THE OCCURRENCE OF ACTINLIKE FILAMENTS IN ASSOCIATION WITH MIGRATING PIGMENT GRANULES IN FROG RETINAL PIGMENT EPITHELIUM

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In the retina of the frog and certain other animals, melanin pigment granules move in response to light so as to shield photoreceptor outer segments. The granules are contained within the cells of the pigment epithelium (PE) which lie as a continuous sheet between the neural retina and the choroid. Moderate illumination of the eye causes the melanin granules to move from a region within a PE cell body into numerous fingerlike extensions of the cell which interdigitate with the receptor outer segments. This migration takes many minutes and is reversed when the light falling on the eye decreases in intensity. Several reviews are concerned with the early descriptions of this phenomenon (6, 30) and with more recent experiments (1, 5, 19).

The mechanism of the pigment granule motion is undetermined although there are studies concerning PE ultrastructure (8, 23, 31), scanning electron microscopy of the fingerlike extensions of the PE cells (27), the role of the PE in photoreceptor phagocytosis (32), the nature of the pigment granules (19), and the action spectrum of the light which induces the migration (16). This study reports the presence of a system of microfilaments associated with the pigment granules in the fingerlike processes of the PE cells. We demonstrate by heavy meromyosin (HMM) labeling that the filaments are actinlike in character and suggest that these filaments could be responsible for the migration of the melanin pigment granules.

MATERIALS AND METHODS

Rana pipiens pipiens (Blue Spruce Biological Supply, Castle Rock, Colo.) were maintained in a 10°C cold room. Frogs were kept overnight at room temperature, either in a fully darkened room, for dark adaptation, or in a chamber lit brightly enough to cause significant pigment migration. Adaptation time was reduced to 2 h for the glycerol extraction-HMM experiments. All tissue remained under approximately constant lighting conditions until initial fixation was completed. (A dim safelight was used when necessary.)

Removal of an eye was accomplished after decapitation. Excess muscle and connective tissue were trimmed from the external surface, the anterior half of the eye was cut away and the lens and vitreous were removed. The remaining eyecup was either placed directly into fixative or was prepared for glycerination by removal of the retina with a dry Q-tip (4). The treatment with glycerol solutions was carried out at 0°C according to a modification of the procedure of Ishikawa et al. (12). The eyecup was initially placed in a standard salt solution (0.1 M KCl and 5 mM $MgCl_2$ in 6 mM Na-phosphate buffer at pH 7.0 [SSS]) for 7 min, next it was treated in a series of glycerol solutions for 7 min each (5, 10, 20, 30, and 40% glycerol in SSS) and then in another series of glycerol solutions for 2 h each (50, 20, and 5% glycerol in SSS). Finally, the eyecup was immersed for 17–20 h at 0°C in a HMM[1] solution (approximately 2.5–5 mg/ml in 15–25% glycerol in SSS) or it was treated with one of the following control solutions in which no binding of HMM to actin should occur (22, 26): (a) SSS alone; (b) HMM (2.5 mg/ml) in SSS containing 10 mM ATP; (c) HMM

(2.5 mg/ml) in SSS containing 10 mM inorganic phosphate. Tissue from control solutions (*b*) and (*c*) was postrinsed in the same solutions without HMM (26) for the 30 min immediately preceding fixation to remove unbound HMM.

Tissue was fixed by immersion of the eyecup in Veronal-acetate-buffered OsO_4, pH 7.9 (7) for 20 min in an ice bath and then for 40 min at room temperature, or in 3% glutaraldehyde in 0.1 M cacodylate-HCl buffer at pH 7.5 for 60 min at room temperature, followed by three 15-min rinses in the buffer and postfixation for 1 h in 1% OsO_4 in the same buffer, all at room temperature. All specimens were then rinsed, dehydrated in a graded series of ethanol solutions for a total of 90 min and then in acetone for 40 min, and were embedded in an Epon-Araldite mixture. In one series of experiments, numerous fixations known to preserve microtubules (13, 25) were carried out. These included Na-phosphate- or Na-cacodylate-buffered 1-8% glutaraldehyde or Na-cacodylate-buffered formaldehyde-glutaraldehyde (1-3% each). These fixations were carried out at approximately 0°C or at room temperature to avoid microtubule depolymerization (28), either in the absence of Ca^{++} or in the presence of up to 1 mM Ca^{++}.

Embedding quality was best after an infiltration time of 30 min with 60% plastic in acetone, 30 min of pure plastic followed by 90 min in pure plastic under vacuum (10 mm Hg), all at 37°C. After polymerization at 60°C, sections were cut with a diamond knife, mounted on bare bar grids or slot grids coated with Formvar and carbon, stained with 5% uranyl acetate in methanol and 4% lead citrate (29), and viewed with a JEM 100B (80 kV) or Philips 300 (60 kV) electron microscope. Optical diffraction was carried out using the techniques and apparatus of McIntosh (17).

RESULTS

The PE of the frog eye is a single layer of cells which lie on a basal lamina. The apical border of each cell is dome shaped with fingerlike cytoplasmic processes protruding from the cell into the extracellular space between the rod and cone outer segments. The processes are generally cylindrical and vary greatly in size but usually range from 0.5 to 1 μm in diameter. They can extend as far as the external limiting membrane. The processes contain pigment granules in a light-adapted eye, which can be found at numerous points, either singly or as clusters of up to six granules making a large bulge in the process. In a dark-adapted eye only a few granules remain in the extensions; the great majority return to the cell body. Observations of numerous longitudinal sections which revealed the presence of processes at all levels of the outer segment region suggest that process length is unaffected by adaptation state. Individual pigment granules are membrane-bound ovoids with an average length of 2 μm and an average width of 0.3 μm. Some small vesicles and particles similar in size to free ribosomes are seen throughout the processes and in apical regions of the PE cells.

At least one bundle of microfilaments is usually seen in any cross section of a fingerlike process (Fig. 1), regardless of adaptation state. Longitudinal section of the processes shows that a bundle of filaments is closely associated with each pigment granule such that the granule lies immediately to


FIGURE 1   A number of fingerlike extensions are seen cut in cross section. Filaments cut perpendicular to their long axes appear as dark points in the processes. Two processes containing pigment granules are shown. These approximately 0.5-μm fingers are shown in the area between photoreceptor outer segments. It should be realized that the photoreceptors are generally 2-4 μm in diameter; and reference should be made to Fig. 2 to directly see the relationship of fingers and photoreceptors. Scale, 0.25 μm. × 41,500.

FIGURE 2 *a* and *b*   Fingerlike processes are shown cut longitudinally to their major axes. Numerous relatively parallel filaments can be seen running within each process. Note the filaments near the membranes that surround the pigment granules in each of the two long processes. A section of a nearby photoreceptor is seen at the top of each figure. Scale, 0.25 μm. × 29,500.

FIGURE 3 *a*   Filaments in a glycerinated preparation decorated with HMM are shown. The typical arrowhead configuration can be seen at numerous places. The individual filaments are contained within a somewhat disrupted region of the PE and are not as tightly packed as those in Fig. 3 *b*. Scale, 0.25 μm. × 63,500.

FIGURE 3 *b*   A fingerlike extension cut in longitudinal section is illustrated. A tightly packed array of individual thin filaments labeled with HMM is contained within the process. Labeling of individual filaments in this glycerinated preparation is clearly seen at the right where the array is disrupted. There is a suggestion of a specific staggered alignment of adjacent filaments within the process. Scale, 0.25 μm. × 63,500.
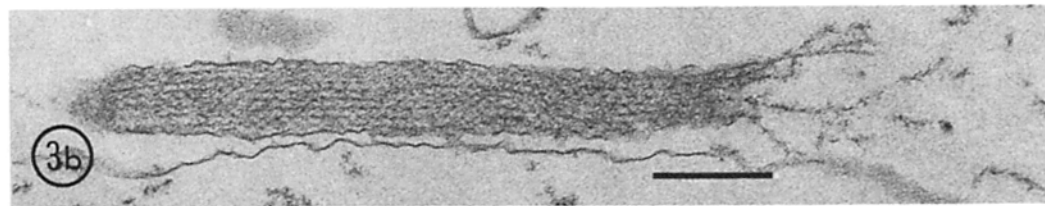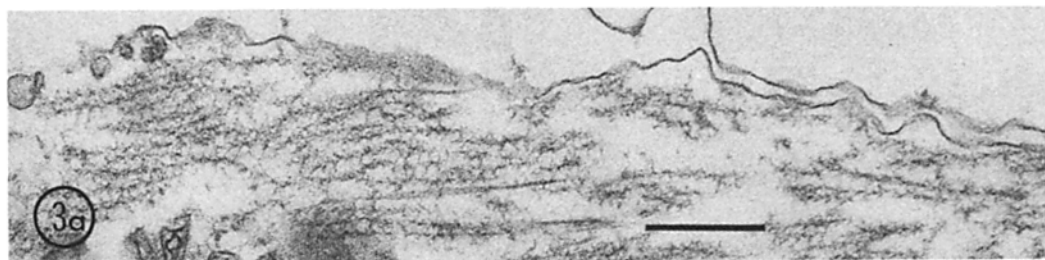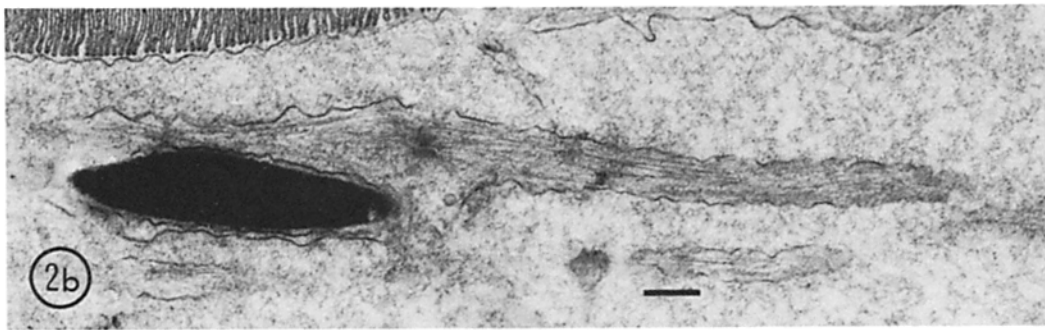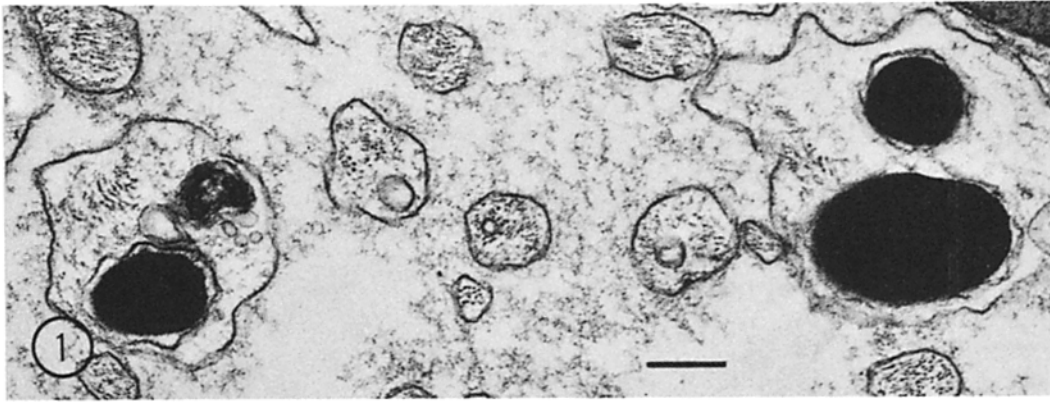
one side of the bundle (Fig. 2). In some sections the filaments appeared to be directly adjacent to the membrane that surrounds a pigment granule. In osmium tetroxide fixations (by which they are best preserved) the filaments had a measured diameter of 8–12 nm; in glycerinated unlabeled preparations the diameter measured 5–8 nm. A second class of thicker filaments was found in PE cell bodies and thus the filaments in the fingers are probably analogous to the so-called thin filaments of other systems (11), although possibly larger in diameter than most other thin filaments described (11, 22). Numerous fixation methods did not reveal any cytoplasmic microtubules in the processes, while simultaneously showing them to be present in large numbers in the inner segments of adjacent photoreceptors.

The apical border of a PE cell was found to contain a dense mesh of thin filaments apparently continuous with those of the processes. This network is so dense that individual filaments could only occasionally be observed, and other cytoplasmic organelles, including agranular endoplasmic reticulum, which are found in the body of the cell's cytoplasm are excluded from this region. Motile pigment granules are only found in the fingerlike extensions or in this region of the cell. This region extends as far as a highly developed junctional complex (9) that connects individual cells at the level of the nucleus. The endings of the filamentous network appear to be associated with the junctional complex.

Glycerinated PE cells appear bloated and disrupted but are still easily recognized since all of the organelles usually found there are present in recognizable form, as are the thin filaments. Treatment of the glycerinated cells with HMM resulted in the labeling of the thin filaments at the apex of each cell, and in the cell processes, with the arrowhead structure (10, 22) characteristic of actin filaments labeled with HMM. In some sections the labeled filaments appeared fuzzy, and individual arrowheads were difficult to discern. The fuzzy appearance of these HMM-labeled filaments is probably due to the filaments lying obliquely relative to the plane of section (22, 26). In many other sections clear arrowheads could be seen either on dispersed filaments (Fig. 3 a) or on filaments lying in a parallel array, closely aligned in bundles (Fig. 3 b). We have no clear evidence concerning the interesting question of whether different filaments have arrowheads pointing in opposite directions. The repeat period of the

arrowheads shown in Fig. 3 a is 22 nm (range 20–26 nm) and is 31 nm (range 28–34 nm) in Fig. 3 b. In similar pictures the arrowhead repeat period was between 26 and 37 nm. The negative of Fig. 3 b was used to make an optical transform diffraction pattern (14) of the filament-containing region shown on the negative. Such a transform yields spacings of layer lines which can be used to determine regular periodicities within the negative. The spacing of the arrowheads, so determined, was 31 nm, in exact agreement with the direct measurement which was based on only the clearest arrowheads that were visible. No other cell structure, including the membranes remaining about individual pigment granules after glycerol extraction, showed the presence of HMM label. Control tissue treated with HMM plus ATP or inorganic phosphate, or without HMM, exhibited thin filaments which were not decorated with the HMM label. The other cell organelles were similarly unlabeled in the control experiments.

DISCUSSION

Thin filaments are the predominant organelle found in the fingerlike extensions and the apical regions of PE cells. These areas of the cells are the same ones occupied by the pigment granules and their paths of migration. The long axes of the filaments run parallel to the migration paths, and bundles of the filaments are intimately associated with the granules. Microtubules are completely absent from these areas of the cells. Thin filaments in these regions "decorate" with HMM under the same conditions which cause the association of skeletal muscle actin and HMM, and the same repeating arrowhead substructure is observed; thus, the filaments are identified as being actinlike. Actual identification as actin (rather than actin-*like*) awaits rigorous biochemical evaluation. All of the above facts, especially the actinlike nature of the filaments, lead us to suggest the hypothesis that the filaments are a part of a system responsible for the migration of pigment granules in the PE of the frog.

Several hypotheses concerning pigment movement have been put forward. Early investigators (see reference 6 for review) thought that the apical processes withdrew from between the rods and cones. This is now known not to occur, although the appearance of withdrawal is sometimes observed in dark-adapted tissue because in that state the retina detaches from the PE rather easily during fixation. Later theories suggest that a

change in endoplasmic reticulum disposition (23), or a pH gradient along the cell processes (19) could be responsible for pigment migration. We now suggest that an association of actinlike filaments with some element in or attached to the membrane that bounds each pigment granule may provide the actual motive force. In this regard we note that the HMM labeling did not indicate the presence of actin in or on the pigment granule membrane. A myosinlike protein could be part of the granule membrane, but this and other possibilities await further experimental proof. A general model of similar nature that accounts for all saltatory particle motion has been proposed by Rebhun (24).

The predominant range of arrowhead repeat distances which were found, 22–31 nm with a few as high as 37 nm, suggests that the actual distance may be smaller than the value of 36–37 nm seen in most other preparations of cytoplasmic actin or actinlike filaments (22). Some shrinkage may occur during fixation and embedding, and some foreshortening is expected because the filaments can lie nonparallel to the plane of section (22,26). In the slime mold *Physarum*, in vivo HMM-labeled sectioned material gave a repeat distance of 20–29 nm for arrowheads on actin filaments (2), while extracted HMM-labeled, negatively stained actin from the same organism yields values of 35–36 nm (21) which are identical to those of muscle actin. These *Physarum* results indicate that the arrowhead spacings for the frog PE preparation reported here are not unreasonable.

Microtubules have been shown to be involved in several systems of intracellular movement. These include pigment migration in melanophores of frog skin (3, 20), and light-activated movement of melanin screening pigment in the lateral eye of the horseshoe crab *Limulus* (18). Thus it was interesting to us that no microtubules were observed in the area of pigment migration in the PE of the frog. This observation was made in material fixed by numerous methods, including many specific for microtubules. Careful attention was paid to the possibility that microtubules in the PE cells might have depolymerized. While PE cells may be peculiar in this regard, there is no basis for believing that the microtubule-specific fixations tried, which in our hands preserved microtubules in adjacent retinal areas as has also been previously reported (15), failed to preserve such tubules only in the PE cells. Therefore, it is concluded that microtubules are not involved in pigment migration in PE cells of the frog retina.

SUMMARY

The cells of the pigment epithelium of the frog eye contain melanin granules which migrate in response to light. The migration occurs within small, fingerlike cell processes which extend into the spaces between the outer segments of the retinal rods and cones. In all adaptation states, thin filaments are found from the apical part of the cell body to the tip of the cell processes. A bundle of thin filaments is associated with the membrane surrounding each pigment granule in a manner suggesting that the granules move along the filaments. Microtubules, though present in other adjacent cell types, are completely absent from the fingerlike processes. Pigment epithelial cells when glycerinated and treated with chicken HMM show the filaments decorated with a characteristic arrowhead structure, demonstrating that the filaments are actinlike in nature. The specificity of the HMM label was shown by the absence of label in samples treated with HMM plus ATP or inorganic phosphate and also by the absence of label on all other structures in samples treated only with HMM. We suggest that the actinlike filaments are involved in moving the pigment granules in the pigment epithelium of the frog eye.

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