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The Distribution of F-actin in Cells Isolated from Vertebrate Retinas

DANA K. VAUGHAN* AND STEVEN K. FISHER

IES-Neuroscience Research Program and Department of Biological Sciences, University of California, Santa Barbara, CA 93106, U.S.A.

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Filamentous actin has been localized in isolated retinal neurons and glia using fluorescent phallotoxin. Photoreceptors, bipolar cells, horizontal cells, amacrine cells and Müller (glial) cells were isolated by gentle enzymatic digestion of frog, lizard, rabbit, rat and cat retinas. The cells were then fixed in paraformaldehyde and stained with rhodamine–phalloidin. The patterns of fluorescence recorded were specific for each cell type. All interneurons had spots of bright fluorescence along dendrites and (or) axon terminals, probably corresponding to synaptic sites. Horizontal cells and Müller cells had a continuous subplasmalemmal layer of fluorescence throughout; this layer was also present in bipolar cells, but only in the region of cytoplasm at the base of the dendrites. Müller cells also had bright fluorescence in their apical microvilli and terminal web and associated with the *zonulae adhaerentes* junctions between Müller cells and photoreceptors. All photoreceptors exhibited fluorescence in their synaptic terminals, in a ring just sclerad to the nucleus (corresponding to *zonulae adhaerentes* junctions formed with Müller cells), and in cables running longitudinally in the inner segment. Frog photoreceptors also had fluorescent calycal processes alongside their outer segments. Rods from mammals and *Xenopus* had distinct spots of fluorescence at the outer segment base in a region that suggests involvement with morphogenesis of new outer-segment disc membrane.

Key words: actin; cytoskeleton; neuron; photoreceptor; retina.

1. Introduction

Filamentous actin (f-actin) has been shown to play important roles in cell motility, regulation of cell shape, and intracellular transport in a wide variety of non-muscle cell types (Stossel, 1984). Indeed, actin can comprise as much as 10% of total protein in certain non-muscle cells (Clarke and Spudich, 1977).

We have used phalloidin to localize f-actin in retinal cells of various vertebrate species. Phalloidin binds only to the filamentous form of actin, so when conjugated to a fluorochrome, this mushroom-derived toxin can be used to localize f-actin at the light-microscope level (Wulf, Deboben, Bautz, Faulstich and Wieland, 1979).

When fluorescent phalloidin is used to label f-actin in cryosections of vertebrate retina, specific patterns of fluorescence are seen in the various cell layers (Drenckhahn and Kaiser, 1983; Vaughan and Fisher, 1985; Drenckhahn and Wagner, 1985). These patterns are difficult to interpret when the goal is to ascertain the subcellular distribution of f-actin. Localization of actin by immunoelectron microscopy is complicated by the facts that only thin sections of tissue are observed and that antibodies to actin label both the monomeric (globular or g-actin) and filamentous forms of actin in the cell.

In this paper, we describe the different cell types isolated from mammalian and amphibian retinas using our method of tissue dissociation, and the patterns of f-actin distribution in whole retinal cells stained with rhodamine–phalloidin.

* To whom correspondence should be addressed.

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2. Materials and Methods

Mammals used in this study were deeply anaesthetized with an intramuscular injection of Rompun and Ketamine and then killed with Nembutal administered by intracardiac puncture. Reptiles and amphibians were decapitated and pithed. Retinas from rabbits, rats, cats and *Xenopus laevis* were dissected in the light; those from *Rana pipiens* and *Sceloporus occidentalis* (the Western fence lizard) were dissected under dim red light after dark adaptation. Retinas were incubated in the appropriate Ca^{2+} and Mg^{2+}-free balanced salt solution (BSS; pH 7·2) supplemented with Nagarse (Sigma) as follows: 0·5 mg ml^{-1}enzyme for 30 min (rabbit, cat and *Xenopus*) or 90 min (*Rana* and *Sceloporus*) or 0·1 mg ml^{-1} enzyme for 15 min (rat). After enzymatic treatment, the retinas were rinsed in BSS containing Ca^{2+} and Mg^{2+} (pH 7·2) containing 0·5 mg ml^{-1} bovine serum albumin (BSA) and then transferred to 2 ml of normal BSS containing 0·5 mg ml^{-1}BSA and 0·1 mg ml^{-1}DNAse I (Sigma). In this small volume, several gentle suctions with a wide-bore pipette were sufficient to separate the cells mechanically. After allowing a few minutes for the settling of undissociated tissue, the cells in suspension were withdrawn and fixed in phosphate-buffered 1% paraformaldehyde (pH 7·2) for 30 min.

Fixed single cells were dried onto polylysine-coated or clean glass slides and then rinsed briefly in phosphate-buffered saline (PBS, pH 7·2). The rinsed cells were incubated in rhodamine-conjugated phalloidin (Molecular Probes, Inc.) diluted 1:10 in PBS plus 0·3% Triton X-100 for 15-20 min at room temperature; as controls for autofluorescence, some cells were incubated in PBS plus Triton X-100 only. Following the incubation, all cells were rinsed twice in PBS, coverslipped, and then sealed in a mounting medium of glycerol plus 5% *n*-propyl gallate to retard photobleaching (Giloh and Sedat, 1982). The stained cells were photographed with a Zeiss Photomicroscope III equipped for epifluorescence. Controls were photographed with exposures equal to those used for experimentals.

3. Results

Isolated cells

Figure 1 shows phase-contrast light micrographs of different types of cells obtained from dissociated rabbit retinas. The morphology of these cells is remarkably well preserved after the dissociation procedure, so that the cell types are readily identifiable from their close resemblance to images derived from Golgi impregnation studies (Ramón y Cajal, 1933; Hughes, 1971). The cell types included: bipolar cells (Fig. 1a); amacrine cells (Fig. 1b); both types of horizontal cells (Type A or axonless, as seen in Fig. 1e, and Type B or axon-bearing; Fisher and Boycott, 1974); the broken axon terminals of B-type horizontal cells (Fig. 1d); photoreceptors (Fig. 1c); and Müller cells (Fig. 1f). Bipolar cells, photoreceptors and Müller cells were easily identified by their distinctive morphologies. A-type horizontal cells were large cells with stout dendrites which bear finer processes ending in aggregates of terminals. B-type horizontal cells appeared similar to the A-type cells, but with slightly finer dendrites and often part of a long axon attached. Axon terminals of the B-type cells were very distinctive, large, tangled structures, usually attached to a short length of axon. When dissociated cells were stained with DAPI (a nucleic acid-specific fluorescent dye), these structures did not stain, whereas all other cell types showed specific nuclear staining. Amacrine cells were of diverse morphology, generally smaller than the horizontal cells and bearing fine processes and no axon.

We often obtained similar examples of the same cell types from rat and cat retinas, but the procedure yielded only photoreceptors from frog and lizard retinas. In all species, cone outer segments were usually absent or not well preserved.

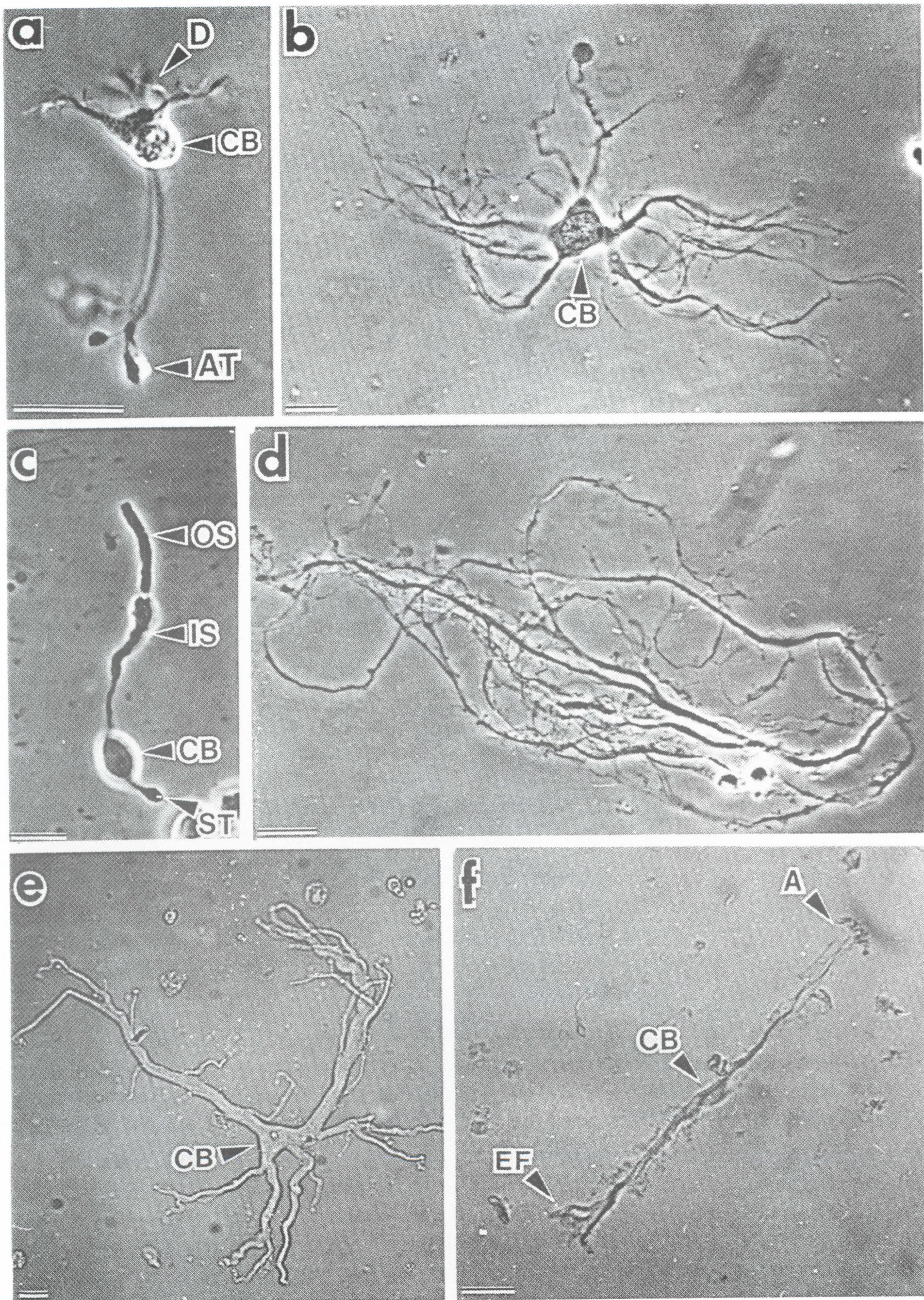

FIG. 1. Types of cells obtained from dissociated rabbit retina (all are phase-contrast images). Scale bars = 10 μm. a, bipolar cell; b, amacrine cell; c, rod photoreceptor; d, axon terminal arborization of axon-bearing horizontal cell (B-type); e, axonless horizontal cell (A-type); f, Müller (glial) cell.

Abbreviations: D = dendrites; CB = cell body; AT = axon terminal; OS = outer segment; IS = inner segment; ST = synaptic terminal; EF = endfoot; A = apex.

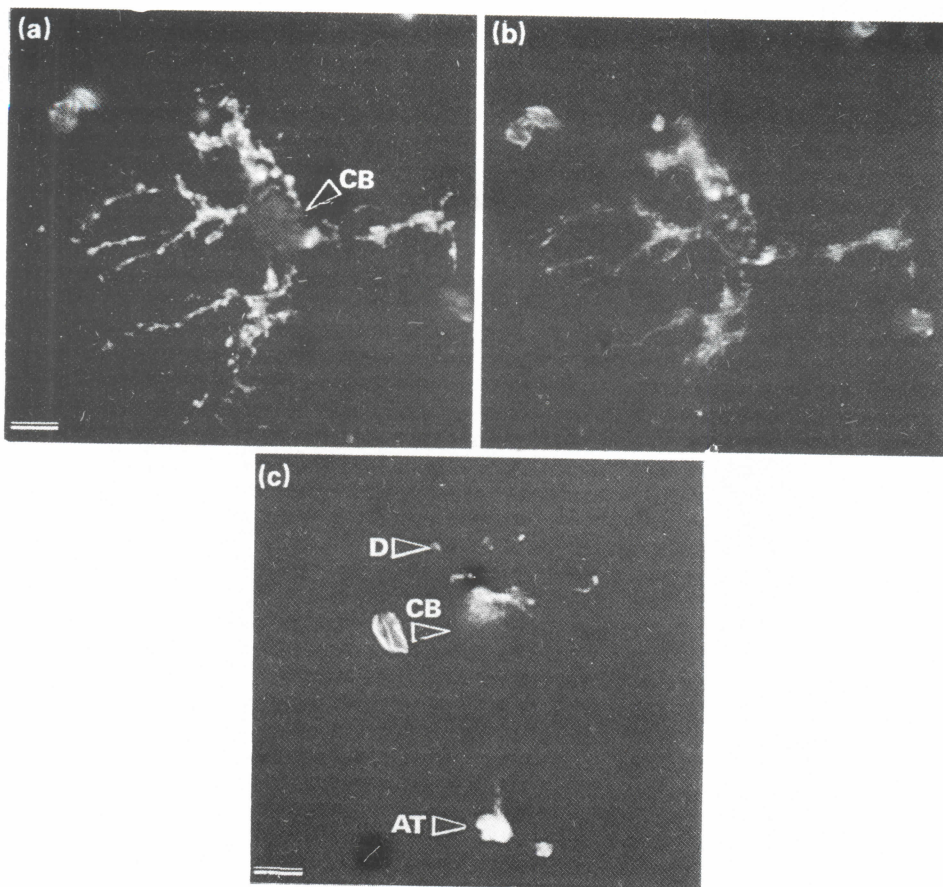

Fig. 2. Cells isolated from rabbit retina and stained with rhodamine–phalloidin (all are fluorescence images). Scale bars = 10μm. a–b, Amacrine cell photographed in two focal planes. a, Fluorescence is spotty along cell processes and, as seen in b, on cell body (CB) itself. c, Bipolar cell. Orientation of cell is identical to that in Fig. 1a. Fluorescence is spotty in dendrites (D) and bright in axon terminal (AT). Some cytoplasmic fluorescence caps the cell body (CB) just beneath the dendrites.

Rhodamine-phalloidin staining of interneurons and Müller cells

Control cells of all types did not fluoresce. Cells exposed to rhodamine–phalloidin showed two general patterns of fluorescence: discrete spots or patches, and (or) a layer underlying the plasma membrane and outlining the whole cell. Amacrine cells had spots of fluorescence on both their neurites (Fig. 2a) and somata (Fig. 2b). Bipolar cells had a small patch of subplasmalemmal fluorescence capping the apical portion of the soma, just under the dendrites and, in addition, bright spots of fluorescence at the ends of their dendrites and axon terminals (Fig. 2c). Horizontal cell somata (Fig. 3a) and the axon terminals of the B-type cells (Fig. 3b) exhibited distinct subplasmalemmal fluorescence so that the whole cell (or terminal) fluoresced brightly. Superimposed on this pattern were bright spots that were particularly evident on the dendrites and fine processes of the axon terminal.

The fluorescence pattern of Müller cells was the same in all species. Each cell had subplasmalemmal fluorescence outlining the whole cell (Fig. 3c), with the brightest fluorescence in the apical microvilli and within the cytoplasm immediately beneath

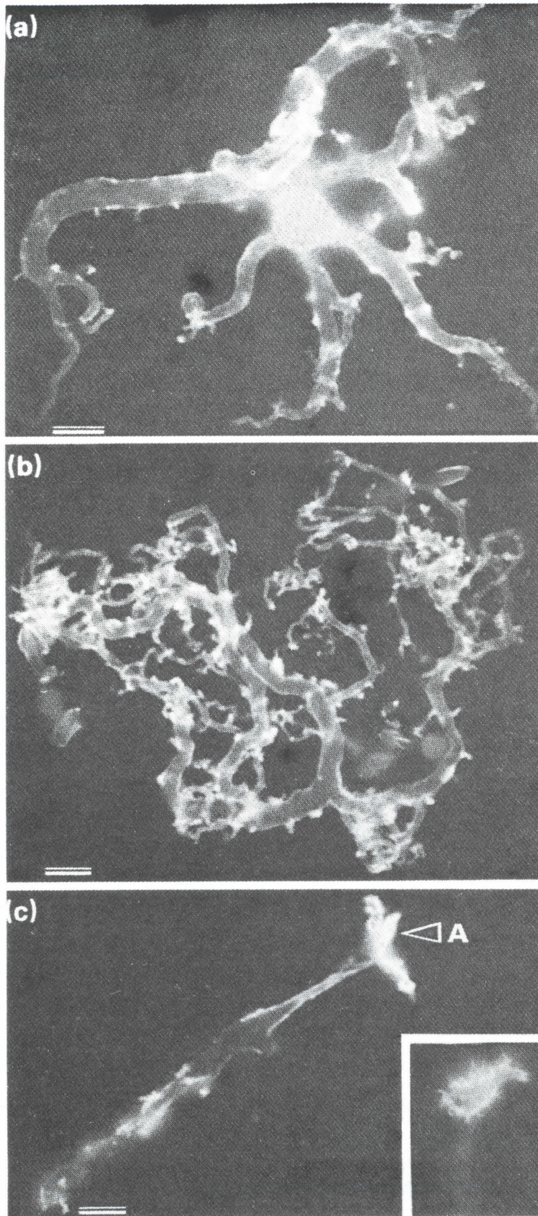

Fig. 3. Cells isolated from rabbit retina and stained with rhodamine–phalloidin (all are fluorescence images). Scale bars = 10 μm. a, Axonless horizontal cell. Bright spots of fluorescence are seen along processes in addition to a cortical layer of cytoplasmic fluorescence. b, Axon terminal arborization of axon-bearing (B-type) horizontal cell. Bright spots of fluorescence are found all along processes; cortical cytoplasmic fluorescence is best seen in thickest processes. c, Müller cell. Orientation of cell is identical to that in Fig. 1f. Fluorescence is bright in lateral processes off cell trunk near endfoot and bright at cell apex (A). Subplasmalemmal fluorescence is also apparent in this plane of focus. Inset: Müller cell apex; high magnification reveals the fluorescence of the apical 'basket' of microvilli.

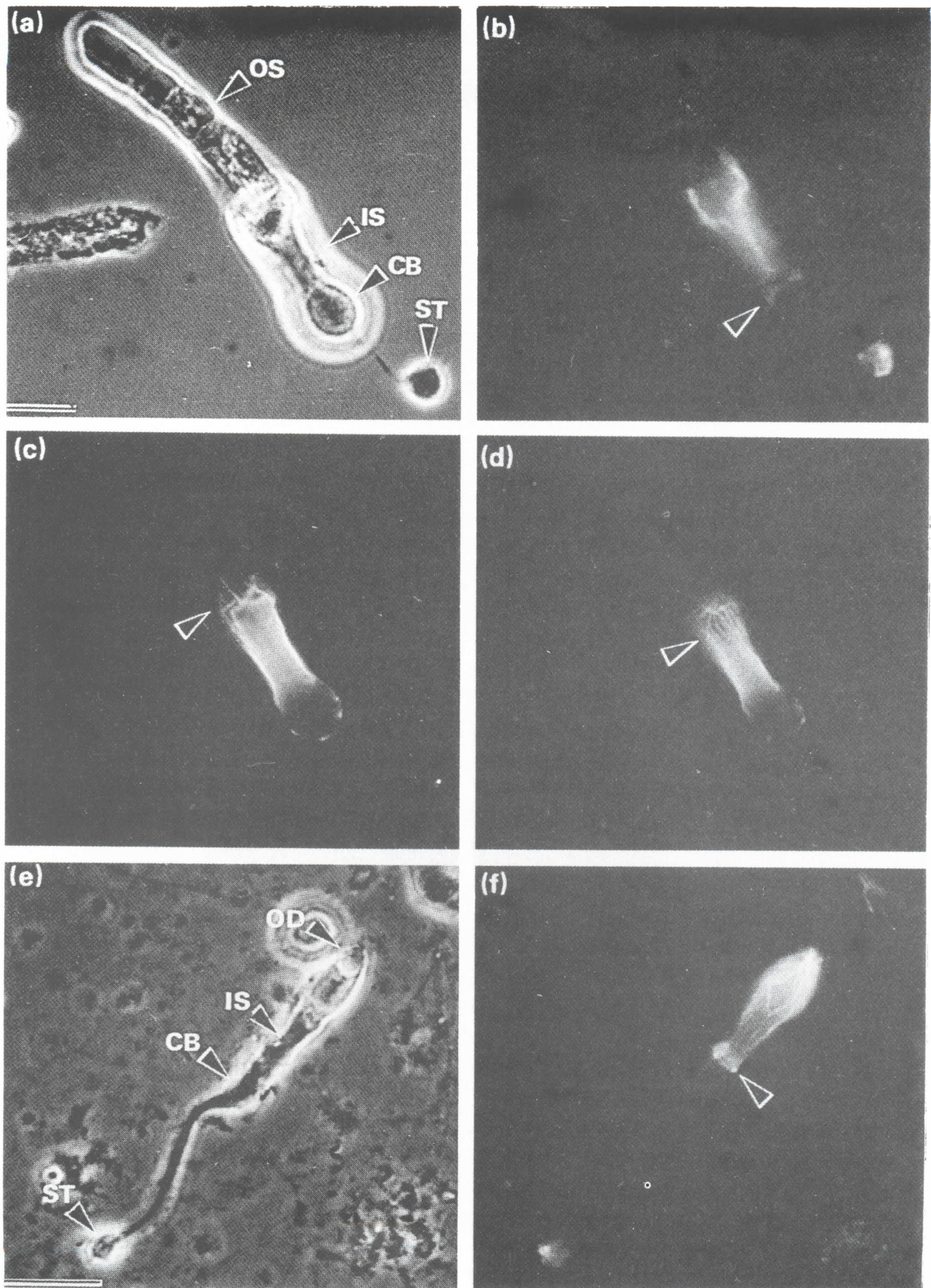

Fig. 4. Isolated photoreceptors from frog (*Rana pipiens*) and lizard retinas stained with rhodamine-phalloidin. Scale bars = 10μm.

a–d, *Rana pipiens* rod. a, Phase image of cell; b–d, fluorescence images of cell through three planes of focus; b, synaptic terminal, collar (arrow) and IS fluoresce; c, short calycal processes fluoresce (arrow), as does IS; d, IS fluorescence can be resolved into cables (arrow).

e–f, *Sceloporus occidentalis* (lizard) cone. e, Phase image of cell; OS is missing; oil droplet (OD) is at distal end of IS; f, fluorescence image of cell. Synaptic terminal, collar (arrow) and IS cables fluoresce.

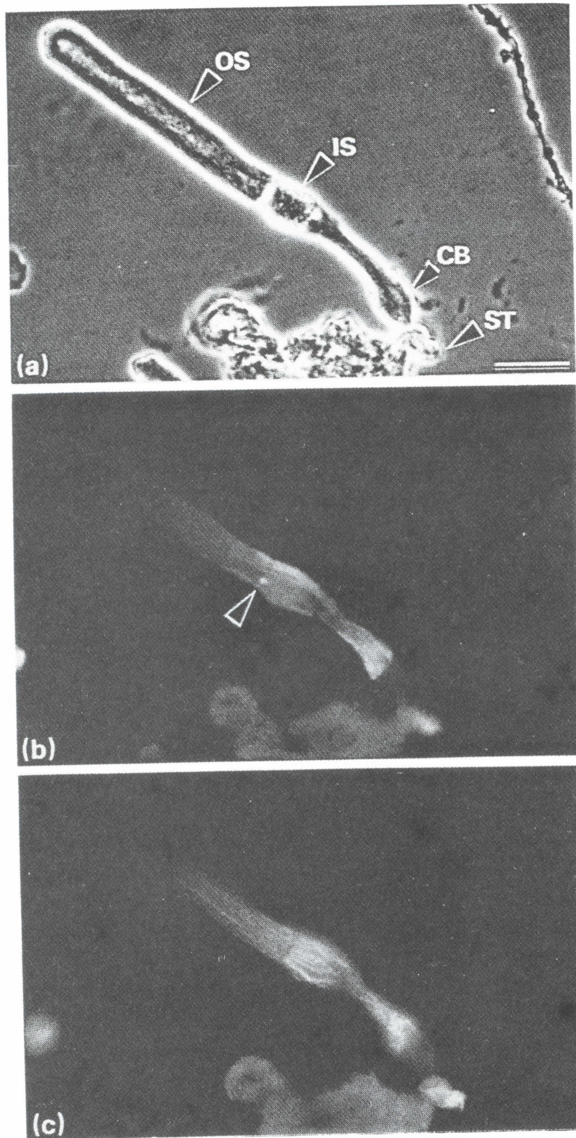

Fig. 5. Isolated rod photoreceptor from frog (*Xenopus laevis*) retina stained with rhodamine–phalloidin. Scale bar = 10 μm. a, Phase image of cell. b–c, Fluorescence images of cell in two planes of focus. b, Fluorescence is seen in synaptic terminal, collar and IS; a bright spot of fluorescence is also visible (arrow) in an area between the IS and the OS; c, fluorescence is seen in synaptic terminal, IS cables, and very fine, tapering streaks along the OS.

them (inset, Fig. 3c). The fine processes extending laterally off the soma also fluoresced.

Rhodamine-phalloidin staining of photoreceptors

In all of the species examined, rods and cones showed virtually the same pattern of staining (Figs 4, 5 and 6): (1) fluorescence in the synaptic terminal; (2) no fluorescence in the axon connecting the soma to the synaptic terminal; (3) a 'collar' of

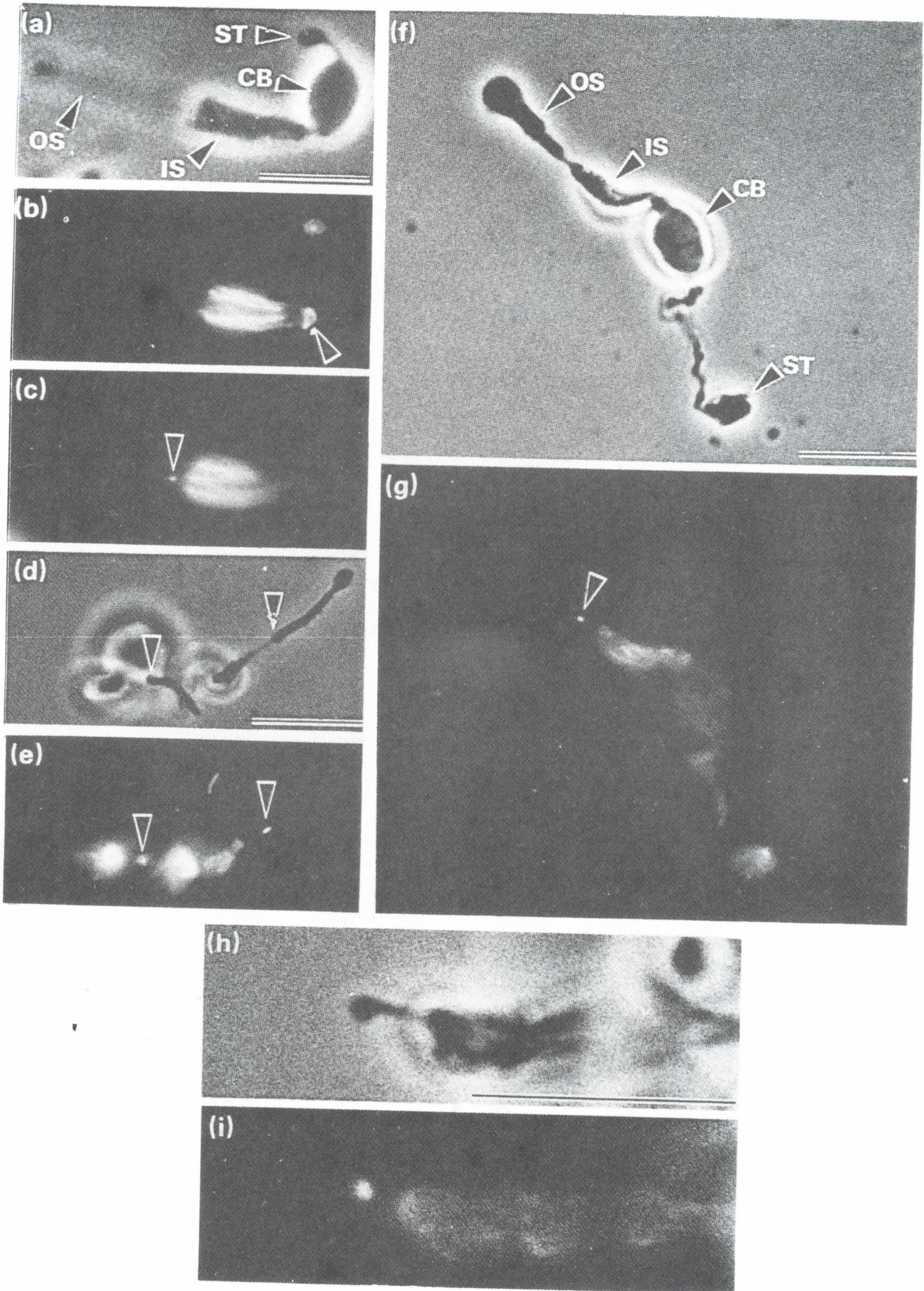

Fig. 6. Isolated rod photoreceptors from mammalian retinas stained with rhodamine-phalloidin. Scale bars = 10μm.

a–c, Rabbit rod. a, Phase image of cell. OS is present, but out of focus. b–c, Fluorescence images of cell in two planes of focus; b, fluorescence is seen in synaptic terminal, collar (arrow), and IS cables; c, plane of focus now reveals a spot of fluorescence at the base of the OS (arrow).

d–e, Rat rods. d, Phase image of two cells complete with OSs (base of OSs are marked with arrows); e, fluorescence image, showing spot at both OS bases (arrows). IS of cell at right also fluoresces.

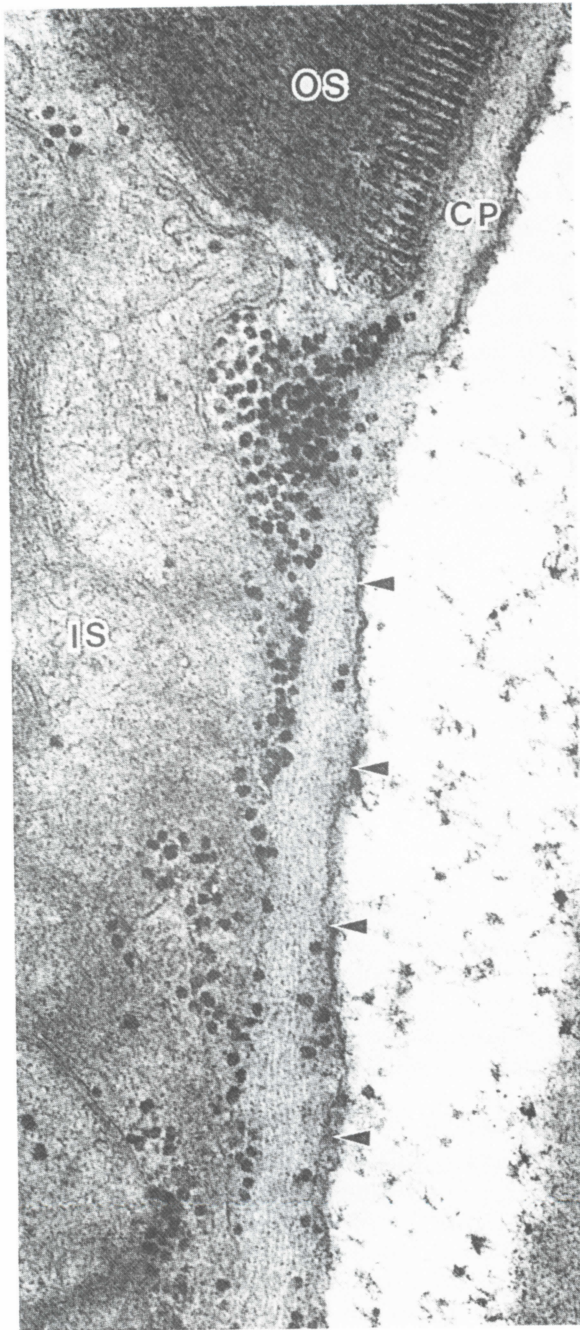

Fig. 7. Electron micrograph of actin filament bundles (arrows) in the inner segment (IS) and calycal processes (CP) of a *Xenopus laevis* rod photoreceptor. A portion of the OS is also shown. × 60000.

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f–g, Cat rod. f, Phase image of cell. OS has curled over on itself. g, Fluorescence image, showing fluorescence in synaptic terminal, IS cables, and spot at OS base (arrow).

h–i, Cat rod. h, Phase image of cell. OS has been torn off, leaving a connecting cilium with a bulbous ending. Fluorescence image, showing spot of fluorescence confluent with bulb. IS fluorescence is out of focus.

fluorescence just sclerad to the nucleus, at the level of the outer limiting membrane (OLM); and (4) fluorescent strands or cables oriented longitudinally in the inner segment (IS). These results are in agreement with similar experiments using teleost cones (Nagle, Okamoto, Taggart and Burnside, 1986). In the isolated rods from *Rana* and *Xenopus*, the IS fluorescence extended in streaks along the vitread portion of the outer segment (OS) (Figs 4c and 5c, respectively). In addition, rods from *Xenopus* and mammals had a small, bright spot of fluorescence at the OS base in a region just sclerad to the connecting cilium (Figs 5b, 6c, e and g). It is not likely that this spot is a product of the isolation procedure, since numerous such spots are seen in cryosections stained with rhodamine–phalloidin (Vaughan and Fisher, 1985). Such spots were seen in *Xenopus* rods collected at light onset, light offset and midday. In some cells the OS was torn away during the isolation procedure, leaving behind a connecting cilium with a fluorescent spot remaining at its tip (Fig. 6i).

In thin sections of *Xenopus* retina examined by EM, there are bundles of fine filaments in the rod IS cytoplasm, lying parallel to the plasma membrane and extending into the calycal processes (Fig. 7). No actin filaments have been observed in photoreceptor OSs examined with electron microscopy (see, for example, Nagle et al., 1986).

4. Discussion

The original goals of this experiment were to determine the distribution of f-actin in retinal cells and to see if this organization could be correlated with some aspect of known structure or function in the cells. Using a relatively gentle dissociation technique, we were able to isolate a variety of cell types from mature vertebrate retinas from several species. A fluorescent, f-actin-specific probe was then used to stain the cells and show the subcellular distribution of this important cytoskeletal protein. Certain consistent patterns did emerge from our data, demonstrating an f-actin distribution pattern unique to each class of isolated cells. The most complex pattern is seen in rod photoreceptors, where four separate actin domains are found; the structural and functional polarization and compartmentalization of this cell type has been well documented (Bok, 1985) and appears to be reflected in the distribution of f-actin. While three separate f-actin domains were observed in Müller cells, their functional roles are difficult to ascertain, since the functions of the Müller cells themselves are not yet fully understood (see Ripps and Witkovsky, 1985, for a review).

Interneurons and Müller cells

In all retinal interneurons examined, rhodamine–phalloidin fluorescence occurred as bright spots associated with the cells' neurites or, in the case of amacrine cells, with the soma. These spots probably represent f-actin associated with pre-synaptic and post-synaptic structures (some possibly adhering to the isolated cells), since both actin antibody and phalloidin label synaptic sites in the brain (Toh et al., 1976; Drenckhahn, Frotscher and Kaiser, 1984; Cohen, Chung and Pfaff, 1985). The most convincing evidence of this from our data is the very precise localization at the ends of bipolar cell dendrites (which are post-synaptic to photoreceptors) and bipolar axon terminals (which are both pre-synaptic and post-synaptic). F-actin has previously been localized to both retinal plexiform layers (Toh, Cragg, Singh and Koh, 1978; Drenckhahn and Kaiser, 1983; Drenckhahn and Wagner, 1985) where the vast majority of retinal synapses occur. In similarly stained cryosections of rabbit retina, we

found most of the bright, discrete spots of fluorescence in the plexiform layers; however, we also found them in significant numbers within the inner nuclear layer (INL) (Vaughan and Fisher, 1984). Although synapses within the INL are not commonly reported, they are known to occur in a variety of species (Fisher, 1972; Boycott, Dowling, Fisher, Kolb and Laties, 1975; Kolb and West, 1977; Linberg and Fisher, 1986), where they are usually associated with cells in the amacrine cell layer. This correlates well with our finding of bright spots of rhodamine–phalloidin fluorescence on the somata of isolated amacrine cells.

Subplasmalemmal fluorescence was observed along the entire plasma membrane of both horizontal cells and Müller cells. Why the major glial cell of the retina should share this characteristic with horizontal cells is not known, but there are reports of other similarities in the cytoskeletons of these two cell types: both contain vimentin in mouse retina (Dräger, 1983) and teleost horizontal cells contain glial fibrillary acidic protein (GFAP) (Linser, Smith and Angelides, 1985), a protein also found in Müller cells in various inherited or induced retinal degenerations (Miller and Oberdorfer, 1981; Eisenfeld, Bunt-Milam and Sarthy, 1984; Erickson, Fisher, Guerin, Anderson and Kaska, 1987). F-actin is well established as a constituent of the cortical cytoskeleton of most cells, where it forms an isotropic matrix. The gel structure of this matrix may exclude some organelles from the peripheral cytoplasm, stabilize that region, or may exert tension on parts of the cell (Stossel, 1984). Indeed, one surprising result of this study is that amacrine, bipolar and photoreceptor cells apparently lack such a layer (bipolar cells have it in only a very limited region).

In addition to the subplasmalemmal fluorescence, Müller cells also stained particularly intensely in their apical microvilli. F-actin is known to form the cores of microvilli and to extend into the underlying cytoplasm, forming the region known as the terminal web (Mooseker et al., 1984). The third distinctive region of rhodamine–phalloidin label occurred just proximal to this region, where Müller cells are connected to each other and to photoreceptors by *zonulae adhaerentes* (forming the OLM of the retina). This is expected, since f-actin is a consistent feature of the endofacial surfaces of two plasma membranes joined by any adhering junction (Geiger, Schmid and Franke, 1983; Bunt-Milam, Saari and Bredburg, 1985). Both rod and cone photoreceptors also had a distinct collar of fluorescence just above their cell bodies, corresponding to their attachment sites to the Müller cells at the OLM.

Photoreceptors

Actin has been localized to the ISs of photoreceptors of a variety of vertebrates (Toh et al., 1978; Burnside and Nagle, 1983; Drenckhahn and Kaiser, 1983; Chaitin, Schneider, Hall and Papermaster, 1984; Drenckhahn and Wagner, 1985; Nagle et al., 1986). Our results also revealed f-actin in photoreceptor ISs of several species, including three mammals, that clearly resemble the bundles of actin filaments observed in photoreceptor ISs of fish; the latter mediate rod and cone contraction and rod elongation (Burnside and Nagle, 1983). While retinomotor movements are not usually attributed to mammalian photoreceptors, subtle movements by these cells may occur, for example in aligning the photoreceptor outer segments with respect to the exit pupil of the eye (Enoch and Birch, 1981). The f-actin cables seen using rhodamine–phalloidin are probably identical with bundles of fine filaments found in the IS and calycal processes of photoreceptors examined by electron microscopy (Fig. 7). Photoreceptors were the only cell type to exhibit clear bundles of f-actin. Such bundles in other cells confer structural stability and rigidity (Tilney and Tilney, 1984;

Mooseker et al., 1984). Recent evidence indicates that such bundles may mediate organelle transport (Kachar, 1985), which is an interesting possibility in the highly polarized photoreceptor. Molecular components used to construct new OS discs are all synthesized in the myoid region of the IS and then transported by unknown means through the IS to the OS (Papermaster and Schneider, 1982). Components of synaptic transmission are, on the other hand, transported from the IS to the synaptic terminal (Young, 1976).

The tapering streaks of fluorescence lying alongside the *Xenopus* OS mark the f-actin contained in the calycal processes arising from the distal IS (Rodieck, 1973). These processes, while shorter, also appeared at the base of the *Rana* rod OS. We have not observed stained calycal processes along the OSs of any mammalian photoreceptors, possibly because they are withdrawn or broken off when the cells are isolated. When we examined isolated rabbit photoreceptors with scanning electron microscopy, no calycal processes were observed around the OSs (Vaughan and Fisher, 1983).

It is interesting that f-actin is observed as a bright, single spot at the base of the rod OS, where the ciliary membrane expands to form new discs (Steinberg, Fisher and Anderson, 1980). When actin is localized immunocytochemically, label is distributed either as a streak extending along the ciliary backbone of the rod OS (Roof and Applebury, 1984) or in a region restricted to the OS base and extending into the nascent discs (Chaitin, Schneider, Hall and Papermaster, 1983; Chaitin et al., 1984; Chaitin and Bok, 1984). Because the actin antibodies recognize both g- and f-actin, our method presumably detects a subset of the total actin within the OS. Preliminary data from retinas treated with cytochalasin D indicate that f-actin may play a regulatory role in disc morphogenesis, since disc growth occurs in the presence of the drug, but is abnormal (Fisher, Williams, Linberg and Vaughan, 1985; Williams, Fisher, Linberg and Vaughan, 1986).

Cone OSs were rarely preserved by the dissociation technique. Teleost cones have been reported to stain with fluorescent phallotoxin (Nagle, Okamoto, Porrello, Taggart and Burnside, 1984), although in a subsequent report the authors indicated that the f-actin may occur only in the accessory cone OS (Nagle et al., 1986).

Our data show that, as expected, all of the retinal cell types examined contain f-actin; moreover, the f-actin distribution pattern typical of each cell type may be correlated with its structural and functional organization. It is likely that f-actin and its accessory proteins perform many important functions in retinal cells, and the first step in elucidating these functions is the localization of the proteins to specific subcellular domains.

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