout the cone, cytoplasmic microtubules are arranged parallel to the long axis of the cell. Within the ellipsoid region, microtubules are found in a sleeve of cytoplasm beneath the plasma membrane, being excluded from the dense mass of mitochondria in the core of the ellipsoid (Fig. 2).

Cone microtubule distribution was examined in normal intact retinas, after cold treatment to disrupt microtubules, and after rewarming to allow microtubule recovery. Microtubule distribution was examined by counting microtubules in transverse sections taken at several different levels along the proximal-distal axis of the cones (Table 1, Fig. 2). The microtubule counts in different parts of the cone are

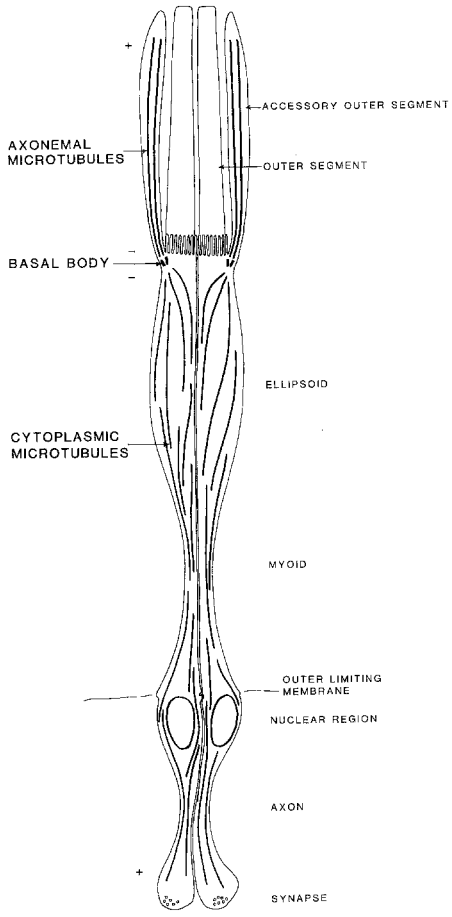

Fig. 1. Microtubule distribution in retinal cones of the teleost Tilapia. The accessory outer segment contains axonemal microtubules oriented with minus ends toward the basal body. Cytoplasmic microtubules of the cone cell body are oriented parallel to the long axis of the cell with minus ends toward the outer segment and plus ends toward the synapse.

population averages; no single cone was followed throughout its length. In untreated cones, the most distal part of the ellipsoid contained about 50 microtubules, while all other ellipsoid levels contained more than 100 microtubules (Table 1).

After incubation at subzero temperature, cones lose all microtubules within the ellipsoid, myoid and perinuclear region (Table 1 and Fig. 2b and e). Only the axonemal microtubules of the accessory outer segment remained (Fig. 2b). Axonemal microtubules of cilia and flagella are also stable in the cold (Lefebvre & Rosenbaum, 1986).

Microtubule recovery proceeded from more distal to more proximal parts of the cone. After 2 min of incubation at 28° C, microtubules could be seen radiating from the region of the basal body into the ellipsoid cytoplasm (Fig. 2c). Most microtubules were found only within the distal one-third of the ellipsoid (Table 1 and Fig. 2c and f). The proximal two-thirds of the ellipsoid exhibited far fewer microtubules, with none in the myoid and nuclear region. After 4 min of recovery, a small number of microtubules could be found in the proximal half of the ellipsoid and in the myoid. Still no microtubules were found in the nuclear region. After 7 min of incubation at 28° C cones possessed a more extensive array of microtubules. The total number of microtubules at all levels of the cell had greatly increased (Table 1 and Fig. 2d and g). Microtubules were found from the basal body through the entire length of the ellipsoid, myoid and perinuclear regions. In one longitudinal section of a cone, a single microtubule was seen to extend from the basal body region to a point two-thirds of the way down the ellipsoid. However, at no level had the microtubule number reached the density of the untreated cone. Thus, microtubules reappeared at all levels of the ellipsoid and myoid even though microtubule numbers had not increased to control levels.

The microtubule counts from electron microscopical studies of whole retinas were correlated with observations obtained by immunofluorescent labelling of cone fragments (CIS-COS) using a monoclonal antibody directed against the beta subunit of tubulin. Immunoblots demonstrated that the antibody recognized a single protein band (Fig. 3B) within homogenates of Tilapia retinas and inner/outer segment fragment suspensions composed of 2% cones and 98% rods (Fig. 3A). A single band was also labelled by this antibody in homogenates of (~95%) CIS-COS that were isolated from green sunfish retinal pigment epithelium (data not shown). The labelled band comigrated with purified calf brain tubulin (Fig. 3B).

Results of immunolabelling CIS-COS with antitubulin antibody were in accord with electron microscopical data. In untreated (T_o) cones, immunofluorescent labelling was extremely heavy throughout the entire ellipsoid and within the accessory outer segment (Figs 4a and b and 5a and b). The fibrous nature of the microtubule array in the ellipsoid and the individual doublets of the accessory outer segment could be discerned in most cones. In the immunofluorescence images, there was a small unstained region near the presumed site of the basal body (Figs 4a and j and 5a and b). Cytoplasmic microtubules seemed to emanate from and/or converge upon this unstained region in the vicinity of the base of the outer segment.

After cold treatment, the fibrous labelling in the cytoplasm disappeared, but the axonemal microtubules of the accessory outer segment were still distinctly labelled (Fig. 4c and d). Upon rewarming, microtubules initially reappeared in the region near the basal body (Figs 4 and 5). After 2 min of incubation at 28° C, a tiny aster of fluorescent fibres could be seen at the junction between accessory outer segment and the cone ellipsoid (i.e. in the vicinity of the basal body); this aster contained the only fibrous fluorescent label within the cone inner segment (Figs 4e and f and 5c

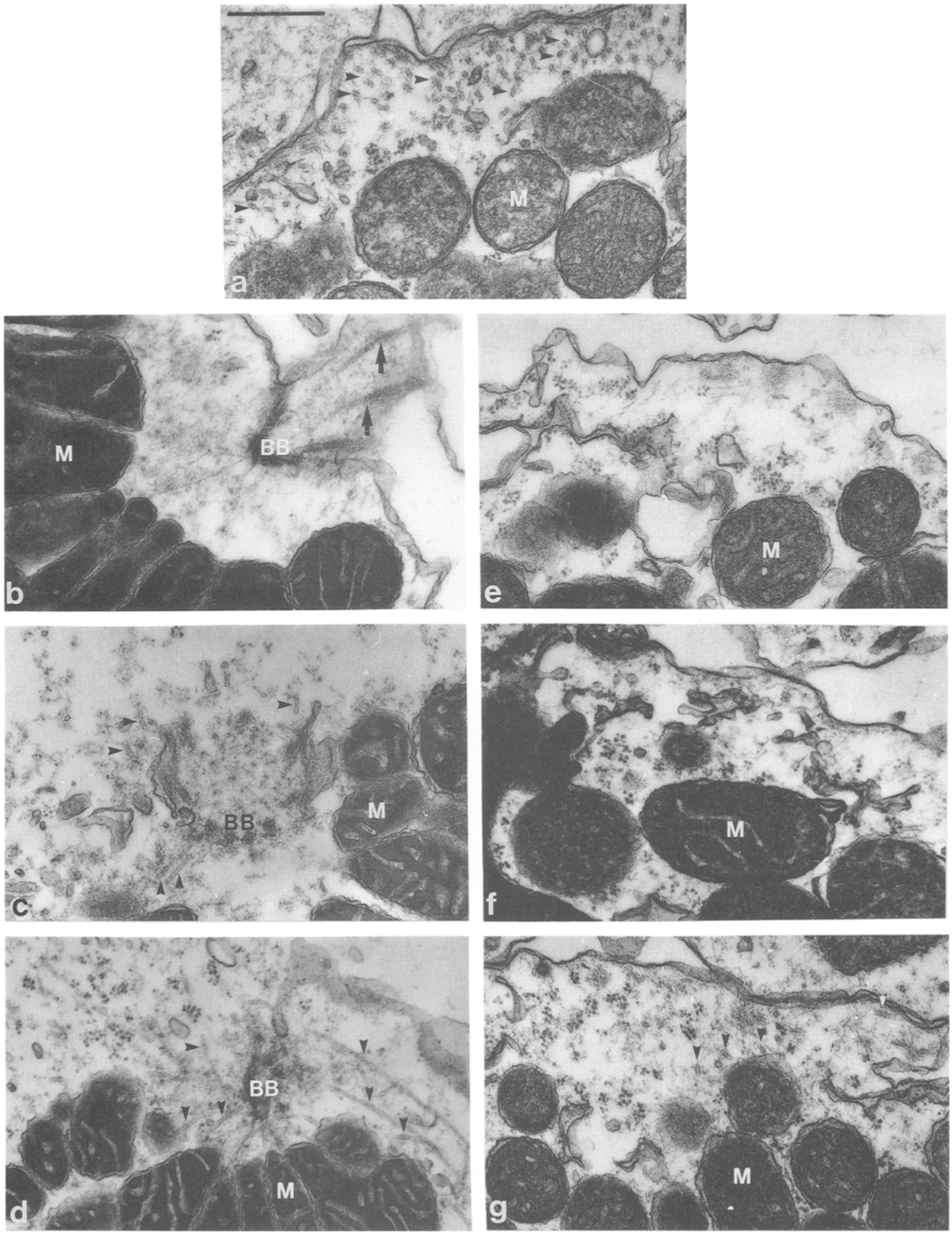

Fig. 2. Cold disruption of cone microtubules and their recovery upon rewarming, as visualized by transmission electron microscopy. In the control cone (a) before cold treatment, the proximal ellipsoid contains many microtubules (arrowheads). Directly after cold treatment, no cytoplasmic microtubules remain either in the basal body region (b) or in the proximal ellipsoid (e); only the axonemal microtubules (arrows) are intact. After 2 min of warm recovery (c), microtubules (arrowheads) have appeared near the basal body (BB), but very few are present in the proximal ellipsoid (f). After 7 min of warm recovery, microtubules (arrowheads) have increased in number in both the basal body region (d) and proximal ellipsoid (g). M, mitochondrion. Scale bar: 0.5 μm.

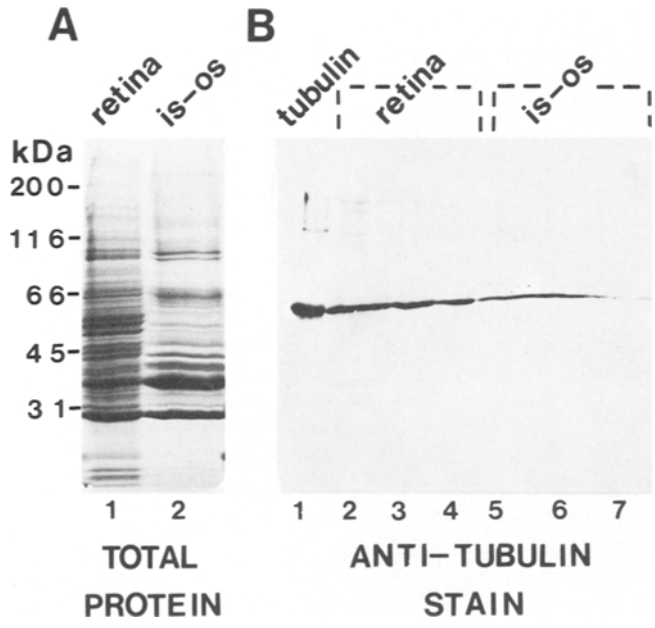

Fig. 3. Cross-reactivity of fish retinal proteins with anti-β-tubulin monoclonal antibody. (A) Total protein from homogenates of retina (lane 1) and isolated photoreceptor inner and outer segments (is-os, lane 2) from Tilapia. Samples were subjected to SDS-gel electrophoresis in a 5–15% gradient minigel and visualized with Coomassie Blue G-250. Each lane contains 30 μg of protein. (B) Anti-tubulin antibody labelling of a tubulin standard purified from calf brain (lane 1) and total protein from retina (lanes 2–4) and is-os homogenates (lanes 4–7). Total protein applied was 30 μg in lanes 2 and 5, 10 μg in lanes 3 and 6, and 3 μg in lanes 4 and 7. Samples were subjected to electrophoresis as in (A), then transferred onto nitrocellulose, and subsequently incubated with culture supernatant that contained monoclonal antibodies against β-tubulin, followed by labelling with an HRP conjugate of an anti-mouse antibody. A single band in the homogenates was labelled by the anti-tubulin antibody.

and d). After 4 min of incubation at 28° C, the aster was larger and fluorescent fibres could be detected at all levels of the ellipsoid (Fig. 4g and h). Since the brightness of some of these fibres is similar to that of the individual axonemal doublet and singlet microtubules, we conclude that they represent individual microtubules or small bundles of microtubules. A few such fluorescent fibres could be traced from the aster to the opposite (proximal) end of the ellipsoid within 4 min, a distance of approximately 25 μm. After 7 min of incubation at 28° C, the amount of fluorescence was increased at all ellipsoid levels (Fig. 4m–q). However, microtubule staining did not reach the intensity of the untreated cones, in agreement with electron microscope observations (Table 1).

Discussion

The results suggest that the basal body region of the connecting cilium in teleost cones can serve as a site for initiation of microtubule formation in the inner segment. The basal body and axoneme of the accessory outer segment were still present after cold treatment that abolished the entire cytoplasmic array of microtubules. Studies using both immunofluorescence and electron microscopy indicated that as cone microtubules began to reassemble upon rewarming, they first appeared in the distal ellipsoid near the basal body. After only 4 min, microtubules or microtubule bundles extended the full length of the ellipsoid. Not until 7 min of rewarming could microtubules be detected in all areas of the inner segment, including the perinuclear region. These observations together with our previous finding that microtubule minus ends are directed toward the basal body (Troutt & Burnside, 1988) suggest that the basal body region acts as an MTOC for the cytoplasmic array of microtubules of the cone.

During the recovery period studied, microtubules appeared in the most distal region of the ellipsoid near the basal body, in the proximal ellipsoid and in the myoid before the control number of microtubules in the distal and mid ellipsoid was reached (Table 1). As shown in Table 1, microtubule numbers were uniform over the entire length of the cone by 7 min after rewarming, but were still far below the numbers in untreated cones. After 4 min of recovery, about 15% of the control number of cytoplasmic microtubules grew throughout the length of the ellipsoid with the growth front (where a substantial drop in microtubule number occurs) in the myoid; after 7 min of recovery an additional 15% (for a total of 30% of the control microtubule number) appeared at all levels of the inner segment and perinuclear region with the growth front beyond the nuclear region. Thus microtubule reassembly is not achieved by immediate and simultaneous initiation of the complete control number of microtubules before elongation to more proximal regions occurs.

All inner segment and perinuclear microtubules in the cone may be nucleated by the basal body and elongate toward the nucleus. Microtubule counts in successive cell regions within each of the recovery periods (Table 1) are similar enough to be consistent with the hypothesis that the microtubules in the proximal regions of the cone are continuous with microtubules in the distal regions, at least during the early stages of microtubule recovery. Furthermore, a few immunofluorescent fibres can be traced from the basal body region to the base of the ellipsoid. If microtubules are indeed continuous over the length of the ellipsoid, a high microtubule growth rate occurs in the cone. The ellipsoid is approximately 25 μm in

Table 1. Microtubule counts in teleost cones at successive stages of recovery from cold treatment. Retinas with cones having myoids approximately 5 μm in length were sectioned perpendicular to the cone axis. Microtubules were counted in populations of cone cross-sections at each level. Taking the most distal 5 μm of the ellipsoid as the first level of the cone cell, each successive level is approximately 5 μm in thickness, with the nuclear region as the most proximal level. Control counts were obtained from cones fixed directly upon dissection of the retina, and are representative of the microtubule numbers in untreated cones. No cytoplasmic microtubules were observed in cones after a 30-min incubation at −3° C. Cytoplasmic microtubules had reassembled after the retinas had been rewarmed to 28° C for 2, 4 and 7 minutes. Data are recorded as mean ± SEM, n ranging from 2 to 6 cones examined in each category.

	≫ *Number of microtubules* ≪						
	Most distal ellipsoid	*Upper ellipsoid*	*Mid ellipsoid*	*Lower ellipsoid*	*Most proximal ellipsoid*	*Myoid*	*Nuclear ellipsoid*
Control	49 ± 13	135 ± 13	126 ± 40	142 ± 25	139 ± 3	92 ± 16	79
−3° C for 30 min	0	0	0	0	0	0	0
−3° C for 30 min; +28° C for 2 min	14 ± 2	4 ± 3	1 ± 1	1 ± 2	2 ± 1	0	0
−3° C for 30 min; +28° C for 4 min	13 ± 6	8 ± 4	16 ± 3	8 ± 4	14 ± 4	13 ± 5	1 ± 1
−3° C for 30 min; +28° C for 7 min	37 ± 3	27 ± 8	33 ± 6	33 ± 10	48 ± 4	45 ± 9	52 ± 9

length, and some microtubules were present in the myoid after only 4 min of recovery time. The total distance from the basal body through the myoid is some 30 μm. If the myoid microtubules nucleated near the basal body, they would have had to elongate at a rate of 7.5 μm per min to reach the myoid by this time. High microtubule growth rates such as this have been observed in living fibroblasts, although average growth rates are generally lower with high statistical variability (Schulze & Kirschner, 1988).

Alternatively, some microtubules in the more proximal regions of the cell may not grow out directly from the basal body, but instead may be spontaneously nucleated at sites distant from the basal body. This possibility cannot be ruled out by our observations. In fact, in the early stages of recovery, although a few fluorescent fibres can be traced from proximal ellipsoid to the basal body region, most fluorescent fibres in the proximal ellipsoid do not appear to extend to the basal body region. Thus some microtubules may be nucleated in the proximal ellipsoid. The low count in the distal ellipsoid of the control cones (Table 1) is consistent with the idea that in the untreated cone many ellipsoid microtubules do not end near the basal body in the distal ellipsoid. However, because the basal body is seated so deeply in the ellipsoid, sections through this region may miss some microtubules actually associated with the basal body. Bre and co-workers (1987) hypothesize that proteinaceous cytoplasmic nucleating factors may promote tubulin nucleation in regions of a cell where tubulin concentration is lower than the usual critical concentration, and where a typical MTOC is absent. Spontaneous nucleation of microtubules may be an inherent quality of very long, asymmetrical cells such as neurons, in which a single microtubule nucleating site may be insufficient to maintain and replace microtubules in cellular extensions that can reach metres in length (i.e., mammalian axons). With the action of spontaneous nucleation, multiple sites of assembly could be employed rather than just one, and an entire microtubule array could be assembled rapidly. A similar scheme had been hypothesized by Zenker and Hohberg (1973) who counted microtubules at different points along axons of motor neurons and suggested that microtubules can form at a distance from the soma in these cells. More recent studies with centrosome-free cytoplasts have shown that microtubules can form in the absence of a distinct MTOC, and suggest that spatial arrangements of microtubules may be varied by shifting the critical concentration for tubulin assembly, changing microtubule end-capping structures, and/or by other cytoplasmic factors (Karsenti *et al.*, 1984; McNiven & Porter, 1988).

Another manner in which an organized array of cytoplasmic microtubules in long cells might be established is by allowing microtubules that contact the original MTOC to detach and subsequently become nucleating sites for more distant microtubules that do not contact the MTOC. For example, microtubules

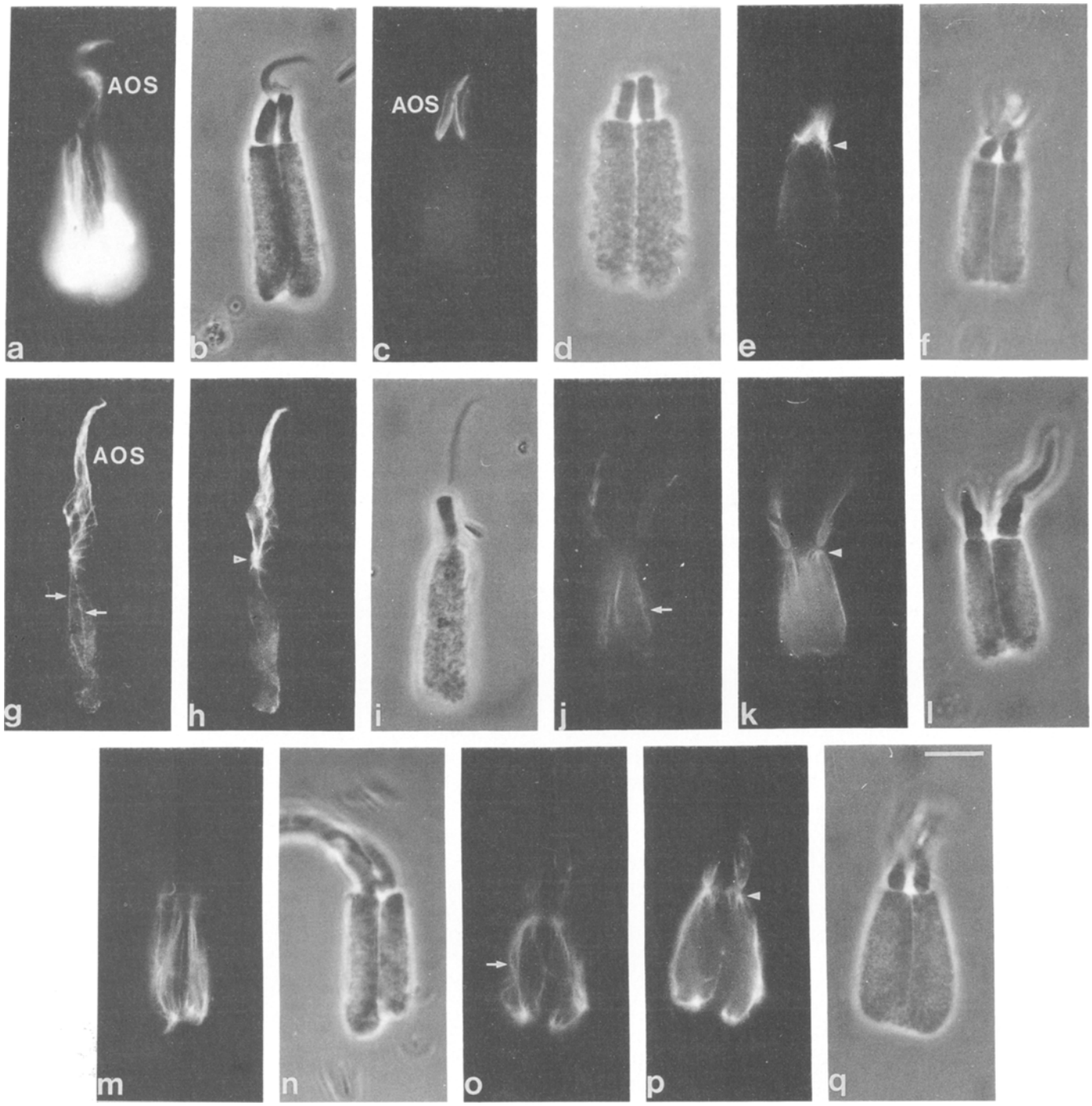

Fig. 4. Cold disruption of cone cytoplasmic microtubules and their recovery upon rewarming, as visualized by immunofluorescence. Cone inner and outer segments (CIS-COS) were labelled with antitubulin antibody after the following treatments: (a) and (b), no cold treatment (control); (c) and (d), 25 min cold treatment (−3° C); (e) and (f), cold treatment followed by 2 min rewarming at 28° C; (g)–(l), cold treatment followed by 4 min rewarming; (m)–(q), cold treatment followed by 7 min rewarming. Each fluorescent image or set of images is directly followed by its corresponding phase image. AOS, microtubules of the accessory outer segment; arrowheads indicate the cytoplasmic microtubule aster in the region of the basal body; arrows indicate individual microtubules or microtubule bundles that extend the full length of the ellipsoid. Scale bar: 10 μm.

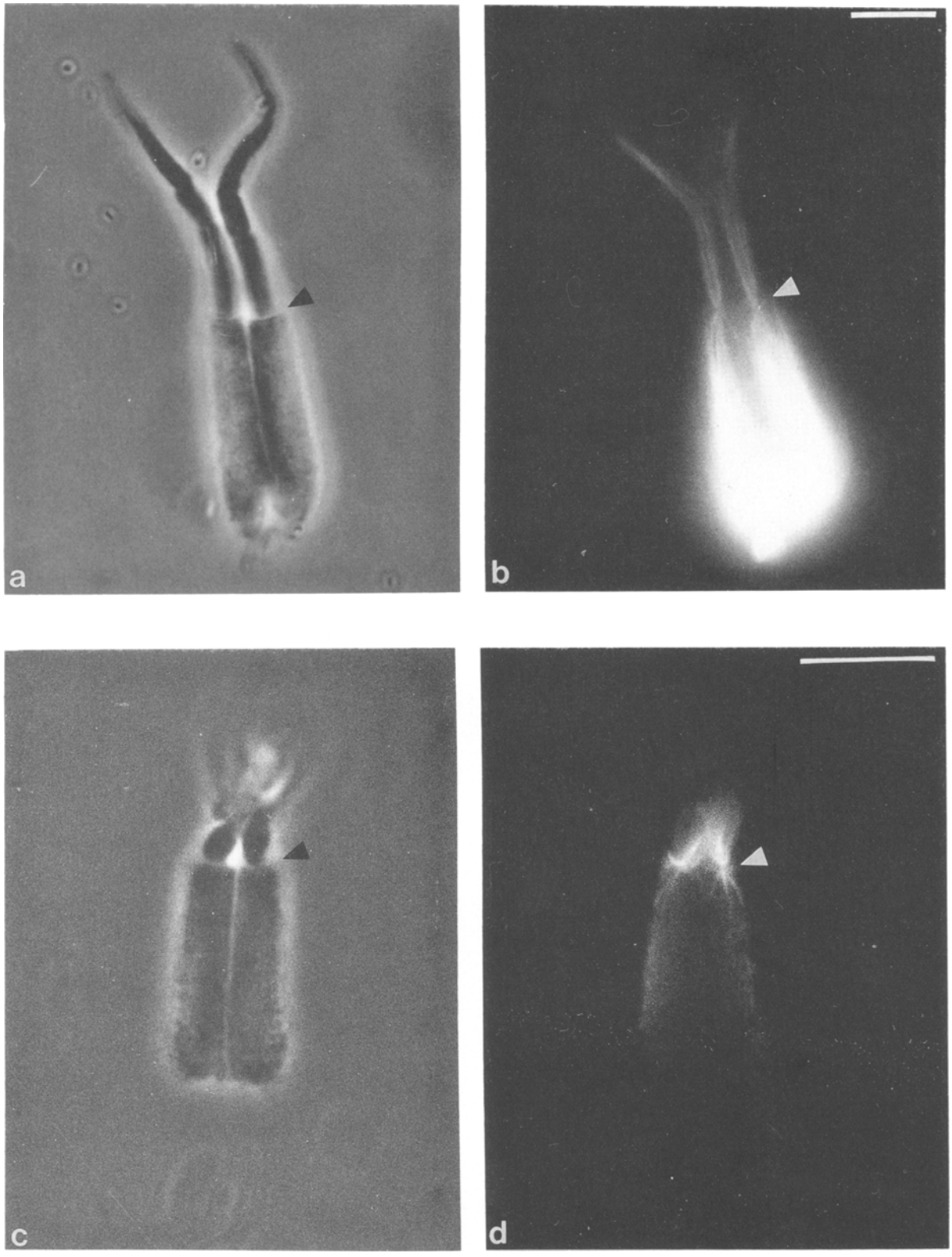

Fig. 5. Microtubule distribution illustrated by immunofluorescence, at higher magnification than Fig. 4, in (a) and (b) control CIS-COS, and in (c) and (d) CIS-COS after 25 min cold followed by 2 min rewarming, (equivalent to Fig. 4e and f). In control CIS-COS, cytoplasmic microtubules converge on an unstained region near the base of the outer segment (arrowhead). Upon recovery, an aster forms at this site (arrowhead). Scale bar: 10 μm.

might be nucleated at an MTOC, elongate and then break, with the distal fragment (still properly oriented) now capable of nucleating further assembly. Alternatively, a microtubule nucleated at an MTOC may become detached and transported some distance away from the MTOC, then be further elongated. In either case, the polarity orientation of microtubules would be automatically conserved.

In summary, the teleost cone provides an example of a cell in which a single MTOC can simultaneously organize two very different types of microtubule arrays. During morphogenesis of the photoreceptor a centriole migrates to the site of outer segment formation and gives rise to the microtubules of the connecting cilium of the outer segment (Kunz *et al.*, 1983), these being in axonemal arrangement (9 + 0) and in the form of doublets. This study suggests that the basal body or associated cytoplasmic material can also serve as an organizing centre for the more numerous singlet cytoplasmic microtubules of the photoreceptor inner segment, which radiate from the basal body and extend down into the ellipsoid and myoid.

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