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Disruption of Microfilament Organization and Deregulation of Disk Membrane Morphogenesis by Cytochalasin D in Rod and Cone Photoreceptors

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ABSTRACT

Morphogenesis of photoreceptor outer segment disks appears to occur by an evagination of the ciliary plasma membrane (Steinberg et al., J Comp Neurol *190*:501–519, '80). We tested if polymerized actin (F-actin) was necessary for the regulation of this postulated process by incubating *Xenopus* eyecups with 5 or 25 μM cytochalasin D for 6–28 hours. During the second hour, the incubation medium contained ^{3}H-leucine. Both concentrations of cytochalasin resulted in: 1) dissolution of the rhodamine-phalloidin labeling pattern of photoreceptors, and 2) collapse of the calycal processes (which are normally filled with actin filaments) and disappearance of the inner segment microfilaments. In addition, the few most basal rod and cone outer segment disks appeared several times their normal diameter. These oversized disks had incorporated ^{3}H-leucine and extended along the margin of the outer or inner segment. The nature of the overgrown disks is consistent only with a morphogenetic process involving evaginations of the ciliary plasma membrane. Deregulation by cytochalasin D was manifest by excessive growth of a few nascent disks rather than normal growth of many. Therefore, the normal network of actin filaments is apparently not necessary for continued evagination of the membrane, but it does seem to be an essential part of the mechanism that initiates the evagination of the ciliary plasma membrane and/or the mechanism that controls how far nascent disks grow.

Key words: actin, membrane turnover

Each vertebrate retinal photoreceptor possesses an outer segment that comprises a stack of phototransductive membranous disks. The outer segment is connected to an inner segment by a ciliary region. It is renewed by the shedding of disks from its distal end and the assembly of new disks near the connecting cilium (Young, '76). Steinberg et al. ('80) proposed that new disks are assembled by *evaginations* of the ciliary plasma membrane. These evaginations grow out to the perimeter of the outer segment. At any one time there are usually several nascent disks of different sizes (Fig. 1).

We considered that such a process of assembly by evagination might be regulated by actin filaments (F-actin) for two reasons. 1) Typically, in outgrowths of plasma membrane—such as lamellipodial formation during locomotion of various types of cells (e.g., cultured fibroblasts, Lazarides and Revel, '79), the acrosomal reaction of sperm (Tilney et al., '73), or advancing growth cones of differentiating neu-

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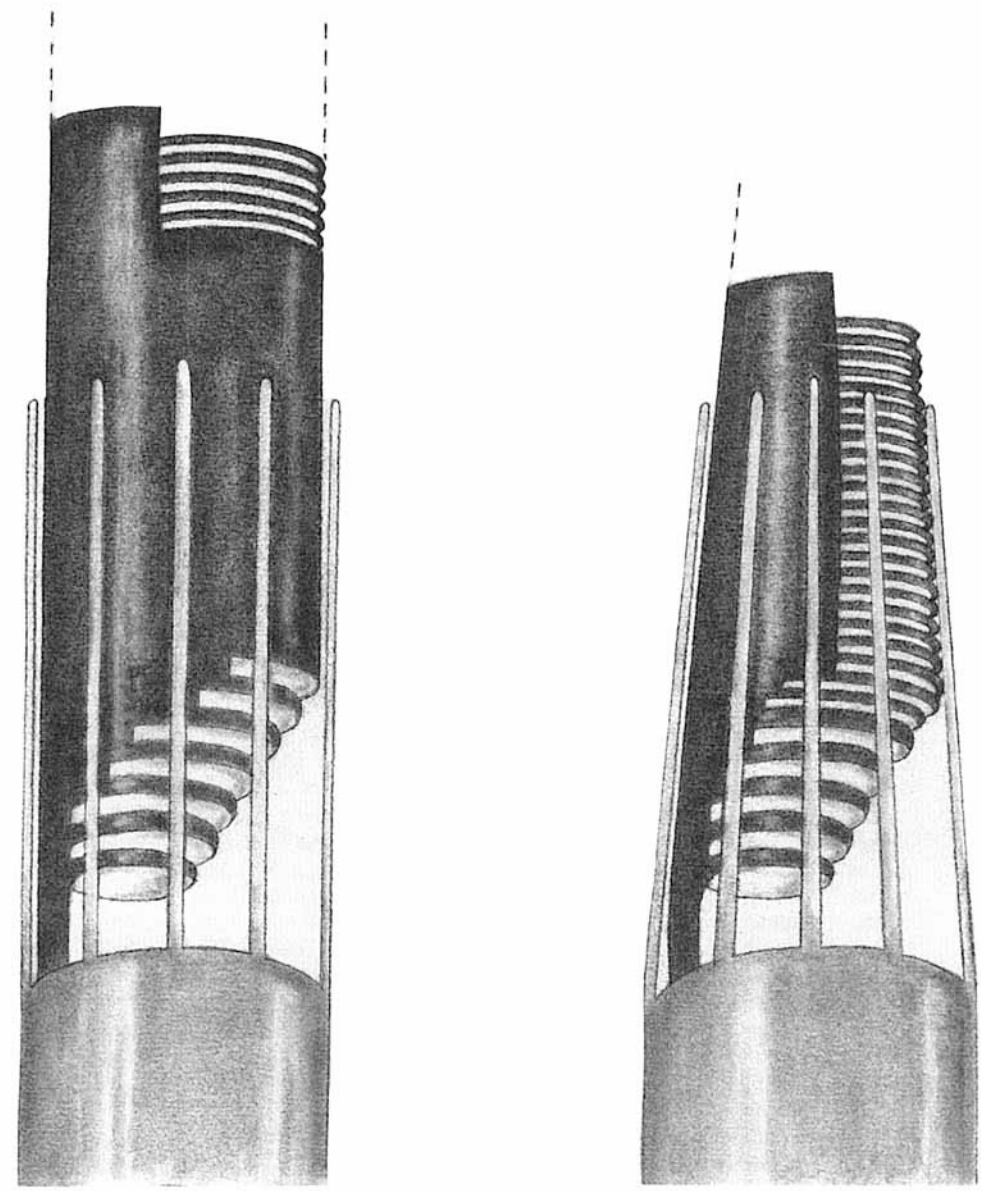

Fig. 1. Diagram (stylized and not drawn to scale) of the basal region of a rod and cone outer segment. Newly developing disks are shown as outgrowths of the plasma membrane of the connecting cilium. The mature disks of the rod outer segment are closed, i.e., they are discrete disks, pinched off from the surrounding plasma membrane. The disks of the cone never become completely closed, however. The basal part of both the rod and cone outer segments is surrounded by a palisade of calycal processes, which extend from the inner segment.

rons (Spooner and Holladay, '80)—actin filaments play a major role (see also Stossel et al., '84). 2) In an immunohistochemical study, actin had been detected in the connecting cilium and nascent disks but not in the mature disks of outer segments (Chaitin et al., '84). Accordingly, we hypothesized that cytochalasins, which disrupt the organization of actin filaments (Brown and Spudich, '79; MacLean-Fletcher and Pollard, '80, Schliwa, '82), might inhibit disk morphogenesis (as they inhibit plasma membrane outgrowths of other cells, such as those above).

In the present study, we have examined the effects of cytochalasin D on actin filament organization and disk morphogenesis in photoreceptors of frog retinae in vitro. We report that the general disruption of actin filaments is associated with a deregulation of disk morphogenesis, although continued evagination of the plasma membrane, and thus growth of already initiated nascent disks, is apparently not impeded.

MATERIALS AND METHODS

Animals and eyecup incubation

Adult South African clawed frogs, *Xenopus laevis*, 3–4 cm long, were kept in aquaria under a 12 hour dark/12 hour

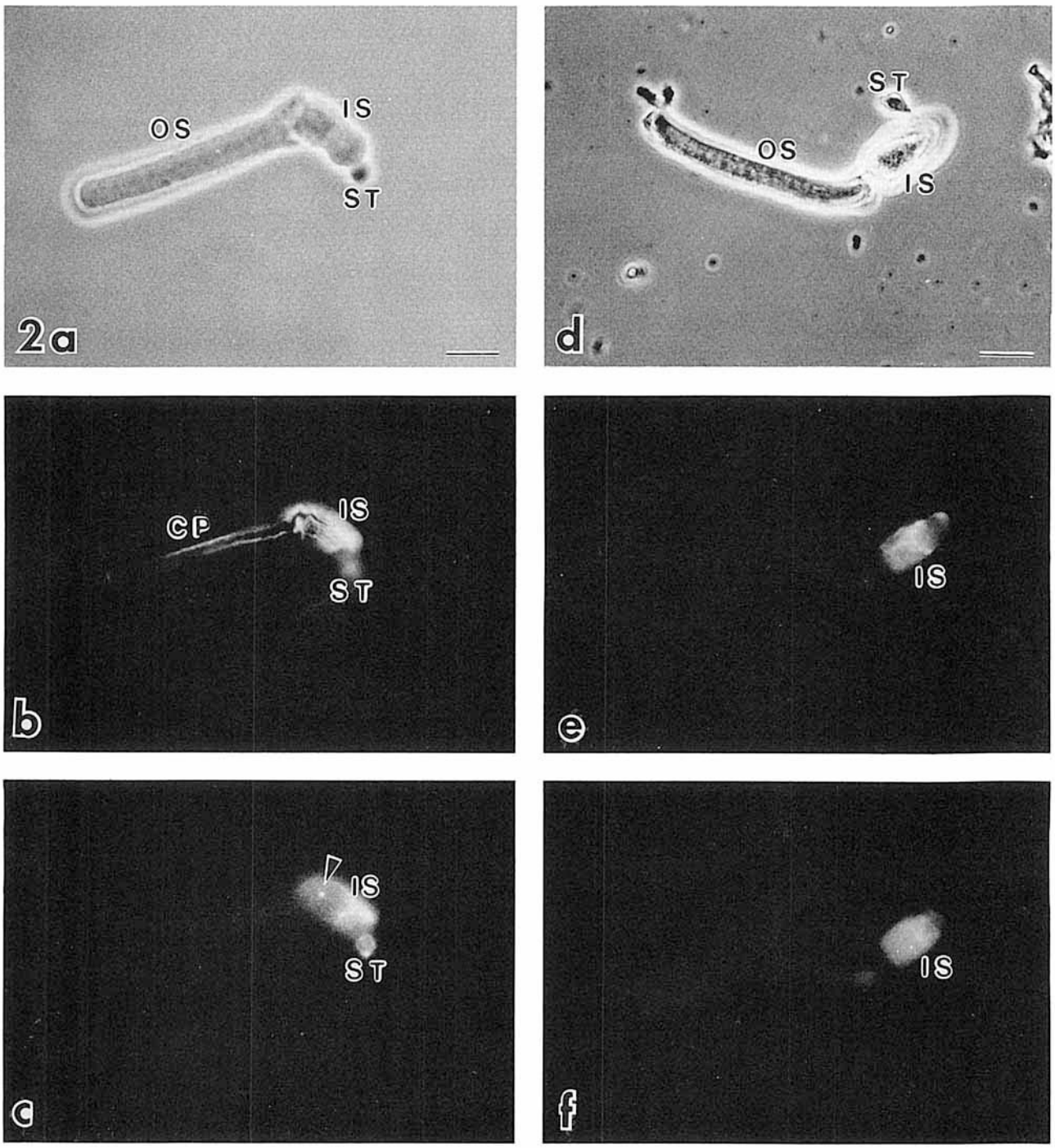

Fig. 2. Rhodamine-phalloidin labeling of isolated rod photoreceptors. (a-c) From an eyecup incubated for 4 hours in medium without cytochalasin D. (d-f) From an eyecup incubated for 4 hours in medium with 25 μM cytochalasin D. (a,d) Phase contrast micrographs. (b,c) and (e,f) Fluorescent micrographs at different depths-of-focus of the same rod as in (a) and (d), respectively. OS, outer segment; IS, inner segment; ST, synaptic terminal; CP, calycal process. Arrowhead in (c) indicates concentrated spot of label at the base of the outer segment. Scale bar = 10 μm.

light cycle, and fed crickets and mealworms for several weeks. Prior to enucleation of their eyes, they were pithed and decapitated. The anterior half of each eye was removed and the resulting eyecups were placed in culture medium. The medium was Wolf and Quimby amphibian culture medium (GIBCO) to which was added $NaHCO_3$ (final concentration, 30 mM) and DMSO (final concentration, 0.1%). Experimental media contained 5 or 25 μM cytochalasin D (Calbiochem), which had been dissolved in the DMSO before being added. The media were gassed with humidified

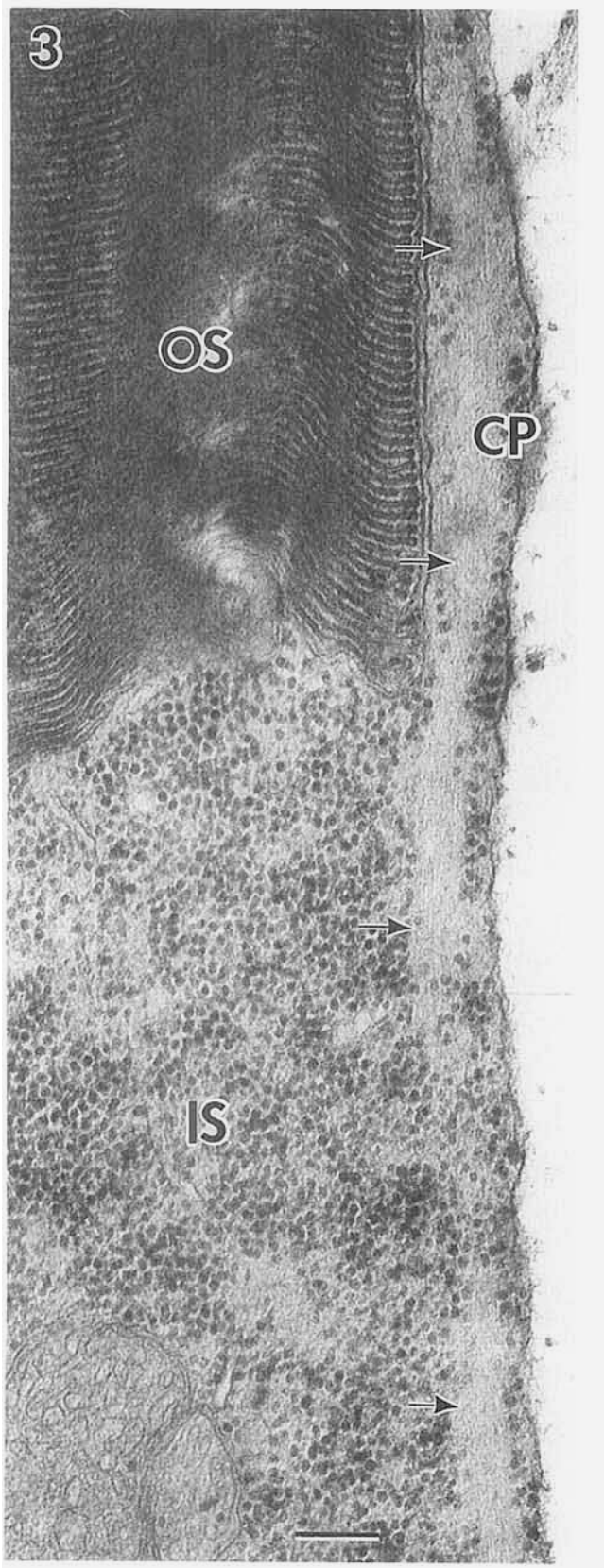

Fig. 3. Electron micrograph of part of a rod inner (IS) and outer (OS) segment in a retina incubated for 28 hours in the absence of cytochalasin. Arrows indicate microfilaments extending along the margin of the inner segment and into a calycal process (CP). Scale bar = 0.2 μm.

95% O_2/5% CO_2 for at least 10 minutes before use. Gassing of the media was continued throughout incubation. Temperature remained at 22–23°C.

Dissections were made between 2 and 3 hours after the onset of light on their daily cycle. The eyecups were collected initially in control or cytochalasin-containing medium, and thus preincubated for 0.5–1.5 hours (average of 1 hour). They were then incubated for 1 hour with 125 μCi/ml ^{3}H-leucine (Amersham: specific activity, 55 Ci/mmol) in the same medium (i.e., with or without cytochalasin D). Finally, they were rinsed several times and incubated in fresh medium without radiolabel for a further 2, 4, 6, 8 or 26 hours. This procedure was repeated on four separate occasions.

Tissue processing

At the end of incubation, eyecups were either fixed and embedded for sectioning, or dissociated for examination of individual photoreceptors with rhodamine-phalloidin labeling.

Retinal sections were examined by electron microscopy and light microscopical autoradiography. Eyecups were fixed in 2.5% glutaraldehyde + 2% formaldehyde in phosphate or cacodylate buffer (pH 7.4) for 3–24 hours. They were postfixed in 1% OsO_4 in the same buffer, dehydrated in ethanol, and embedded in Araldite. Ultrathin sections were collected on formvar-coated grids for electron microscopy, and 1-μm thick sections (red-green interference color) were collected on glass slides for light microscopical autoradiography. Autoradiography was carried out by dipping the slides in 50% (v/v) Kodak NTB-2 emulsion at 43°C. After suitable exposure at 4°C, the emulsion was developed in full strength D-19 for 2 minutes at 20°C, stopped, and fixed. Sections were stained with Azure II.

To free individual photoreceptors, retinae were detached and placed in Barth's balanced salt solution (Vaughan and Fisher '87), without Ca^{2+} or Mg^{2+} for a few minutes. Retinae were then incubated with 0.5 mg/ml Nagarse (Sigma) for 35 minutes in the same salt solution. Gentle suctions with a wide-bore pipette separated the enzymatically-treated retinal cells, which were then fixed in 1% paraformaldehyde for 30 minutes. Isolated cells were dried on glass slides and then incubated for 15 minutes with rhodamine-phalloidin (Molecular Probes Inc.) in phosphate-buffered saline (PBS), for detection of F-actin. After rinsing in PBS, the labeled cells were mounted in a 1:1 mixture of 5% n-propyl gallate in glycerol (Giloh and Sedat, '82) and PBS, and then examined under epifluorescence.

RESULTS

Organization of F-actin

In rods from control retinae, labeled actin filaments were evident in the synaptic terminal, around the nucleus, in the inner segment (Fig. 2b), concentrated in a spot at the base of the ROS (Fig. 2c), and in the calycal processes (Fig. 2b). Exposure to 5 or 25 μM cytochalasin D abolished this

Fig. 4. Transverse sections near the base of a rod outer segment in a control retina (**a**) and in a retina exposed to 5 μM cytochalasin for 6 hours (**b**). In (b), calycal processes are absent from the upper right margin of the outer segment. Here, overgrown nascent disks are apparent in cross section (arrowheads). (**c**) Higher magnification of part of a cytochalasin-treated outer segment where the calycal processes (arrows) are still evident. Note their dilated and irregular shape. Scale bars = 1.0 μm.

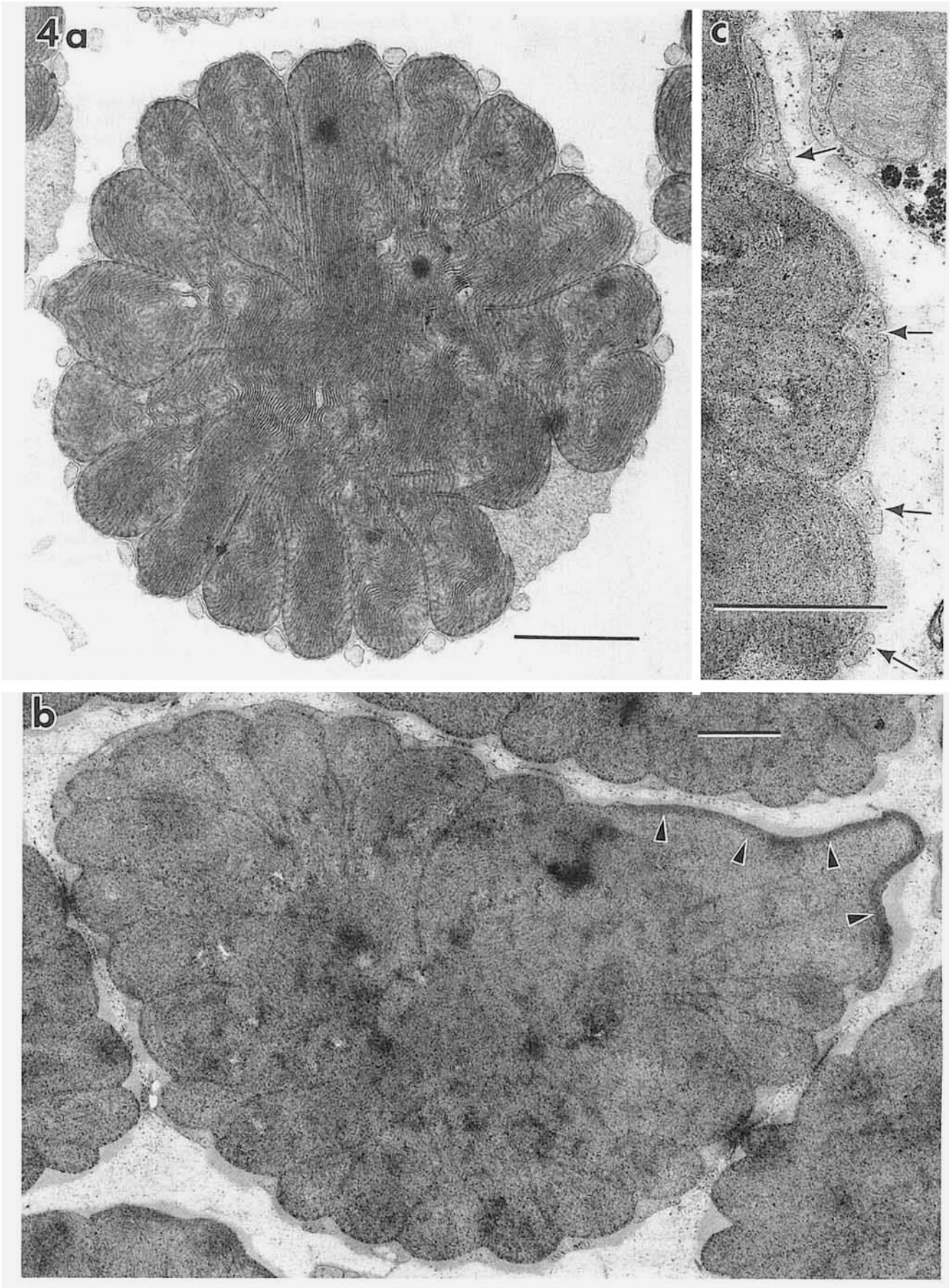

Figure 4

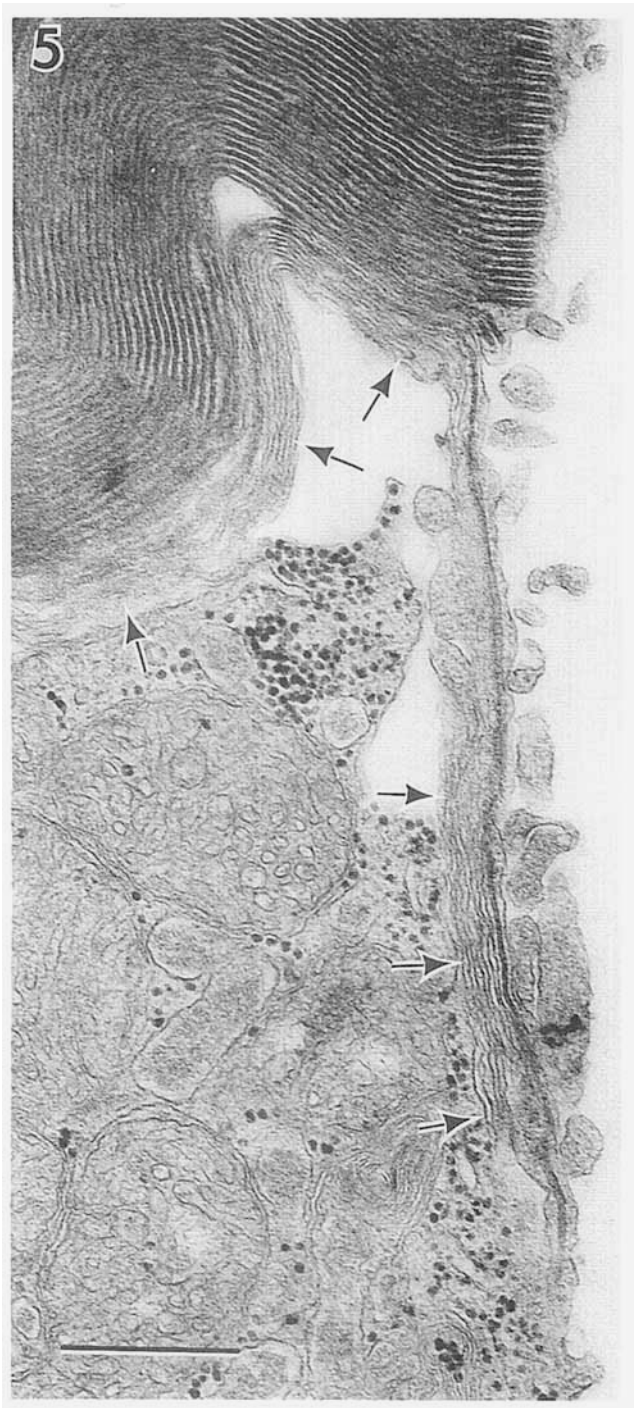

Fig. 5. Electron micrograph of part of a rod photoreceptor in a retina incubated for 6 hours in the presence of 25 μM cytochalasin D. Arrows indicate nascent disks. These disks are still open and have been characteristically preserved as wavy and less-organized structures in comparison to the closed (i.e., mature) disks (shown just above them). Exposure to cytochalasin has induced the nascent disks to grow beyond the margin of the outer segment. Scale bar = 0.5 μm.

labeling pattern in all regions except around the nucleus, indicating that cytochalasin D causes dissolution of most of the F-actin network (Fig. 2e,f).

With electron microscopy, bundles of filaments, about 7 nm in diameter (the commonly reported size of actin filaments), were evident in the rods and cones of control retinae. As reported by Drenckhahn and Groeschel-Stewart ('77), they were found along the margin of the inner segments and in the calycal processes (Fig. 3). They were not observed in retinae exposed to cytochalasin D for any of the tested lengths of time.

Effects of cytochalasin D on outer segment morphology

Rod and cone outer segments of control retinae exhibited characteristically normal structure, even after 28 hours in vitro. Most notably, the nascent disks did not extend beyond the calycal processes (Figs. 1, 3), which formed a regular array around the basal third of each outer segment (Fig. 4a). Exposure to cytochalasin D disrupted this organization markedly.

After all times sampled (6–28 hours) with 5 or 25 μM cytochalasin, the basal disks appeared very overgrown. They often appeared several times their normal width, extending beyond the rims of the normal mature disks, and along the margin of the inner segment, or, less commonly, along the outer segment (Figs. 4b, 5–13). Occasionally, they appeared to have burrowed into the inner segment (Figs. 6, 7, 10, 11). In the rods, these overgrown disks were still open after 6 hours (Fig. 5), but in later samples they had complete rims, and thus were discrete disks (see Steinberg et al. '80). In the cones (Fig. 12), they remained open.

Most rods and cones were affected. In transverse sections of the inner segments, the overgrown disks can be seen clearly around their inner segments in low power light microscopy (Fig. 10). Table 1 shows the proportion of affected rods thus observed.

No nascent disks were apparent proximal (vitread) to the overgrown disks, indicating that no new evaginations had occurred as the overgrown disks continued to grow. The calycal processes were short with irregular configurations, or completely absent; they appeared to have collapsed (Figs. 4, 13). After 28 hours of 5 μM or 25 μM cytochalasin, some of the overgrown disks of a few retinae were partially vesiculated.

Additional morphological effects

With light microscopy, cytochalasin D appeared to have no deleterious effect on the morphology of the inner retina. However, the RPE of some retinae, particularly of those exposed to 25 μM cytochalasin for 28 hours, was vacuolated in places. Consistent with reports by other researchers, the myoids of the rods, and especially the cones were narrowed and extended (see O'Connor and Burnside, '82), and the RPE generally contained fewer phagosomes (Besharse and Dunis, '82) than in control eyes.

TABLE 1. Rods With/Without Overgrown Disks Around Their Inner Segments

Treatment	With	Without	% With
Control	0	278	0
Cytochalasin D[1]	170	99[2]	63

[1] 5 μM for 8 hours.
[2] Note that many of these had nascent disks that had overgrown around their outer segments.

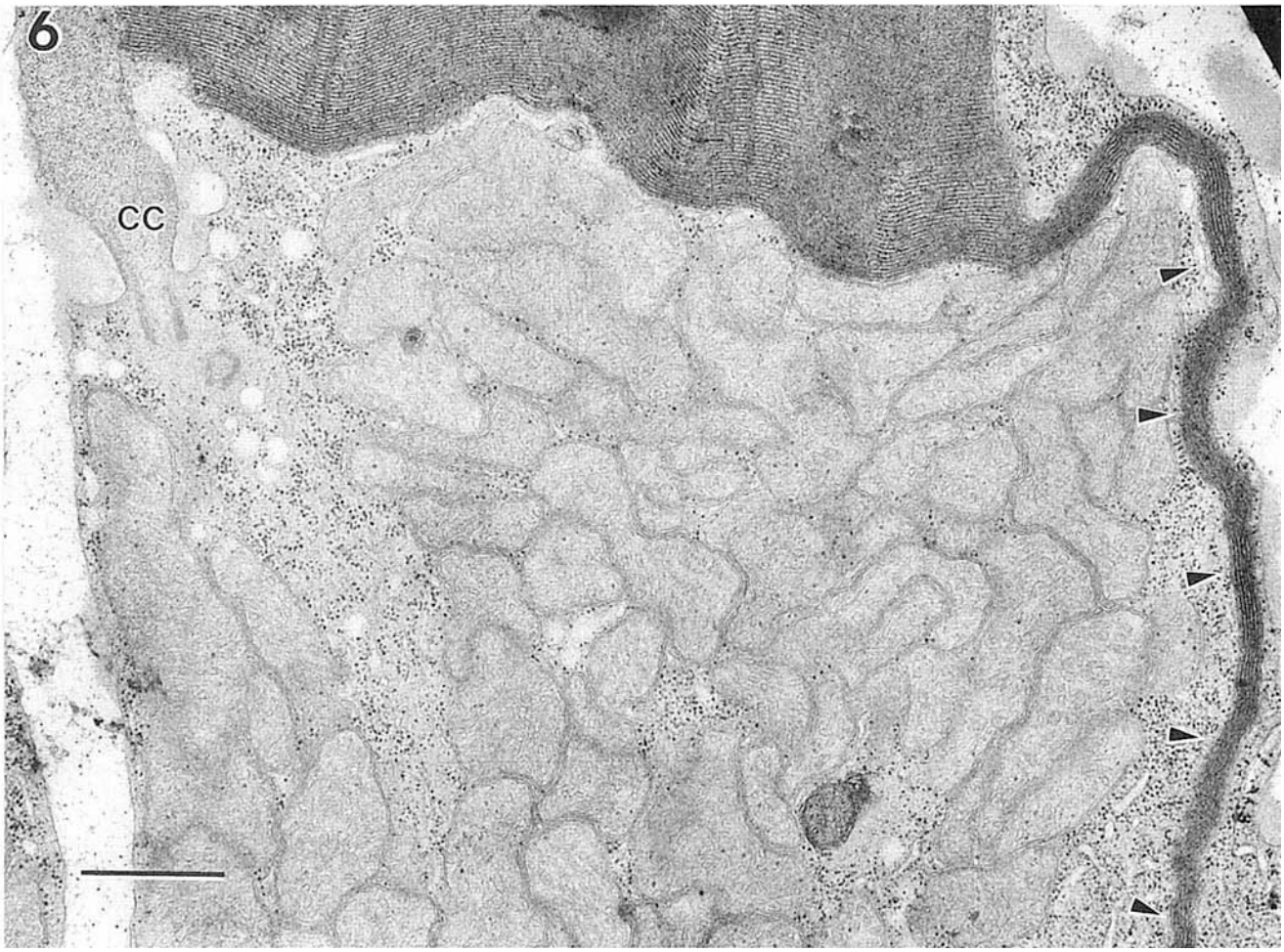

Fig. 6. Electron micrograph of part of a rod photoreceptor in a retina incubated for 10 hours in the presence of 25 μM cytochalasin D. Overgrown new disks have burrowed (arrowheads) into the inner segment. CC, connecting cilium. Scale bar = 1.0 μm.

^{3}H-leucine labeling

Rod outer segments in control retinae all possessed a band of radiolabel at their bases (Fig. 14a), indicating that their nascent disks had incorporated ^{3}H-leucine and must have been assembled in vitro (see Young '67). In cytochalasin D-treated retinae, radiolabel was less concentrated in a band at the rod outer segment bases; it was more evident along the sides of many inner segments and the occasional outer segment (Fig. 14b). By refocusing the microscope, "threads" that stained with the same intensity as the outer segments—indicating that they represented overgrown disks—could usually be discerned beneath the label along the sides of the inner segments. Thus it appears that the overgrown disks contained ^{3}H-leucine.

DISCUSSION

As expected, cytochalasin D disrupted most of the network of F-actin. In association with this disruption of actin filaments, the nascent disks grew excessively, extending well beyond the normal margin of the outer segment. Only the regulation of nascent disk growth seemed to be perturbed, however. The overgrown disks were labeled with ^{3}H-leucine, indicating that membrane containing newly synthesized protein was still transported and added to the ciliary plasma membrane, despite the disruption of F-actin. The amount of this new protein incorporated into the nascent disks was probably also unaffected. The rate of incorporation of radiolabeled amino acid into rhodopsin was not found to be affected significantly when bovine retinae were incubated in 21 μM cytochalasin B for 3 hours (Dr. Paul O'Brien, personal communication). From our autoradiographs, we have no evidence to suggest that there was less radiolabel incorporated into new disk membrane in the cytochalasin-treated frog retinae. The different labeling pattern in the cytochalasin-treated rods could be explained by the spread out of label in a few overgrown disks, rather

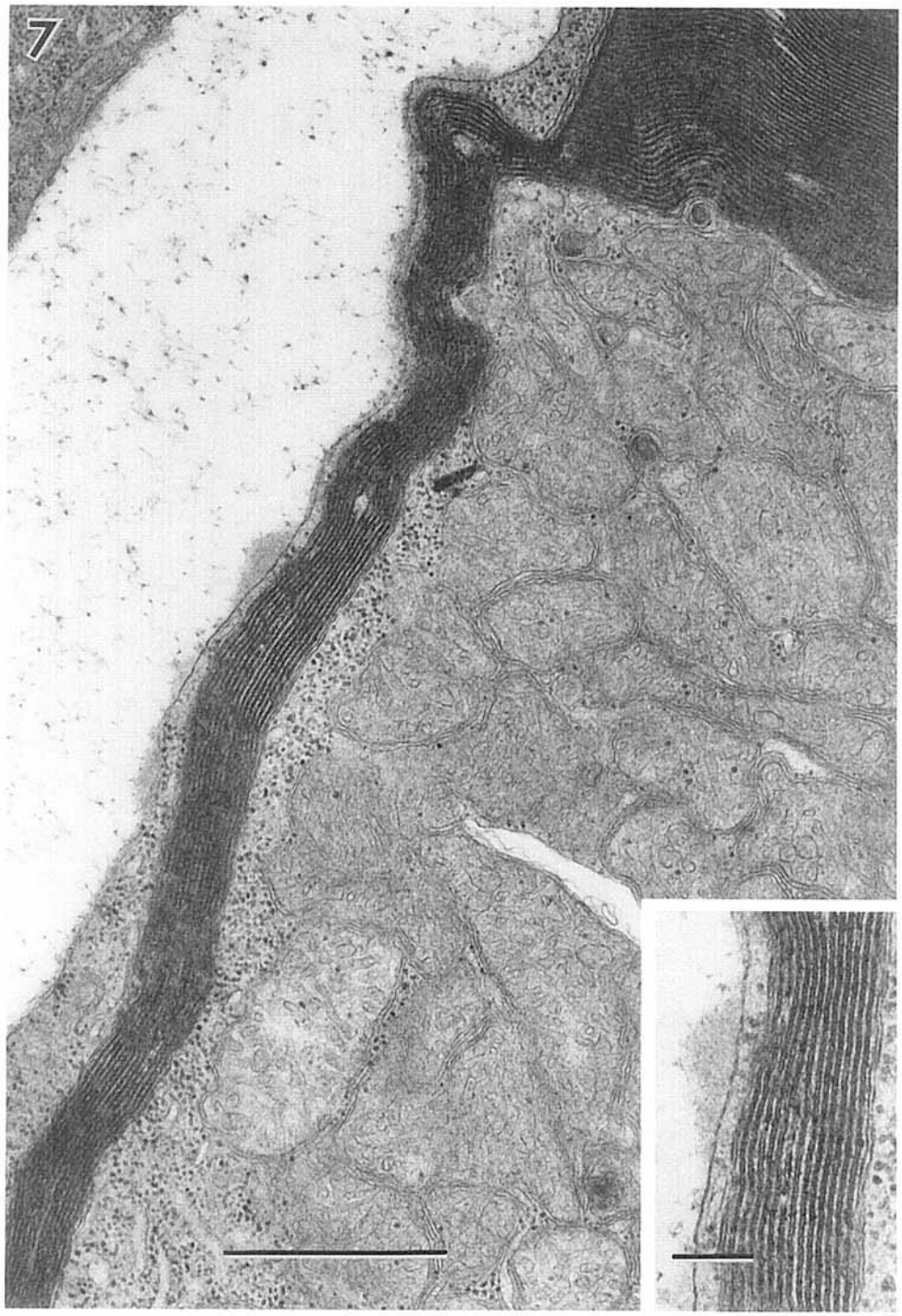

Fig. 7. Electron micrograph of part of a rod photoreceptor in a retina incubated for 10 hours in the presence of 25 μM cytochalasin D. The new disks have grown beyond the margin of the outer segment and appear to have burrowed into the inner segment. Scale bar = 1.0 μm. Inset: Higher magnification of the overgrown disks, which are still extracellular. Scale bar = 0.2 μm.

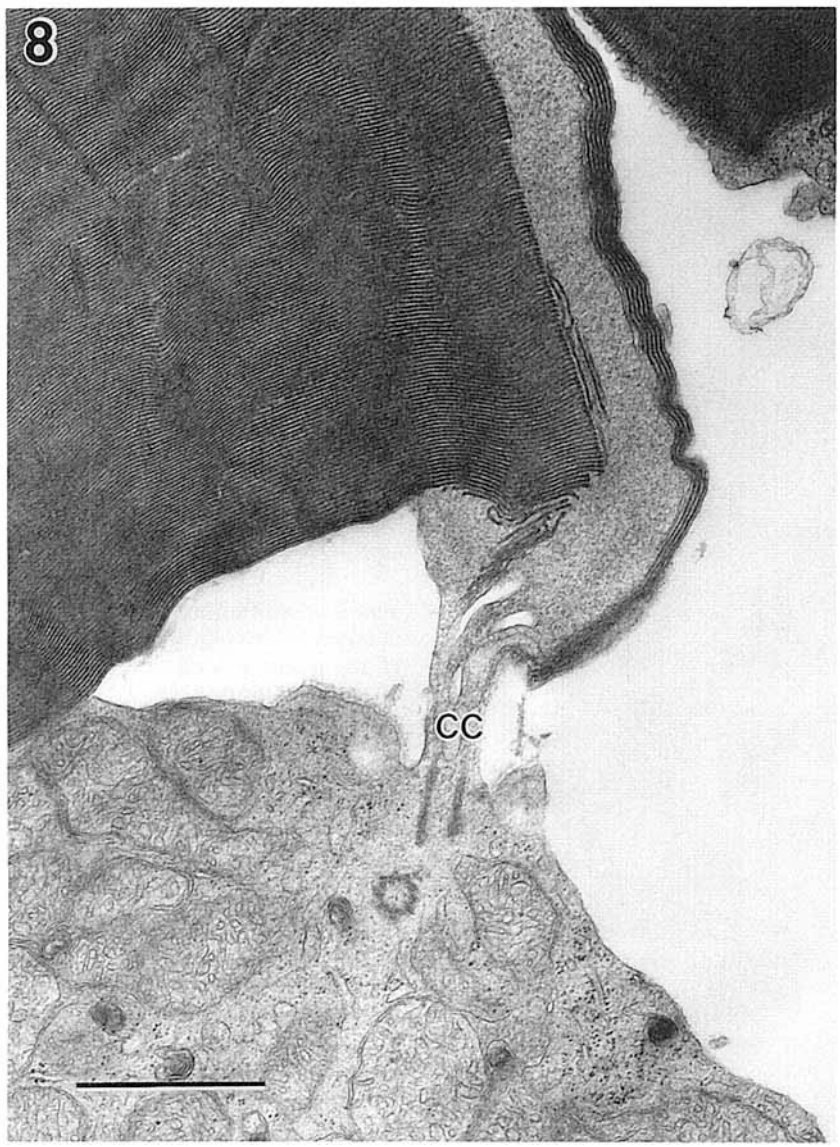

Fig. 8. Electron micrograph of part of a rod photoreceptor in a retina incubated for 10 hours in the presence of 5 μM cytochalasin D. Overgrown new disks are evident distally alongside the connecting cilium (CC) and outer segment. Scale bar = 1.0 μm.

than in a deeper layer of many normal-size disks compacted at the base of each outer segment.

Support for disk morphogenesis by evaginations of the plasma membrane

Our observation of overgrown disks provides the first experimental support for the hypothesis that disk morphogenesis occurs by evaginations of the ciliary plasma membrane (Steinberg et al., '80), and not by invaginations, as was proposed by earlier microscopists (e.g., Sjostrand, '61; Nilsson, '64). If the disks developed by invaginations, then overgrowing nascent disks would have to push out the plasma membrane of the opposing side of the outer segment, and would thus be surrounded by it. By examining the cone disks, which remain open, it can be seen unequivocally that they are not surrounded by an extra membrane; the only membranes apparent are those of the overgrown open disks themselves (Fig. 12b inset).

Continued evagination after disruption of actin-filaments

We had considered the possibility that new disks might not form at all in the presence of cytochalasin D, since

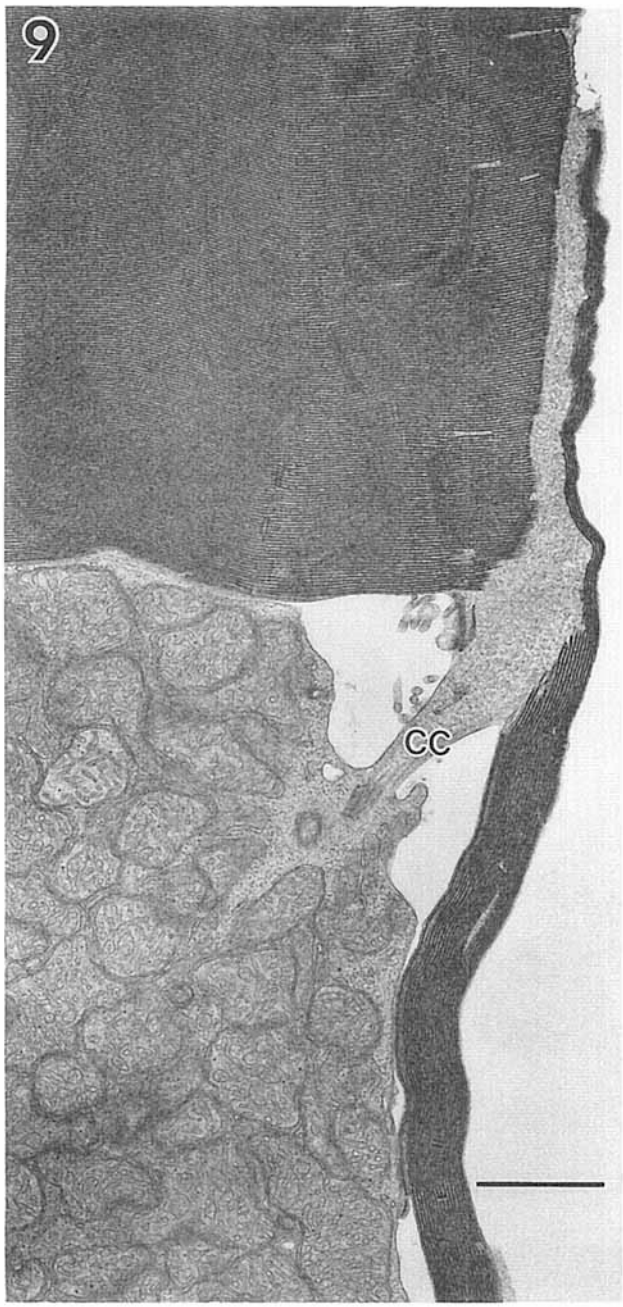

Fig. 9. Electron micrograph of part of a rod photoreceptor in a retina incubated for 28 hours in the presence of 25 μM cytochalasin D. Overgrown nascent disks are seen adjacent to the connecting cilium (CC) and alongside both the inner and outer segments. Scale bar = 1.0 μm.

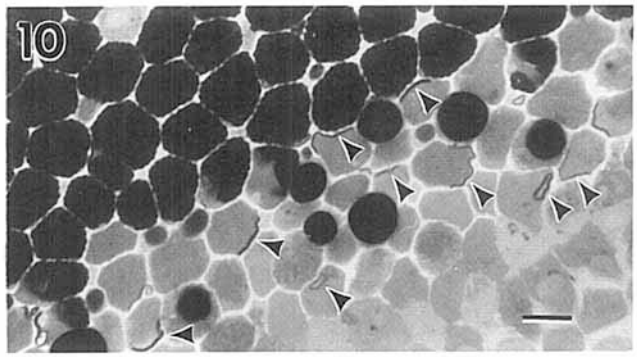

Fig. 10. Light micrograph of oblique section through rod outer segments (upper left) and inner segments (diagonally, upper right to lower left) of a retina incubated for 8 hours with 5 μM cytochalasin D. The rod outer segment disks have stained more densely (with Toluidine Blue). Overgrown nascent disks that extend proximally around their lighter staining inner segments are therefore manifest (e.g., arrowheads). Scale bar = 10 μm.

outgrowth of the plasma membrane in various other types of cells are inhibited by cytochalasin. It is clear, however, that at least the continued growth of new disks was not inhibited, so that this process does not seem to be dependent on actin filaments. It is notable that each new disk grows out in apposition with a more mature disk, distal (sclerad) to itself. Moreover, in nearly all cases (there were a number of exceptions among the cones), the overgrowing disks followed the margins of the inner or outer segment once beyond the size of the more distal normal-size disks. These observations support the idea of an interaction between a nascent disk and extant plasma membrane. Thus, perhaps, rather than employing actin filaments, new disk growth is directed by an interaction with its more mature neighbor, which acts as a template. The possibility of such a mechanism involving sugar residues is supported by the observation that tunicamycin (which blocks N-linked glycosylation) causes nascent rod disks to develop into a disordered array of tubules and vesicles (Fliesler et al., '85). However, a mechanism in which the disk rim provides the template for morphogenesis, as speculated by Corless and Fetter ('87), seems unlikely in view of the neatly-apposed, overgrown disks observed in the present study.

Perturbation of the normal morphogenetic process

Overgrowth of the nascent disks (summarized in Fig. 15) indicates that F-actin is involved in regulating disk morphogenesis, but is not required for the continued evagination. The overgrowth was characterized by the excessive growth of just a few disks. These disks may have been initiated before exposure to cytochalasin. They appeared to grow at the expense of newer, more basal evaginations. There are two possible, not necessarily exclusive, explanations for the effect of cytochalasin: 1) the mechanism that initiates a new evagination was disrupted, so that new membrane added to the ciliary plasma membrane was channelled into nascent disks that were already initiated, perhaps overriding a mechanism that normally might have signaled the disks to stop growing, and 2) the mechanism limiting how far nascent disks grow was affected, so that the few existing new disks grew beyond their normal size, leaving no membrane available for the initiation of further disks.

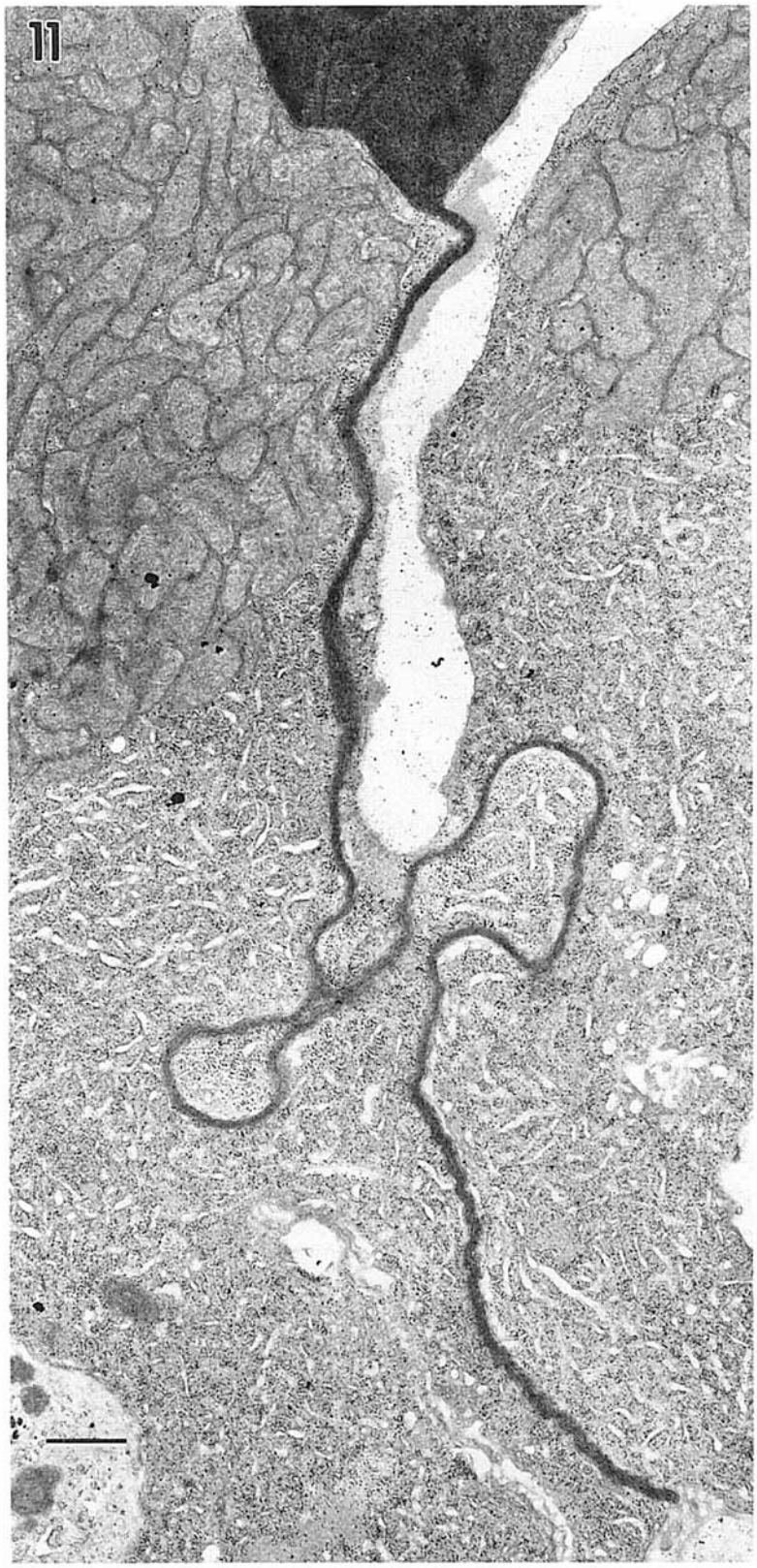

Fig. 11. Electron micrograph of part of a rod photoreceptor in a retina incubated for 10 hours in the presence of 25 μM cytochalasin D, illustrating an extreme example of excessive growth of the nascent disks. Scale bar = 1.0 μm.

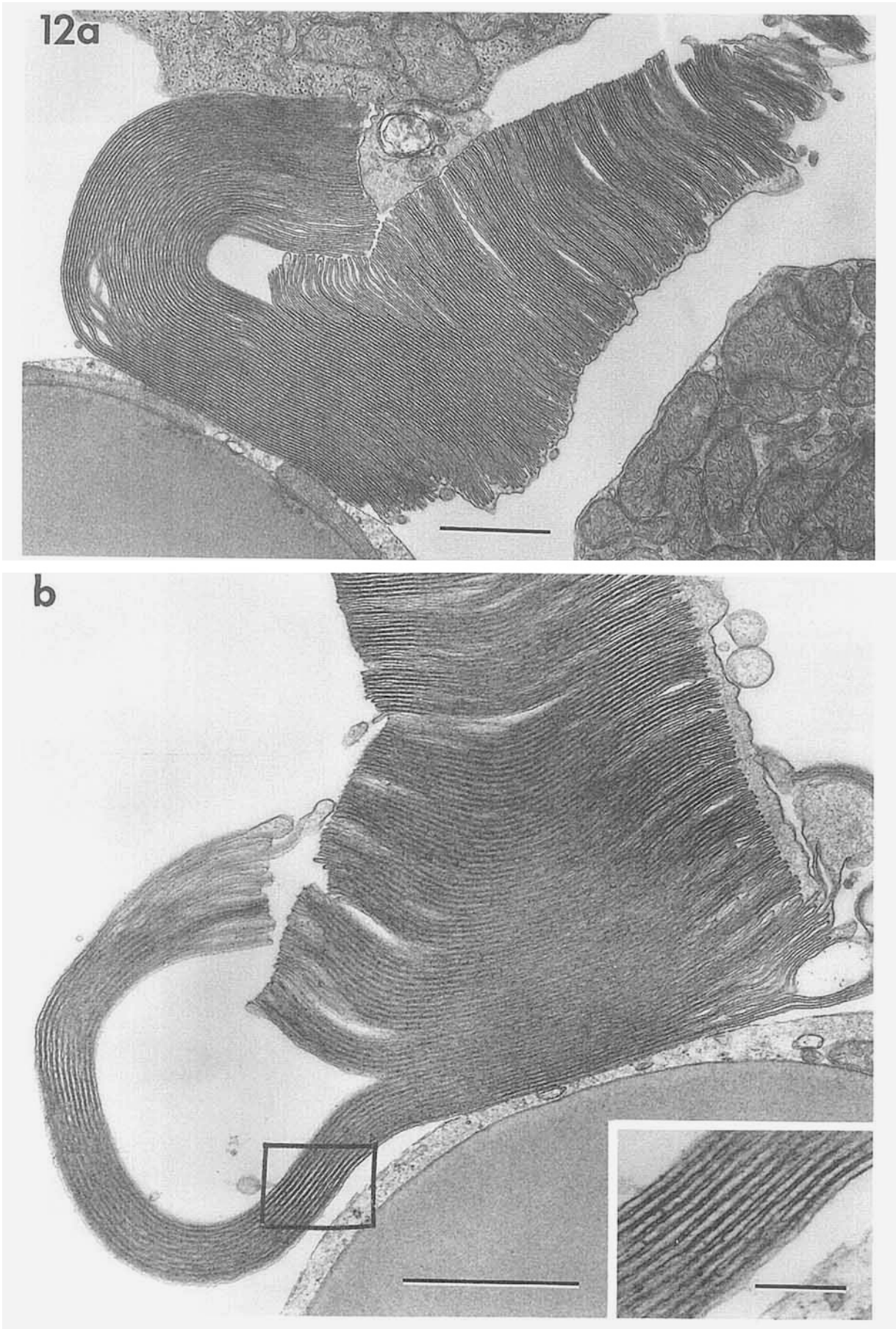

Figure 12

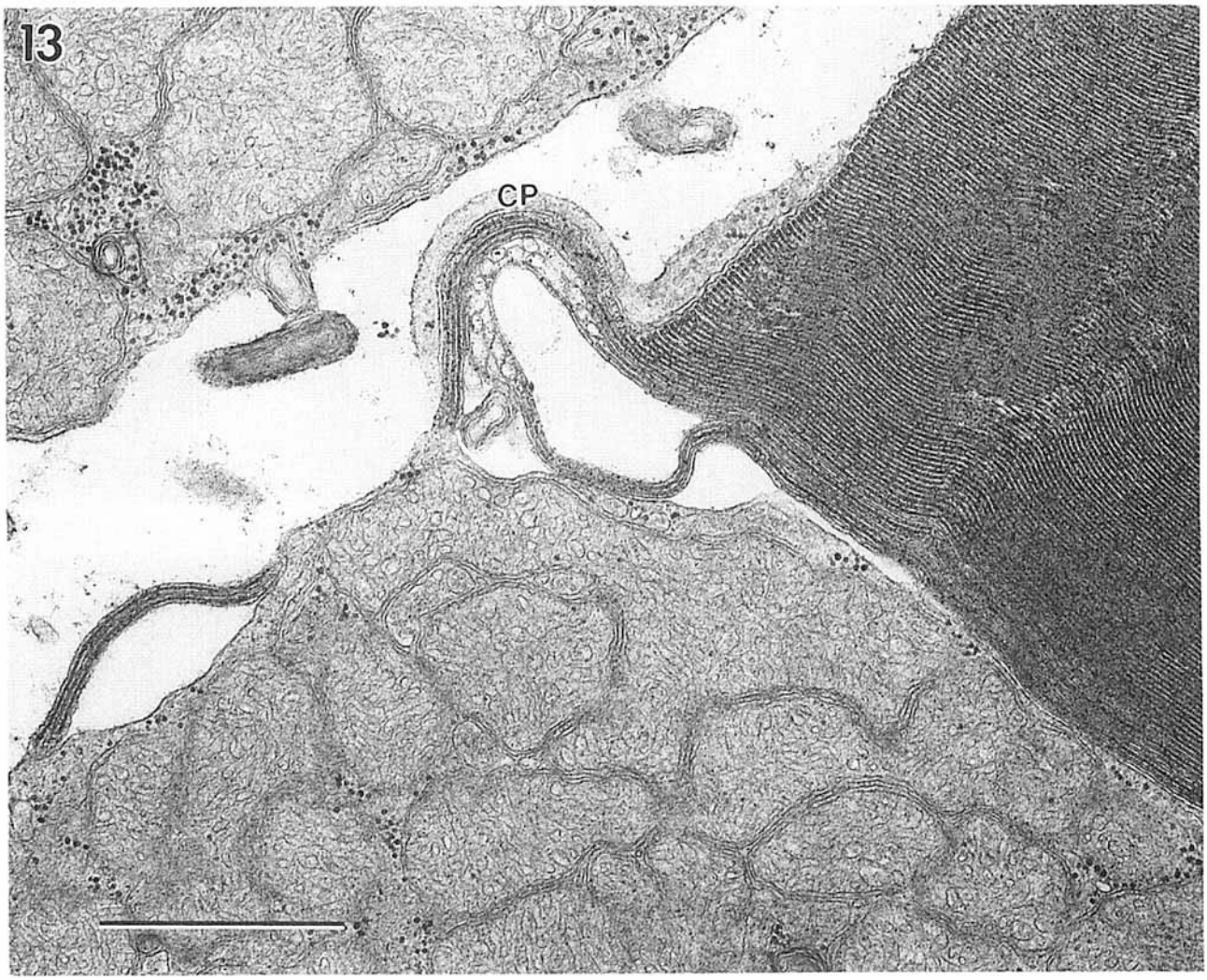

Fig. 13. Electron micrograph of part of a rod photoreceptor in a retina incubated for 6 hours with 5 μM cytochalasin D. The disks have overgrown and the calycal process (CP) is distorted. Scale bar = 1.0 μm.

Support for the first explanation comes from the finding of a concentration of actin (Chaitin et al., '84), which we have shown to contain F-actin (Fig. 1c; Vaughan and Fisher, '87), in the ciliary region at the base of the outer segment. Even if this actin plays no role in the continued outgrowth of the membrane during disk formation, it might still be important for the initiation of an evagination of the ciliary plasma membrane.

Support for the second explanation comes from examination of the calycal processes, which infers a role for them in regulating nascent disk growth. Cytochalasin induced the calycal processes, which are normally filled with actin filaments, to become disordered and collapse (Figs. 4,13). Perhaps the ensuing loss of the structural framework around the bases of the photoreceptor outer segments effectively eliminated the means by which the growth of the nascent disks is normally terminated.

Fig. 12. Electron micrographs of parts of cone photoreceptors from different retinae incubated for 26 hours with 5 μM cytochalasin D. Scale bars = 1.0 μm. Inset of (**b**): Higher magnification of area marked in (b) showing the disk membranes, which are not surrounded by any other membrane. The intradiskal space is considerably greater than the space between adjacent disks. Scale bar = 0.2 μm.

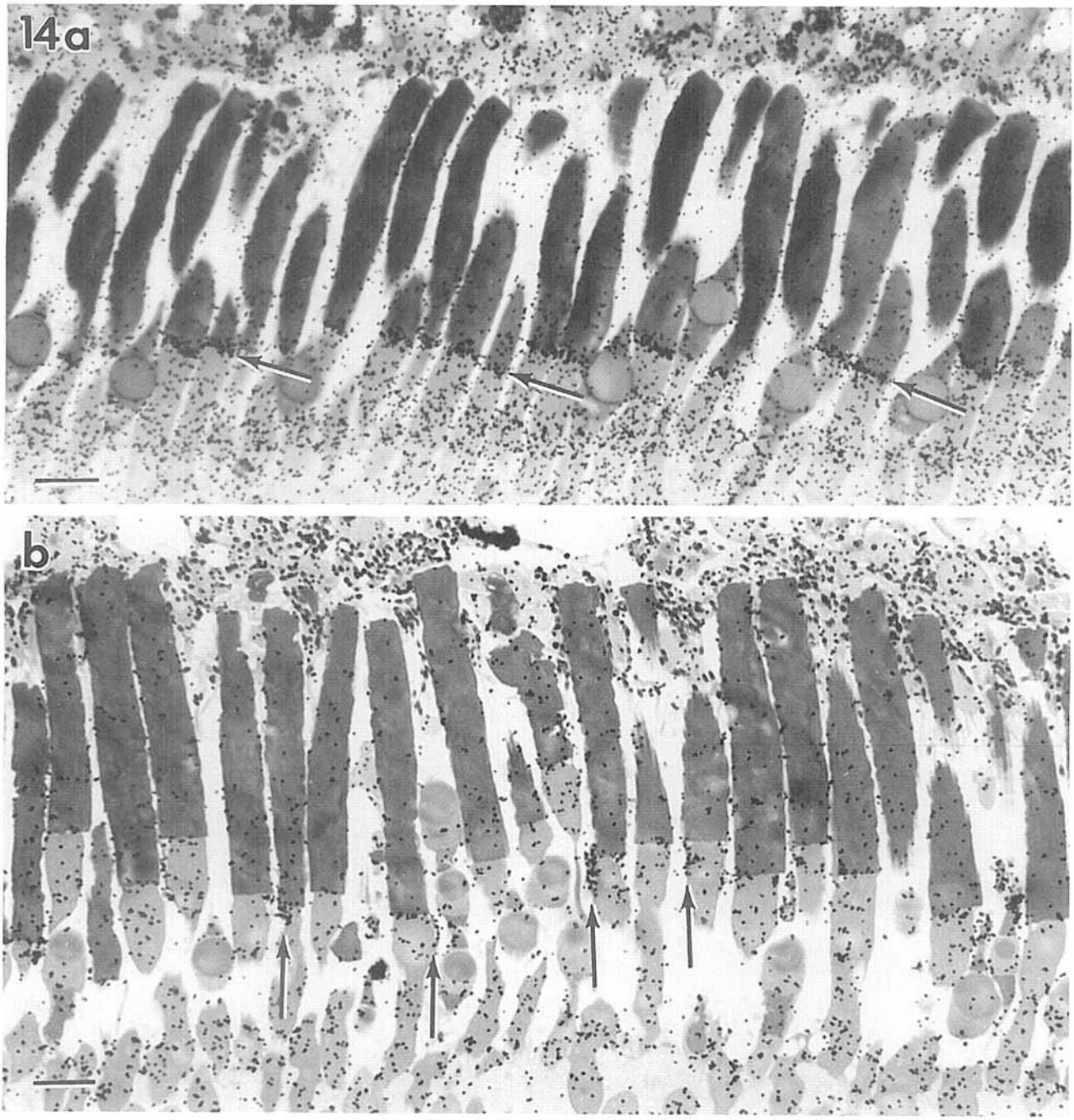

Fig. 14. Light microscopical autoradiographs of the photoreceptor layer from retinae incubated for 10 hours in control medium (**a**) and medium containing 25 μM cytochalasin D (**b**). In (a), a band of label is evident at the bases of the rod outer segments (e.g., arrows). In (b), label is less concentrated here; instead it appears more concentrated along the sides of some rod inner segments (e.g., arrows). The contractile myoid regions of the cytochalasin-treated photoreceptors have elongated because the actin filaments have also been disrupted in this region (see O'Connor and Burnside '82). Scale bar = 10 μm.

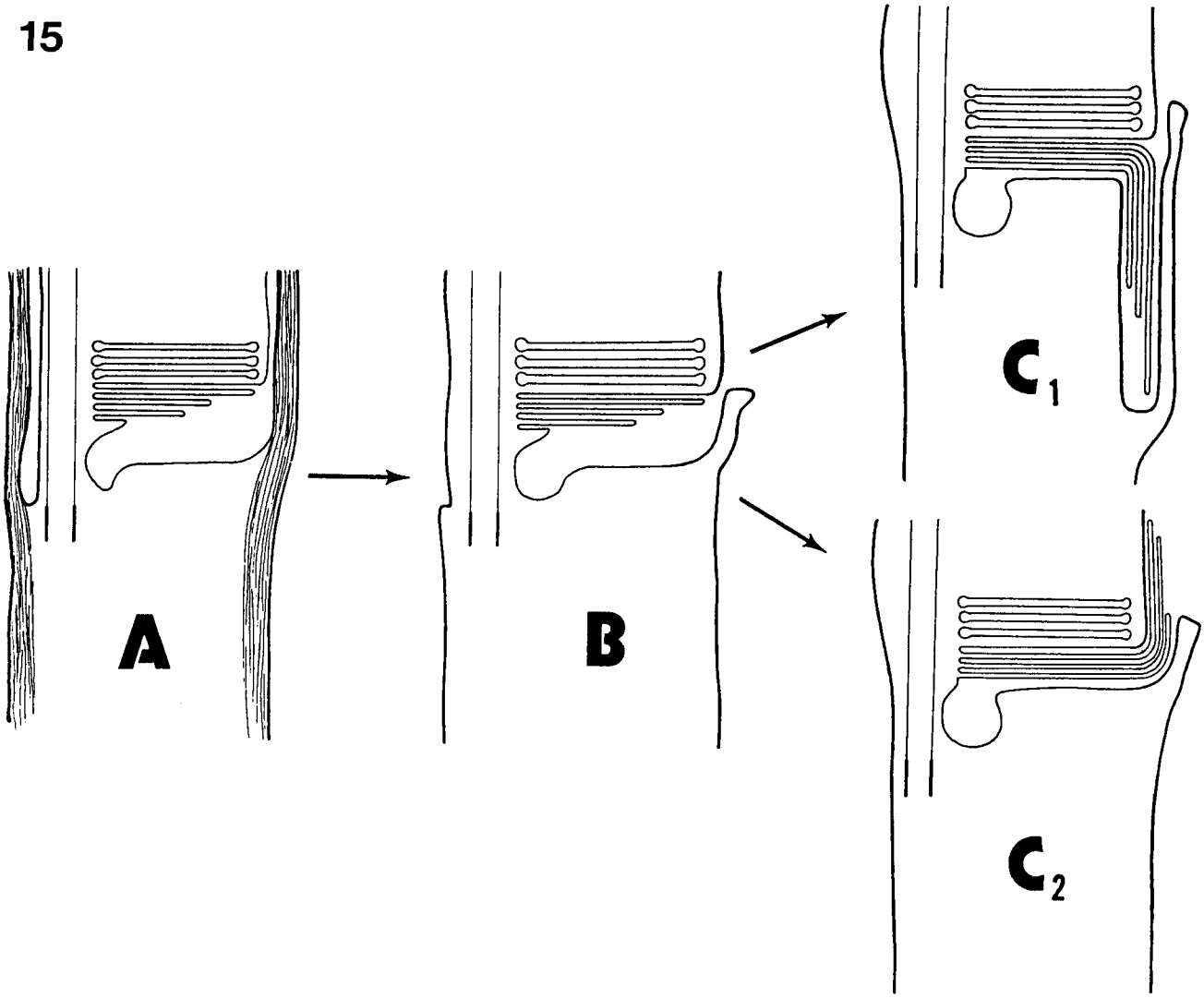

Fig. 15. Summary of the observed effect of cytochalasin D on disk morphogenesis in a rod photoreceptor. **A.** Before exposure to cytochalasin (normal). **B.** Disruption of microfilaments and collapse of calycal processes. **C.** Overgrowth of nascent disks, either proximally (C_1) or distally (C_2), without the initiation of further nascent disks. Note that in normal photoreceptors F-actin was also detected by rhodamine-phalloidin in the distal connecting cilium (Fig. 2c). However, microfilaments were not observed in this region with electron microscopy, so that the organization of this F-actin is not known, and thus not shown in panel A.

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