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Evidence that Microtubules Do Not Mediate Opsin Vesicle Transport in Photoreceptors

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Abstract. The organization of the rod photoreceptor cytoskeleton suggests that microtubules (MTs) and F actin are important in outer segment (OS) membrane renewal. We studied the role of the cytoskeleton in this process by first quantifying OS membrane assembly in rods from explanted *Xenopus* eyecups with a video assay for disc morphogenesis and then determining if the rate of assembly was reduced after drug disassembly of either MTs or F actin. Membrane assembly was quantified by continuously labeling newly forming rod OS membranes with Lucifer Yellow VS (LY) and following the tagged membranes' distal displacement along the OS. LY band displacement displayed a linear increase over 16 h in culture. These cells possessed a longitudinally oriented network of ellipsoid MTs between the sites of OS protein synthesis and OS membrane assembly. Incubation of eyecups in nocodazole, colchicine, vinblastine, or podophyllotoxin disassembled the ellipsoid MTs. Despite their absence, photoreceptors maintained a normal rate of OS assembly. In contrast, photoreceptors displayed a reduced distal displacement of LY-labeled membranes in eyecups treated with cytochalasin D, showing that our technique can detect drug-induced changes in basal rod outer segment assembly. The reduction noted in the cytochalasin-treated cells was due to the abnormal lateral displacement of newly added OS disc membranes that occurs with this drug (Williams, D. S., K. A. Linberg, D. K. Vaughan, R. N. Fariss, and S. K. Fisher. 1988. *J. Comp. Neurol.* 272:161–176). Together, our results indicate that the vectorial transport of OS membrane constituents through the ellipsoid and their assembly into OS disc membranes are not dependent on ellipsoid MT integrity.

POLARIZED cells sort various plasma membrane components and transport them to specific and distinct parts of the cell. The vertebrate photoreceptor provides an excellent model system for studying these phenomena. This highly specialized neuron (Fig. 1) supports not only its synaptic terminal, which connects to second-order neurons in the retinal circuitry, but a highly specialized outer segment (OS)[1] at the opposite end of the cell. Photoreceptor OS renewal, in particular, presents a uniquely suitable model system for studying vectorial protein transport in polarized cells (Young, 1967). Opsin makes up >90% of the total OS membrane protein (Hall et al., 1969), it has been well characterized biochemically (Hargrave, 1982), and it is known to be transported from its site of synthesis to its site of plasma membrane insertion by vesicles (Papermaster et al., 1985).

We wondered if microtubules (MTs) might mediate opsin vesicle transport in photoreceptors, perhaps by a mechanism akin to fast axonal transport (Allen et al., 1985; Miller and Lasek, 1985; Vale, 1987). We used an organ-cultured eyecup preparation from *Xenopus* to pursue this question. *Xenopus* has been used in numerous studies of photoreceptor OS renewal (revised in Besharse, 1986). Its photoreceptors are relatively large and lend themselves well to light microscope–level examination, and OS renewal continues unabated for hours when *Xenopus* eyecups are maintained in culture medium (Besharse et al., 1980). *Xenopus* photoreceptor inner segments contain a population of microtubules in their ellipsoid region that are arranged in such a way as to make them reasonable candidates for mediating vesicular opsin transport. They are specifically and readily disassembled by antimicrotubule drugs (Sale et al., 1988). Fluorescent dyes added to the culture medium become progressively entrapped within assembling, basal rod outer segment (ROS) discs (Laties et al., 1976; Kaplan et al., 1982; Matsumoto and Besharse, 1985), providing a convenient means whereby ROS assembly may be measured (Fig. 2).

We reasoned that if microtubules were responsible for the transport of opsin vesicles to their site of insertion in the distal basal rod inner segment, then disrupting them by drug treatment (Dustin, 1984) for a period of hours would result in significantly reduced rates of ROS assembly. However, the

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1. *Abbreviations used in this paper*: CB, cacodylate buffer; IS, inner segment; LY, Lucifer Yellow; MT, microtubule; OS, outer segment; RIS, rod inner segment; ROS, rod outer segment.

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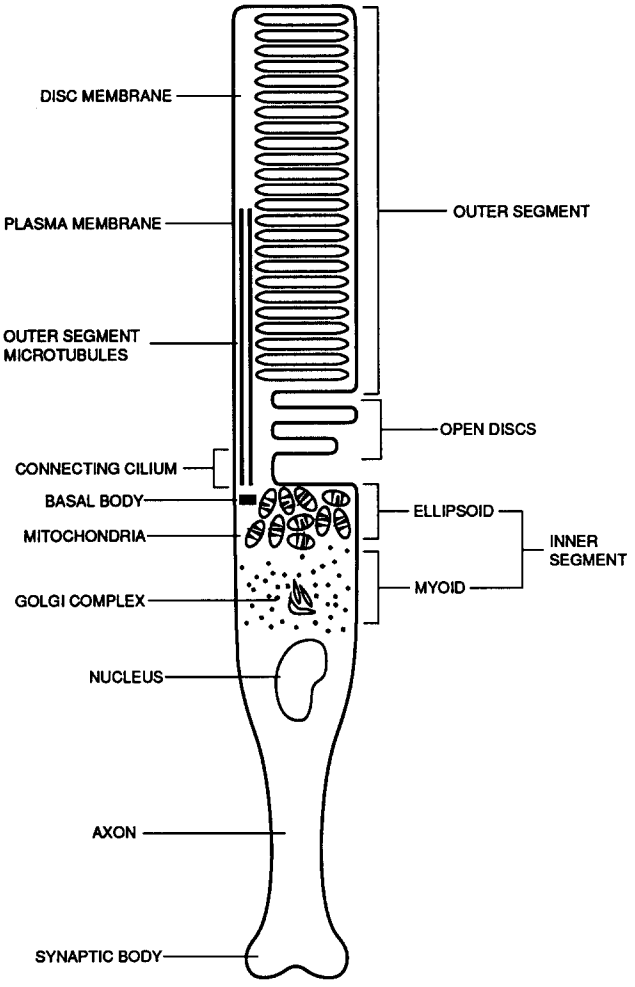

Figure 1. Diagram of the *Xenopus* rod photoreceptor (not to scale). Opsin destined for the ROS is synthesized and glycosylated in the myoid region of the proximal RIS and then transported, in vesicular form, through the ellipsoid region to the RIS cytoplasm at the base of the connecting cilium. ROS assembly is initiated when new vesicular opsin is incorporated into the distal RIS plasma membrane; it is next transferred to the ROS by an unknown mechanism. Evaginations of the ciliary plasma membrane create immature, open discs. These subsequently seal off into separate, flattened sacks (mature discs) that are added to the ROS stack from the base. In contrast, synaptic membrane components (including synaptic vesicles) synthesized in the RIS are transported from the myoid region through the cell body and axon to the synaptic terminal region, creating two distinct pathways of vectorially transported components in this specialized neuron.

data presented in this study indicate that normal rates of ROS assembly persist despite drug disassembly of ellipsoid microtubules, thereby eliminating microtubules as the substrate for vesicular opsin transport.

Materials and Methods

Experimental Animals

Postmetamorphic (7–8 cm) *Xenopus laevis* (Charles River Breeding Laboratories, Wilmington, MA) were maintained at 21–22°C and entrained on a 12-h light/12-h dark photoperiod for at least 3 wk before use. Under dim red safelights, animals were killed in the dark just before normal light onset. The eyes were enucleated, the anterior segments were removed, and the resulting eyecup preparations were placed into culture medium. The lights were then turned on at the normal time of light onset in the 12/12 cycle.

Organ Cultures

The basal medium for eyecup incubations was a modified Wolf and Quimby amphibian tissue culture medium (Gibco Laboratories, Grand Island, NY) supplemented with sodium bicarbonate (final concentration, 35 mM; Besharse et al., 1980) and gassed with 95% O_2/5% CO_2. Eyecups were incubated in a continually gassed atmosphere with gentle rotation at a temperature of 22–23°C. Ambient illumination was provided by fluorescent bulbs giving 32 ft-c illuminance. Except as noted, Lucifer Yellow VS (Sigma Chemical Co., St. Louis, MO) was included in all culture media at a concentration of 0.1%. Photoreceptors were treated with anticytoskeletal drugs by including nocodazole (Sigma Chemical Co.; 16.5 μM), cytochalasin D (Calbiochem-Behring Corp., La Jolla, CA; 25 μM), colchicine (Sigma Chemical Co.; 20 μM), vinblastine (Sigma Chemical Co.; 10 μM), or podophyllotoxin (Aldrich Chemical Co., Milwaukee, WI; 10 μM) in the culture medium. Drug stocks were prepared in DMSO, whose final concentration was normalized to 0.25%. Control medium contained only DMSO. In the cases where eyecups were cultured >6 h, fresh medium was substituted every 6 h.

Immunofluorescence Microscopy

Microtubule distribution was documented by immunofluorescence with an mAb specific to beta tubulin. Eyecups were first incubated in drug-supplemented or control (DMSO only) media for 2 or 11 h. Retinas were then carefully detached from the eyecups and immediately fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer (CB; pH 7.2) for 45 min. Retinas were rinsed in CB and then incubated overnight in a primary antibody solution consisting of PBS, 0.5% BSDA, 0.1% Triton X-100, and an mAb that recognizes beta tubulin (1:1,000; Chu and Klymkowsky, 1987). Retinas were rinsed again and then incubated overnight in a secondary antibody solution containing a rhodaminated rabbit anti-mouse IgG (1:20; Cappel Laboratories, Malvern, PA). After a final rinse, retinas were embedded in JB-4 resin (Polysciences, Inc. Warrington, PA; Matsumoto et al., 1987). A dry glass knife was used to obtain 2-μm-thick sections, which were mounted in glycerol plus *n*-propyl gallate (Giloh and Sedat, 1983). Antitubulin labeling in rod photoreceptors was viewed with an image processing system described below using a rhodamine filter set of an epifluorescence Photomicroscope III (Carl Zeiss, Inc., Thornwood, NY).

The Lucifer Yellow Band Height Assay for ROS Membrane Assembly

The process by which the fluorescent dye, Lucifer Yellow (LY), labels basal ROS disc membranes is illustrated in Fig. 2. To visualize the LY bands at the bases of ROSs (Matsumoto and Besharse, 1985), eyecups were removed from culture and immediately fixed with cold 4% paraformaldehyde plus 0.1% glutaraldehyde in CB for 30 min, followed by overnight fixation in 4% paraformaldehyde in CB; rinsing in CB; quenching the background fluorescence with 0.5 mg/ml $NaBH_4$ in CB for 10 min; rinsing in CB; dehydration in ethanol; and embedment in Spurr's resin (Spurr, 1969). 1-μm-thick sections were taken along the midsaggital plane of each eyecup, so that aligned ROSs from ventral-to-dorsal peripheries were available for measurement.

LY labeling of ROSs in the 1-μm sections was viewed through the fluorescein filter set of the photomicroscope (Carl Zeiss, Inc.) equipped for epifluorescence; the objective lens was a 100× Zeiss Plan-Neofluar oil immersion lens. Images were recorded onto high resolution VHS videotape (TDK Hi-Fi, T-120; Port Washington, NY) for 30 s per field of view, using a silicon intensified target camera (model No. 66, Dage-MTI, Inc.; Michigan City, IN) and a video cassette recorder (Quasar; Glenview, IL). The analogue-recorded images were then digitized and displayed with an image processor (MegaVision, Inc., Santa Barbara, CA). Fifteen consecutive frames were averaged for noise reduction. Contrast enhancement and digital filtration were used to improve the image edge sharpness.

Interactive measurement software (written by Steven A. Bernstein) was used to measure the proximal-to-distal dimension of each basal LY band (in microns) along the center of every well-aligned ROS in each field of view (Fig. 2 *E*). This quantity is referred to as "LY band height." In all experiments, LY band heights from 30–150 ROSs were measured per eyecup. The numerical data were imported into a spreadsheet program (Lotus Development Corp., Cambridge, MA) and analyzed with the graphics and statistical functions.

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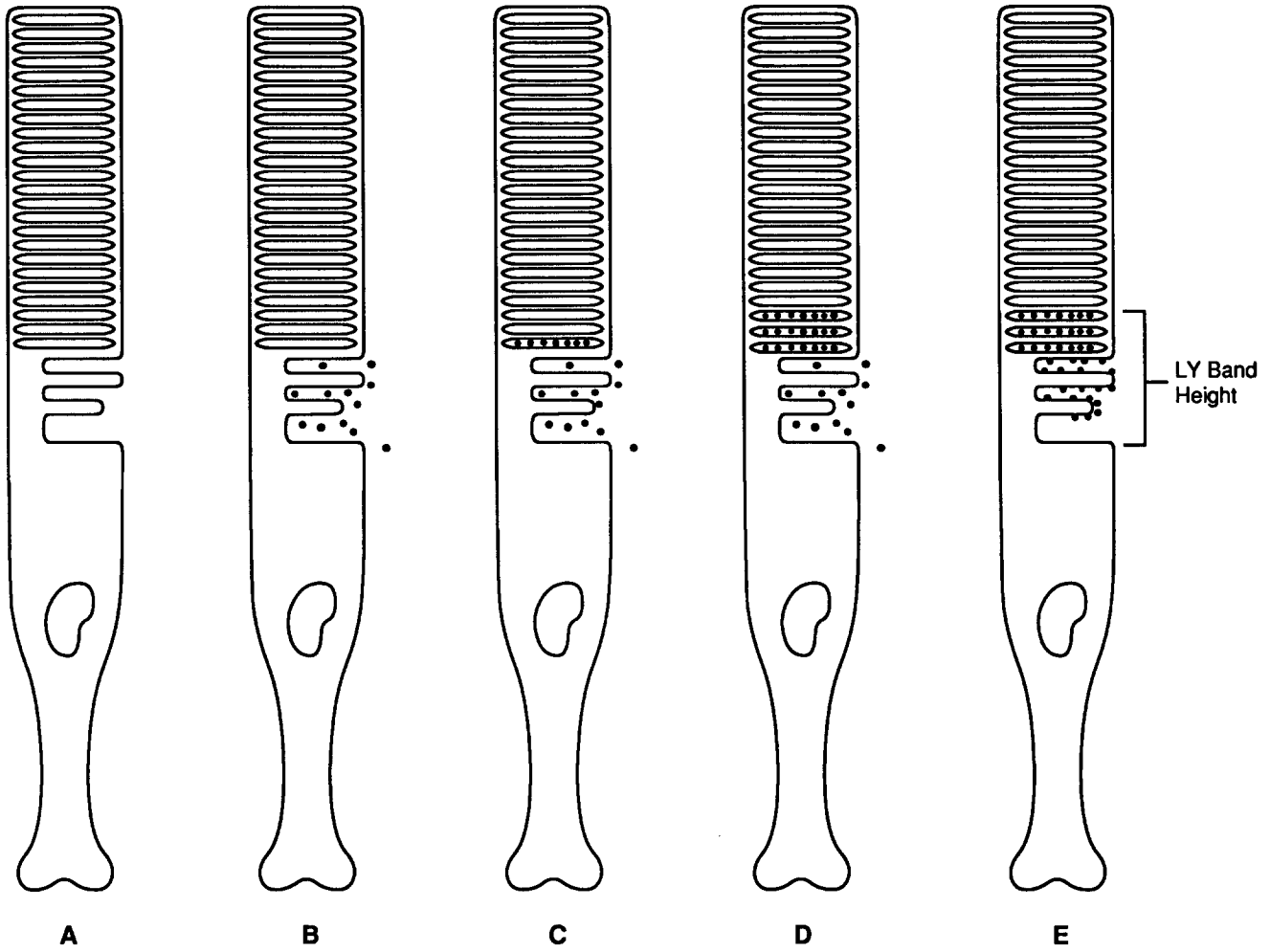

Figure 2. (*A–D*) Diagram of LY dye entrapment within maturing ROS discs. LY dissolved in the culture medium binds to exposed membrane proteins and becomes concentrated within the intradiscal space as successive immature evaginations seal off into separate, flattened-sack discs. LY-labeled discs are shifted distally in the ROS stack in an orderly fashion as ROS assembly continues. (*E*) Explanation of LY band height measurements. In the continuous presence of the dye, a band of yellow fluorescence (the LY band) develops. Over time, its proximal-to-distal dimension (height) within the stack increases and it is this dimension that is measured (*brackets*).

For the purpose of this study, the LY band height assay was used in two ways, whose details follow.

Time Course of ROS Membrane Assembly

The LY band height assay was used to quantify ROS membrane assembly over time in culture. In these experiments, eyecups were incubated in basal medium plus LY for 1, 2, 4, 8, 11, and 16 h after light onset. Whereas the 16-h eyecups would normally have experienced light offset at 12 h, the lights remained on for the duration of the incubation. Four eyecups (from different frogs) were used per time point. After the designated incubation, the eyecups were fixed and LY band heights were measured as described above. At 2 and 4 h in culture, a small number of ROSs failed to exhibit visible LY bands; these were assigned a LY band height value of 0.00 and were included in calculations for mean LY band heights of those two time points. Data were plotted as mean LY band height as a function of time.

Anticytoskeletal Drugs and ROS Membrane Assembly

The LY band height assay was used to quantify the effects of anticytoskeletal drugs on ROS membrane assembly. In these experiments, eyecups were incubated for 10 or 11 h in either control (no drug) or experimental (with drug) medium, followed by fixation and LY band height measurement as described above. All anticytoskeletal drug and control incubations were performed on two separate occasions, using three to four eyecups per treatment (all from different animals). Mean LY band height data with experimental and control media were analyzed for variance followed by a Dunnett's *t* test (Winer, 1962).

Light Microscope Autoradiography

As an adjunct to the LY band height assay for ROS membrane assembly, autoradiography of radiolabeled ROS opsin was performed after the methods of Hollyfield et al. (1982). Eyecups were pulsed with tritiated leucine by incubation for 15 min in a balanced salt solution supplemented with [^{3}H] leucine at 0.2 mCi/ml. They were then transferred to control (0.26% DMSO) or experimental (0.2 mM colchicine in 0.26% DMSO) medium and incubated for a further 6 h. Eyecups were removed from culture and fixed overnight in 3% paraformaldehyde plus 3% sucrose in 0.1 M phosphate buffer (PB; pH 7.2). They were then washed in PB plus 3% sucrose; post-fixed for 1.5 hr in 2% osmium tetroxide in PB; dehydrated in a cold, graded ethanol series; infiltrated with propylene oxide; and embedded in Araldite. 1-μm-thick sections were cut on a microtome (MT-2B; Sorvall, Inc., Newton, CT) and dried down thoroughly onto clean glass slides, which were then dipped in NTB-2 emulsion (Eastman Kodak Co., Rochester, NY), dried, boxed, and refrigerated in the dark for 7 d. The autoradiograms were developed in D-19 (Eastman Kodak Co.), fixed, and stained with toluidine blue for light microscopic examination of silver grain distribution in the ROSs.

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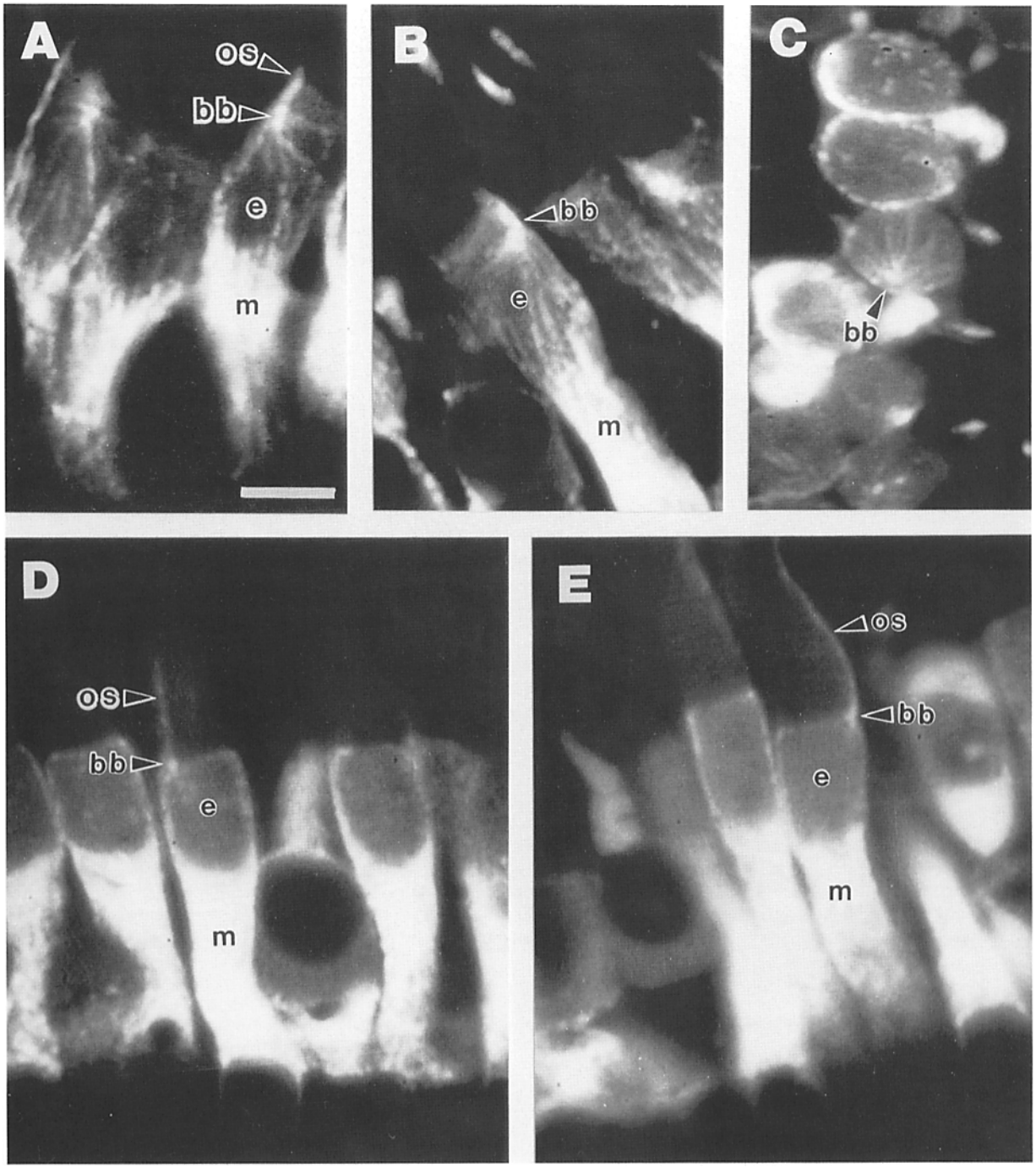

Figure 3. Tubulin immunofluorescence of rod photoreceptors from eyecups cultured for 11 h. (*A* and *B*) Control medium (LY plus DMSO only). In longitudinally sectioned material, antitubulin label reveals microtubules in the OS (*os*), connecting cilium and its basal body (*bb*), the ellipsoid (*e*), and the myoid (*m*); they are especially numerous in the myoid. MTs are also present in the photoreceptor axon (not shown). Scale bar, 10 μm. (*C*) Control medium (DMSO only). In horizontally sectioned material, antitubulin label reveals a "funneling" of microtubules from the RIS ellipsoid to the basal body (*bb*). (*D* and *E*) Experimental medium (LY plus DMSO-carried nocodazole). In horizontally sectioned material, labeled MTs have been eliminated from the ellipsoid region (*e*), but persist in the OS (*os*), myoid (*m*), and basal body (*bb*). The plane of section here is comparable to that of *A* and *B*, as evidenced by the location of the basal body (*bb*).

Electron Microscopy

Eyecups were incubated in either basal medium or basal medium plus LY for 11 h and then removed from culture and prepared for transmission EM. They were fixed in 1% paraformaldehyde plus 1% glutaraldehyde in 0.1 M CB; rinsed in CB; postfixed in 1% osmium tetroxide in CB; rinsed in CB; dehydrated in a graded ethanol series (including a 1-h en bloc staining step in 1% uranyl acetate in 70% ethanol); infiltrated in propylene oxide; and embedded in Spurr's resin (Spurr, 1969). Silver or gold thin sections were examined and photographed in an electron microscope (CM 10; Philips Electronic Instruments, Mahwah, NJ).

Results

Tubulin immunofluorescence revealed the distribution of

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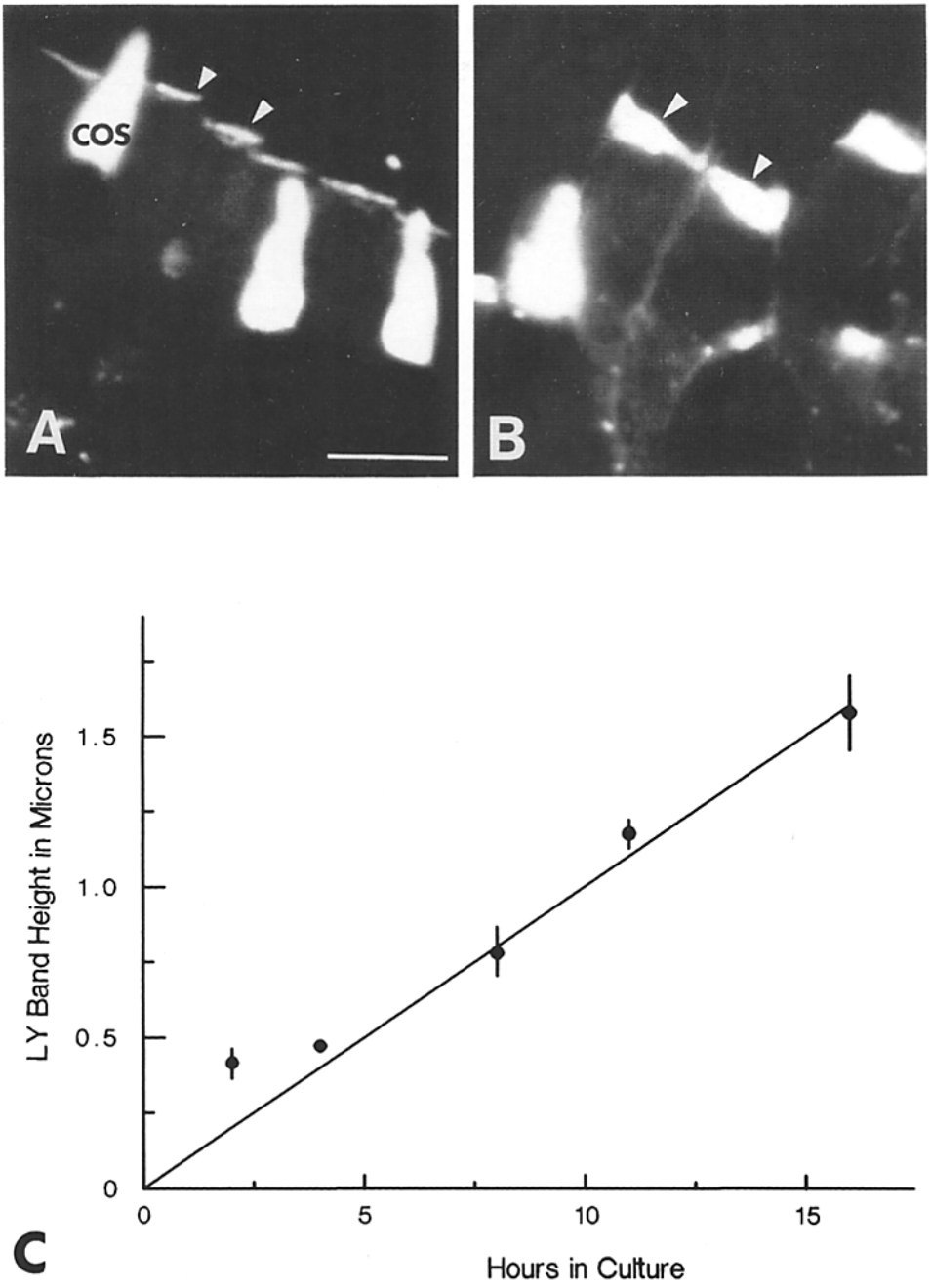

Figure 4. Relationship between LY band height and time in culture. (*A*) LY label in photoreceptors from an eyecup cultured for 2 h. The brightly labeled triangular profiles are cone OSs (*COS*), which label in their entirety. The narrow bands at the rod OS bases (*arrowheads*) are LY-labeled disc membranes assembled during the 2-h culture period. Scale bar, 10 μm. (*B*) LY label in photoreceptors from an eyecup cultured for 11 h. The LY bands at the ROS bases (*arrowheads*) have increased in "height" compared with the 2-h time point. Cone OS label is complete, as before. Small spots of label in the proximal rod IS correspond to LY uptake by pinocytosis. (*C*) Plot of mean LY band height as a function of time in culture. Time 0 corresponds to the beginning of the LY incubations at normal light onset. Each bar represents two SEMs. After 4 h, the increase in LY band height is linear.

microtubules in *Xenopus* photoreceptors maintained in culture medium supplemented with LY (Fig. 3). In rods maintained in control (DMSO) medium (Fig. 3 *A–C*), labeled MTs were seen in the ROS, in the connecting cilium and its basal body, in the ellipsoid, and in the myoid (where they were the most numerous). When immunolabeled rods were viewed in cross-section (Fig. 3 *C*), ellipsoid MTs were seen to "funnel" from the RIS to the basal body, corresponding to the region of the cell where membrane-bound opsin is transferred to the distal RIS plasma membrane for eventual assembly into new OS discs. In rods from eyecups incubated for 2 h in media containing a MT-depolymerizing drug (nocodazole, colchicine, vinblastine, or podophyllotoxin), labeling of the ellipsoid microtubules was lost (Fig. 3, *D* and *E*); ellipsoid labeling was still absent after 11 h incubation in each drug (not shown). The basal bodies, connecting cilium, and MT in the RIS myoid region were still labeled at both 2 (Fig. 3, *D* and *E*) and 11 h of treatment with MT-depolymerizing drugs.

Ellipsoid MTs were not reliably visualized by transmission EM of normal photoreceptors (four animals), due to their low number and the dense packing of ellipsoid mitochondria.

Eyecups cultured in LY for at least 2 h exhibited distinct bands of yellow fluorescence in their basal ROSs (Fig. 4 *A*), corresponding to dye that had become progressively entrapped within the newly assembled disc membranes (Fig. 2). Qualitatively, LY band height gradually increased with longer incubations (Fig. 4 *B*). This can be demonstrated

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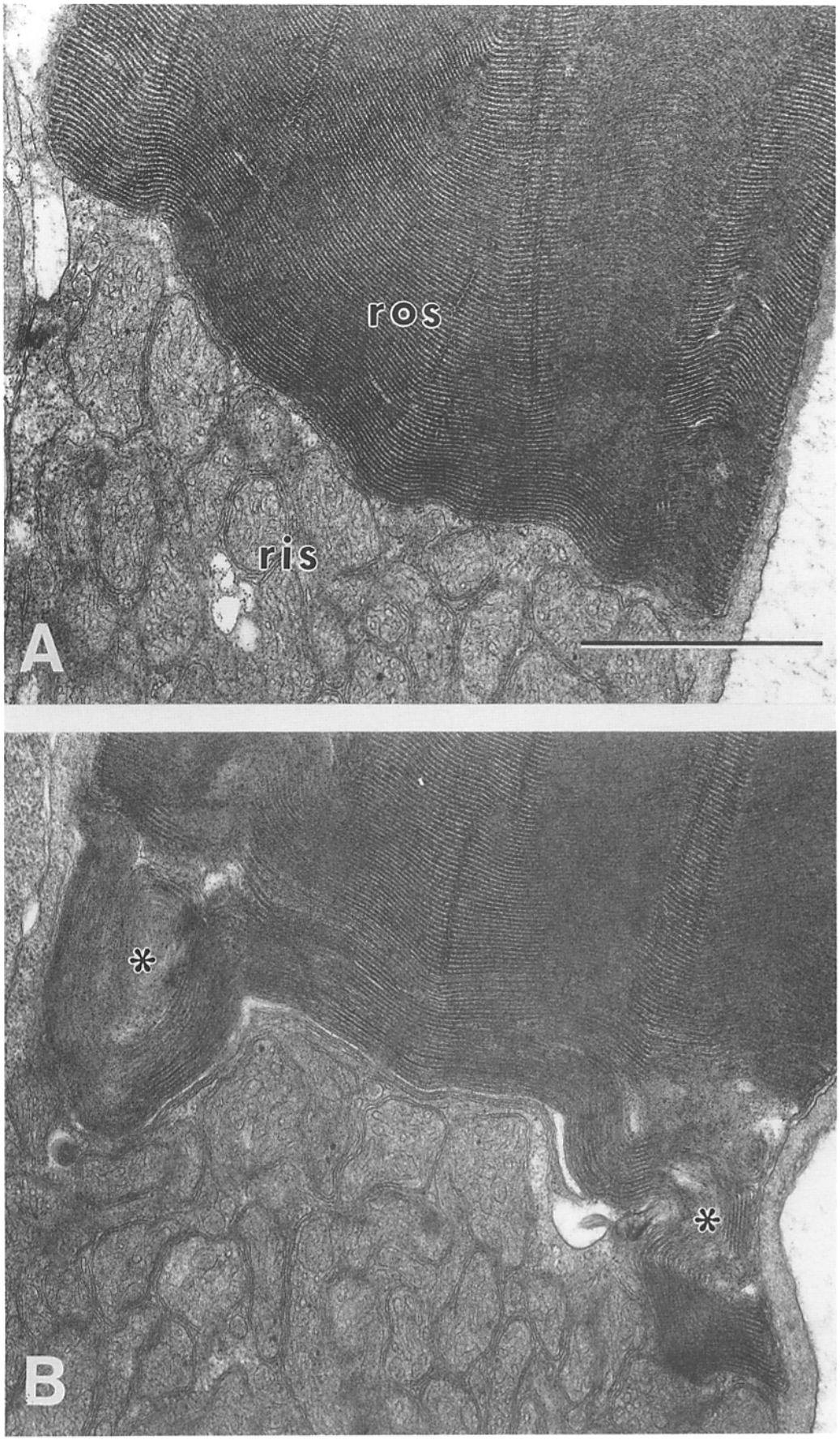

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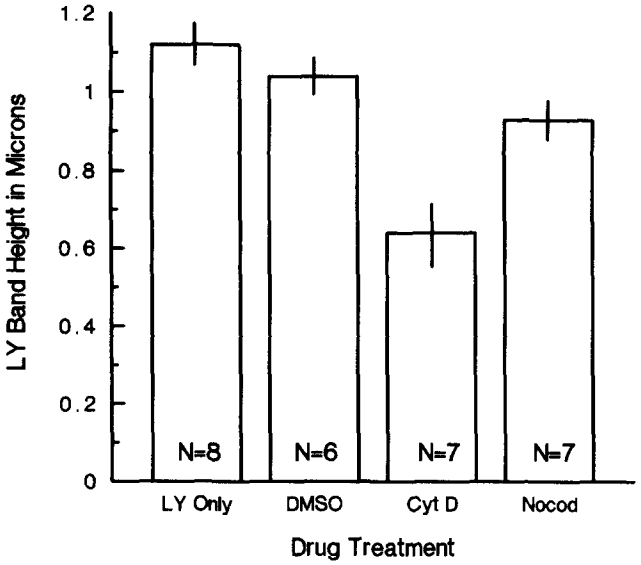

Figure 6. The effect of anticytoskeletal drugs on ROS assembly as assayed with LY. The bar graph shows mean LY band height values (and SEs) obtained when eyecups were cultured for 11 h in different control or drug-supplemented culture media (all media contained LY). *N*, number of eyecups sampled. There was no significant difference between the means obtained when eyecups were cultured in the presence or absence of DMSO, the carrier solvent for all drugs used in this study. Cytochalasin D treatment significantly reduced the mean LY band height value compared with control values ($P < 0.005$). Nocodazole treatment had no significant effect.

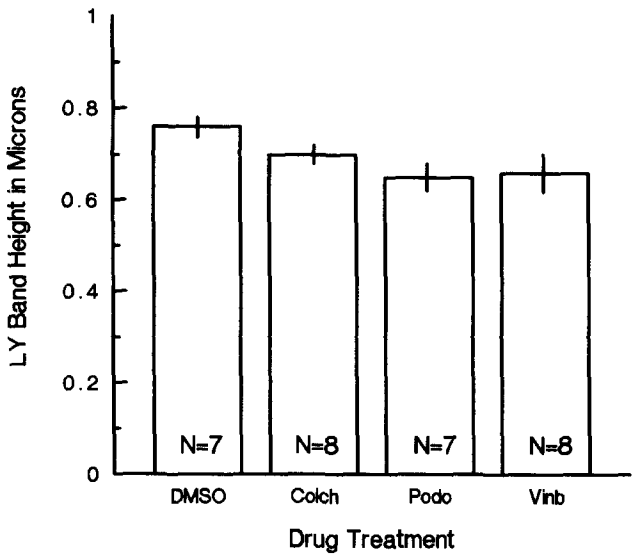

Figure 7. The effect of additional antimicrotubule drugs on ROS assembly as assayed with LY. The bar graph shows mean LY band height values (and SEs) obtained when eyecups were cultured for 10 h in control or drug-supplemented culture media (all media contained LY). *N*, number of eyecups sampled. There was no significant difference between the means obtained from control (DMSO only) or experimental (DMSO-carried colchicine, podophyllotoxin, or vinblastine) media.

quantitatively by the least squares line constructed from mean LY band heights calculated for time points ranging from 2–16 h in culture (Fig. 4 *C*). The increase in LY band height was linear after 4 h in culture.

While measuring LY band heights from both control and experimental eyecups, we occasionally observed ROSs with unusual, wedge-shaped basal LY bands. When thin sections from eyecups incubated for 11 h in LY were examined in the electron microscope, some basal ROS discs (assembled in the presence of LY) did indeed exhibit irregularities at their edges; an example is shown in Fig. 5 *B*. Such disc edge irregularities were not observed in ROSs incubated in basal medium without LY (Fig. 5 *A*). Importantly, even in our worst-case examples (as in Fig. 5 *B*), membrane irregularities did not appear in the center of the basal ROS discs where LY band height measurements were made (Fig. 2 *E*).

The LY band height assay was used to detect differences in ROS assembly in eyecups cultured in the antimicrotubule drugs, nocodazole, colchicine, vinblastine, and podophyllotoxin; in the antimicrofilament drug, cytochalasin D; or in control medium (DMSO only). The results of these experiments are shown in Figs. 6 and 7. Mean LY band heights in the presence or absence of DMSO, the carrier present in both control and experimental media, were not significantly different, indicating that DMSO had no deleterious effect on ROS assembly. Mean LY band heights from eyecups cultured in the four different antimicrotubule drugs were also not significantly different from the mean value derived from control eyecups. In contrast, mean LY band height in eyecups cultured in cytochalasin D was only 60% of the control value (Fig. 6), indicating that this inhibitor of actin polymerization had a significant effect on ROS disc assembly as measured by this technique.

Autoradiography of control eyecups cultured in DMSO only for 6 h yielded ROSs with distinct bands of silver grains over their basal membranes (Fig. 8 *A*). Equivalent accumulations of silver grains were seen in autoradiograms of experimental eyecups cultured in DMSO-carried colchicine for 6 h (Fig. 8 *B*).

Discussion

Xenopus rods assemble about 78 new discs, or the equivalent of 7,800 μm^2 of ROS membrane, per day (Kinney and Fischer, 1978). This remarkable assembly process requires an equally impressive mechanism to support delivery of ROS membrane precursors to the site of their incorporation into

Figure 5. Transmission electron micrographs of basal ROS membranes from eyecups cultured for 11 h. (*A*) Culture medium did not include LY. Membranes are stacked regularly and in register (*ros*, rod outer segment; *ris*, rod inner segment). Ellipsoid MTs are not easily detected in thin sections because of their low number in this region of the RIS. Scale bar, 2 μm. (*B*) Culture medium included LY. This is a worst case example of basal membrane irregularities observed in some cells. The edges of the newest disc membranes are curled (*asterisks*), which may have caused the irregular LY band shapes occasionally observed in our experiments (see text). However, the central portion of the stack of new discs (which was used for the LY band height measurements) is unaffected.

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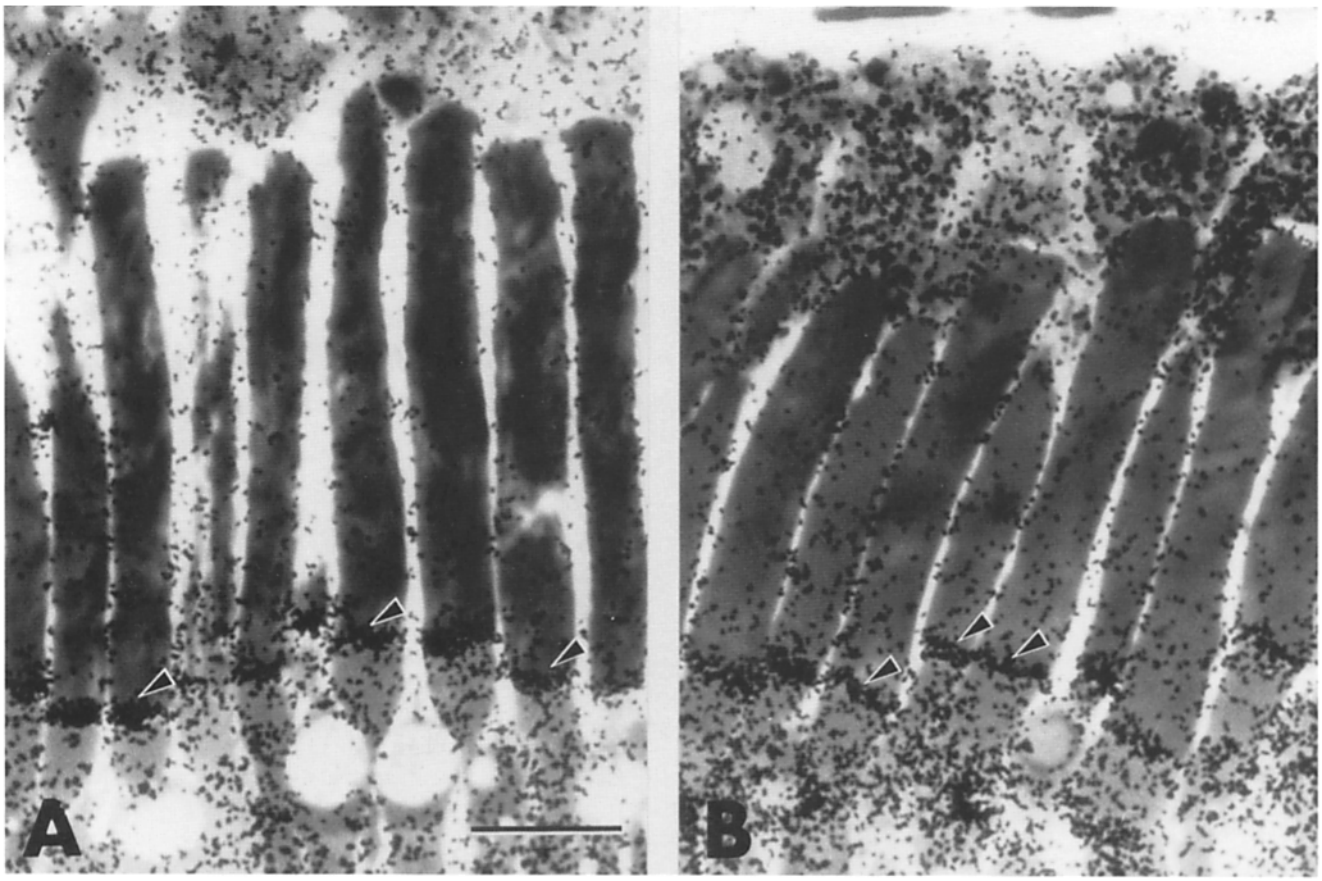

Figure 8. The effect of the anti-MT drug, colchine, on ROS assembly as assayed with light-microscope autoradiography of radiolabelled ROS protein. Cultured eyecups were pulsed with tritiated leucine and then incubated for 6 h in control or experimental medium. Neither culture medium contained LY. (*A*) Cultured in control medium (DMSO only). Distinct bands of silver grains (arrowheads) corresponding to newly assembled, basal ROS membranes are visible in all rod photoreceptors. Scale bar, 20 μm. (*B*) Cultured in experimental medium (DMSO-carried colchicine). As in the control eyecups, distinct bands of silver grains (*arrowheads*) are visible in all rod photoreceptors, indicating that colchicine did not affect delivery of protein to the site of ROS membrane assembly.

maturing ROS discs. Opsin, the apoprotein of the visual pigment (rhodopsin), is membrane-associated from the time time it is first synthesized in the RIS (Papermaster and Schneider, 1982; Papermaster et al., 1985). It has been proposed that opsin vesicles (Kinney and Fisher, 1978; Papermaster et al., 1985) are transported from the Golgi body through the RIS to a specialized region at the base of the connecting cilium, where they fuse with the RIS plasma membrane (Peters et al., 1983). The nascent membrane constituents can then form the ciliary plasma membrane evaginations that initiate ROS disc morphogenesis (Steinberg et al., 1980).

Opsin delivery to the distal RIS has been experimentally inhibited at its starting point by incubating frog retinas in monensin, a Na^+-H^+ ionophore that selectively disrupts the organized structure of the Golgi apparatus (Matheke et al., 1984; Matheke and Holtzman, 1984). These studies suggest that opsin must pass through an intact Golgi to be correctly "addressed" and subsequently delivered to the distal RIS. We are interested in the next step, i.e., in the transport mechanism whereby the addressed vesicles are delivered to the distal RIS. Based on the well-established role for MTs in axonal transport (Vale, 1987), it seemed reasonable to hypothesize that opsin vesicles are transported along this pathway via an MT-dependent mechanism. To test our hypothesis, we needed to detect and disrupt the population of photoreceptor microtubules that are likely candidates for this role. We also needed to measure ROS assembly, because any change in transport of membrane-bound opsin is presumably reflected in the amount of ROS assembly.

The results of our tubulin immunofluorescence studies established the existence and arrangement of RIS ellipsoid MTs, which are uniquely situated to provide a possible mechanism for membrane-bound opsin transport through the RIS. Although epifluorescence microscopy does not have the resolution of EM, it has been shown to reliably detect single MTs labeled by immunofluorescence (Osborn and Weber, 1982) and has been used to study the dynamics of single microtubules (e.g., Schulze and Kirschner, 1986). The ability to sample a large number of cells by light microscopy provides us with confidence that the majority, if not all, of the MTs radiating from the basal body into the ellipsoid are disassembled by drug treatment. Studies using EM to visualize microtubules cannot reliably demonstrate drug disassembly of these structures because of the limited region of cytoplasm that can be examined in thin sections. Even in the region of the basal body, where the MTs are the most abundant, the absence of MTs can represent either an unfortunate plane of

section or the result of MT depolymerization by drug treatment. Although it is probably not possible to prove that all the microtubules in the ellipsoid were completely depolymerized, we are confident, based on our data (Fig, 3, *D* and *E*) and that of others (Sale et al., 1988), that the vast majority of potential tracts for MT-dependent transport were effectively disrupted by drug treatment. This would be expected to disrupt MT-dependent vesicular transport in turn.

We developed the LY band height assay as an improved method for measuring membrane assembly in rod photoreceptor OSs, which is more traditionally measured by following the movement of radiolabeled OS disc proteins by autoradiography. Young and Droz (1968) were the first to demonstrate that such labeled photoreceptors form a band of radioactive protein at the ROS base that is then apically displaced as new membrane is added to the ROS. The rate of radioactive band displacement reflects the rate of new disc assembly. Hall and co-workers (1969) later demonstrated that the bulk of radioactivity in the outer segment membrane is in the visual pigment apoprotein, opsin. Because opsin is an integral membrane protein that does not diffuse out of the rod disc membranes, radioactive band displacement provides an accurate assay for opsin renewal in the ROS (Young, 1967). In our study, autoradiographic measurement of radioactive bands was not attempted, because of the technique's limited precision (Rogers, 1979). The exposed silver grains that define the presence of radioactivity are physically separated from the labeled proteins, causing a geometric error between the position of the silver grains and the radioactive source (Salpeter et al., 1969). Additionally, the size of the silver grains makes it difficult to precisely define the borders of the radioactive band in the ROS. These sources of error can be eliminated if the nascent disc membranes are labeled directly. Laties et al. (1976) demonstrated that fluorescent dyes can be used to stain newly assembled disc membranes and suggested the use of this staining as an assay for monitoring membrane assembly. Our data demonstrate the applicability of LY staining as a direct measure of membrane assembly in normal eyecups and in those undergoing drug treatment.

Our data further confirm earlier studies that demonstrated that opsin biosynthesis and membrane assembly occur in culture (Basinger and Hall, 1973). Fluorescent band measurements indicate a linear displacement of disc membranes from 4 to 16 h in culture, permitting us to use intermediate time points of 10 or 11 h for evaluating the effects of anticytoskeletal drugs on ROS assembly.

In the controls for the drug treatment experiments, we demonstrated that the carrier solvent, DMSO, had no significant effect on ROS assembly as measured by the LY band height assay. In contrast, the LY assay detected a 40% reduction in ROS assembly when eyecups were cultured in the antimicrofilament drug, cytochalasin D. This can be explained by the fact that cytochalasin D causes newly forming ROS discs to overgrow their normal diameter, so that new disc membranes extend laterally into the extracellular space, beyond the normal diameter of the ROS (Williams et al., 1988; Vaughan and Fisher, 1989). What we have detected here as a decrease in the height of new membrane added to the ROS is apparently due to the distribution of new membrane into the overgrown portions of new discs. In fact, LY-labeled overgrown ROS membranes were frequently observed in our cytochalasin D-treated eyecups (data not shown). This experiment therefore served as a positive control for the ability of the LY assay to detect drug-induced changes in ROS assembly over the time period studied.

Treatment with four different anti-MT drugs, which specifically disrupted the ellipsoid MTs, had no significant effect on ROS assembly, as measured by the LY band height assay (Figs. 6 and 7). These results were further supported by autoradiography, which showed the formation of radiolabeled bands at the base of the ROSs in both control and colchicine-treated retinas (Fig. 8). Together, these data provide a strong argument against a role for MTs in the delivery of opsin to the site of opsin incorporation and eventual ROS membrane assembly. Microfilaments, an alternative possibility (Kachar, 1985), are also eliminated by experiments showing continued incorporation of radiolabeled protein into ROSs in the presence of cytochalasin D (Williams et al., 1988).

Our study raises the question of what mediates the intracellular transport of membrane-bound opsin from its site of synthesis in the proximal RIS to its site of incorporation into the distal RIS plasma membrane. Using anti-MT drugs, vectorial transport of plasma membrane proteins has been shown to be MT-dependent in some polarized cells (Eilers et al., 1989). In others, vesicular transport of plasma membrane proteins continues in the presence of anti-MT drugs, but the polarity of their eventual distribution is lost (Rogalski et al., 1984). In still other reports, vectorial transport of plasma membrane proteins is microtubule-independent (Rogalski and Singer, 1984; Salas et al., 1986). Clearly, in photoreceptors and in other cell types, some transport mechanism exists that is distinct from the known MT- or microfilament-based systems.

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