Isolation and Characterization of Circumferential Microfilament Bundles from Retinal Pigmented Epithelial Cells

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ABSTRACT   Chicken retinal pigmented epithelial cells have circumferential microfilament bundles (CMBs) at the zonula adherens region. We have isolated these CMBs in intact form and characterized them structurally and biochemically. Pigmented epithelia obtained from 11-d-old chick embryos were treated with glycerol and Triton. Then, the epithelia were homogenized by passing them through syringe needles. Many isolated CMBs were found in the homogenate by phase-contrast microscopy. They formed polygons, mostly pentagons and hexagons, or fragments of polygons. Polygons were filled with meshwork structures, i.e. they were polygonal plates. Upon exposure to Mg-ATP, isolated CMBs showed clear and large contraction. The contraction was inhibited by treatment with N-ethylmaleimide-modified myosin subfragment-1.

  After purification by centrifugation with the density gradient of Percoll, CMBs were analyzed by SDS PAGE. The electrophoretic pattern gave three major components of 200, 55, and 42 kdaltons and several minor components. Electron microscopy showed that the polygons were composed of thick bundles of actin-containing microfilaments, and the meshworks were composed primarily of intermediate filaments.

The structural organization of contractile proteins in nonmuscle cells is usually very complex and labile. However, in epithelial cells, which have rather stable morphology, the contractile proteins constitute cytoskeletons of regular structures (3, 24, 28).

  The retinal pigmented epithelium is a single cuboidal epithelium of homogeneous polygonal cells. Each cell in the epithelium has thick circumferential microfilament bundles (CMBs) at the zonula adherens region (9, 12). These bundles are necessary to maintain the epithelial structure because destruction of the bundles by cytochalasin B causes breakdown of epithelial structure. Recently, we found that upon exposure to Mg-ATP the glycerinated pigmented epithelium shows characteristic folding movement and transforms a planar sheet to a three-dimensional cuplike shape (29). This movement was considered to be due to contraction of the CMB of each cell.

  In this work we have isolated the CMBs from the glycerinated pigmented epithelium in intact form. We describe here the method of isolation of the CMBs and their contraction by Mg-ATP, and we report some results of biochemical and ultrastructural studies.

MATERIALS AND METHODS

Pigmented Epithelia

  Retinal pigmented epithelia were prepared from 11-d-old chick embryos and glycerinated as previously described (29). Briefly, after removal of the vitreous body and neural retina, the posterior halves of eyes were treated with a glycerol solution (50% glycerol, 0.1 M KCl, 5 mM EDTA, 10 mM sodium phosphate buffer, pH 7.2) at 4°C for 24–48 h and stored in fresh glycerol solution at −20°C. The glycerinated materials, if stored at −20°C, can be used for experiments even after 1 yr. Pigmented epithelia were isolated from the glycerinated specimen in 10 mM sodium phosphate buffer (pH 7.2) containing 0.1 M KCl. Contaminated small fragments of neural retina were carefully removed.

Preparation of CMBs

  The glycerinated epithelia were washed with 10 mM sodium phosphate buffer (pH 7.2) containing 0.1 M KCl for 30–60 min at 0°C and treated with a Triton solution (1% Triton X-100, 0.1 M KCl, 10 mM sodium phosphate buffer, pH 7.2) for 10 min at 0°C. These epithelia were successively passed through syringe needles of 25, 27, and 29 gauge 3–5 times each to isolate CMBs. For purification of CMBs in the homogenate, the centrifugation was carried out successively with a self-forming and a discontinuous density gradient of Percoll.

Percoll Density Gradient Centrifugation

  Percoll, colloidal silica coated with polyvinylpyrrolidone, was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The methods of centrifugation in

Percoll have been outlined by Pertoft et al. (31). For the self-forming gradient centrifugation, the homogenate was mixed with a Percoll solution in a 10 mM sodium phosphate buffer (pH 7.2) containing 0.1 M KCl. The final concentration of Percoll was 50%. The solutions were spun at 18,000 g for 45 min using a Sorvall rotor SS34 with adapters for a small-volume sample (DuPont Instruments, Inc., Newtown, CT). The gradient of Percoll was calibrated by the Density marker beads (Pharmacia Fine Chemicals). For the discontinuous gradient centrifugation, the gradients were made in five steps from 50% to 10% of Percoll in the same buffer solution. The samples were layered on the top of the gradient and spun at 1,000 g for 7 min.

Electron Microscopy

A drop of the homogenate was put on a grid covered with carbon-coated colloidion film, using a Pasteur pipette. After 1 min, the specimen was rinsed and negatively stained with three drops of 1% uranyl acetate or 2% potassium phosphotungstate. After removal of the excess solution with filter paper, the grid was dried quickly under an electric bulb. In the case of uranyl acetate, quick drying of the specimen was required because the CMBs were disintegrated in a low pH solution. Sometimes, the CMBs were fixed with 0.1% glutaraldehyde in a 50 mM sodium phosphate buffer (pH 7.2) containing 0.1 M KCl for 10 min at room temperature before staining. The negatively stained CMBs were examined with a JEM 100C electron microscope at 80 kV.

Other Methods and Materials

SDS PAGE was performed according to the method of Laemmli (22). Myosin subfragment-1 (S1) was prepared by the method of Weeds and Pope (37). N-ethylmaleimide-modified S1 (NEM-S1) was prepared by the method of Cande (7).

RESULTS

Isolation and Morphology of CMBs

The glycerinated pigmented epithelia obtained from 11-d-old chick embryos showed a similar appearance as before glycerination (Fig. 1 a). They have a typical epithelial shape, in which each cell adheres to surrounding cells and keeps many pigmented granules. After treatment with Triton, the epithelia were homogenized by passing through syringe needles. In the homogenate, CMBs, their fragments, nuclei, partially destroyed cells, and many pigmented granules were observed with a phase-contrast microscope (Fig. 1 b). Although the homogeneity of isolated CMBs varied from preparation to preparation depending on shearing force, the ratio of the CMBs and their fragments to nuclei to intact and partially destroyed cells was roughly 1:2:0.3 by proper homogenization. Homogenization with a Teflon homogenizer or by sonication did not result in good yields of CMBs.

The CMBs in the homogenate were purified by successive Percoll density gradient centrifugations. The CMBs and their fragments, and most of nuclei, were concentrated and aggregated at a density of ~1.038 in the self-forming gradient centrifugation. Before the next centrifugation, this fraction was passed through a 27-gauge syringe needle twice. After the discontinuous gradient centrifugation of this fraction, the CMBs were found at the interface between 30 and 40% of Percoll, whereas most of nuclei remained at the interface between 10 and 20% of Percoll. Fig. 2A shows the CMB fraction after this centrifugation. The CMBs were still contaminated with a small number of nuclei, and some nuclei were associated with the CMBs. Pigmented granules tended to reattach to the CMBs during the density gradient centrifugation. The ratio of the CMBs and their fragments to nuclei to partially destroyed cells was 1:0.2:0.1 in this stage as the average of five different preparations. The glycerinated pigmented epithelium from an eye, which contained about 0.15 mg of protein, yielded
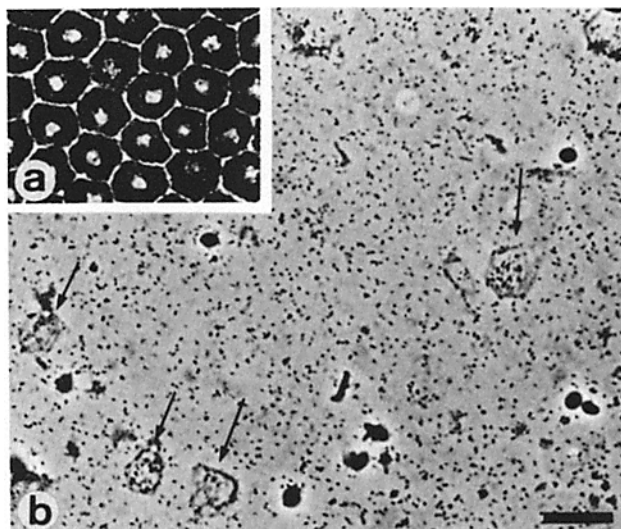

FIGURE 1 Isolation of CMBs from pigmented epithelial cells by homogenization. (a) A phase-contrast photomicrograph of a glycerinated pigmented epithelium. The zone of cellular apposition shows a clear space. (b) An example of phase-contrast photomicrographs of the homogenate of the pigmented epithelial cells treated with glycerol and Triton. Arrows show isolated typical CMBs. Note that these CMBs and the pigmented epithelial cells have almost the same dimension. Fragmented CMBs, nuclei, and many pigmented granules are also observed. Bar, 20 μm. × 450.
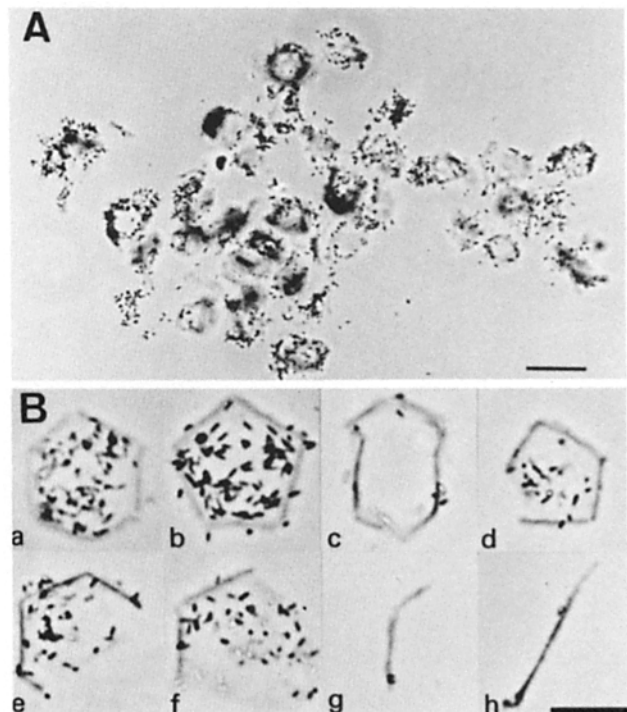

FIGURE 2 Purified CMB fraction. (A) An example of phase-contrast photomicrographs of purified CMB fraction. Pigmented granules reattach to the CMBs during density gradient centrifugation. Bar, 20 μm. × 400. (B) Typical examples observed in the purified CMB fraction. Photographs were taken after passing the fraction through a syringe needle. Hexagonal (a) and pentagonal (b) CMBs filled with meshworks and their fragments (d, e, f) are dominant in the fraction. The CMBs without meshworks (c) are sometimes observed. Heavily fragmented CMBs (g, h) become dominant if the homogenization is too severe. Each of them is contractile, except the meshwork. Bar, 10 μm. × 1,040.

about 0.013 mg of the purified CMB fraction. Fig. 2 *B* shows typical examples of the CMBs observed in the purified CMB fraction, which were passed through a 27-gauge syringe needle twice to remove reattached pigmented granules. Most of the CMBs were pentagons or hexagons, and fragments of each. Heptagons and octagons were also observed in small numbers.

Contraction of CMBs

To examine the contractility of isolated CMBs, we perfused them with a Mg-ATP solution (3 mM ATP, 3 mM MgCl$_2$, 0.1 M KCl, 10 mM sodium phosphate buffer, pH 7.2) and observed them with a phase-contrast microscope. The rate and the extent of shortening of CMBs varied from specimen to specimen, mainly depending on the interaction of the CMBs with a cover glass or a slide glass. The CMBs tightly adhering to the glass shortened slowly and poorly. Photographs of Fig. 3 were taken successively after perfusion of Mg-ATP. The CMBs in these photographs showed fast and extensive shortening, with retention of polygonal shape. The total length of one of the CMBs in Fig. 3 was measured and plotted against the time after
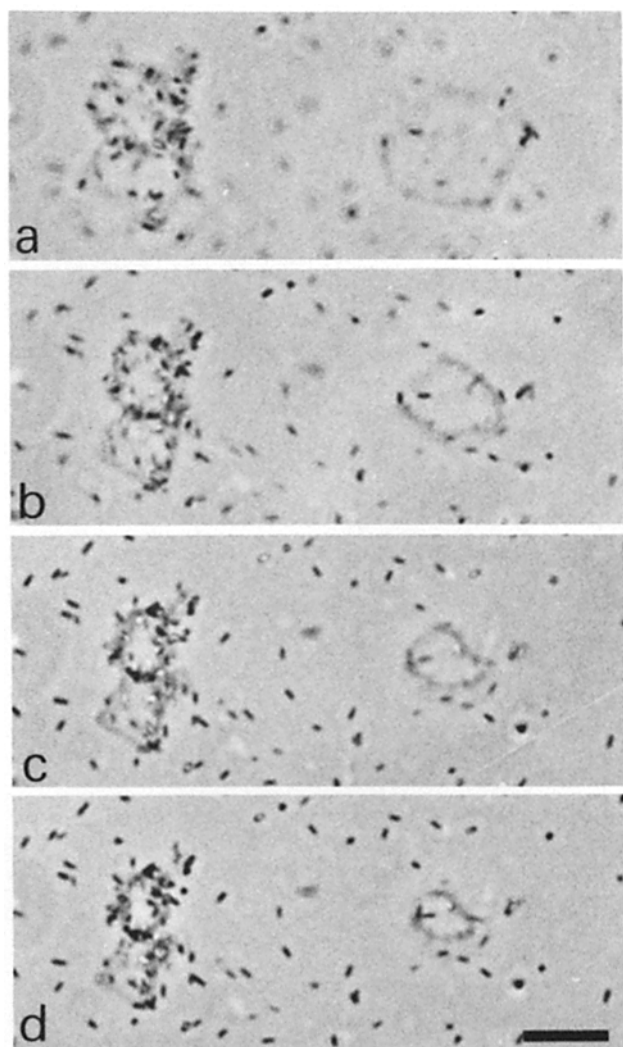
perfusion of Mg-ATP (Fig. 4). This CMB showed one of the most rapid and extensive contractions observed. In this case, the final length was ~40% of the original length, and the shortening rate during the first 2 min was ~8 μm/min at room temperature. Fragments of the CMBs also showed shortening in Mg-ATP. The contraction of CMBs showed no Ca^{2+}-sensitivity.

N-ethylmaleimide-modified myosin subfragment-1 (NEM-S1) is a specific inhibitor of actin-myosin interaction. The incubation of the CMBs with 3 mg/ml NEM-S1 for 5 min at 0°C completely inhibited the shortening in Mg-ATP (Fig. 4 *b*).

Polypeptide Composition of CMBs

The CMB fraction was analyzed by SDS-gel electrophoresis using 7.5% polyacrylamide gels. Fig. 5 shows the electrophoretic pattern of the pigmented epithelial cells treated with glycerol and Triton and the pattern of the CMB fraction. Three major bands and several minor bands were observed in both
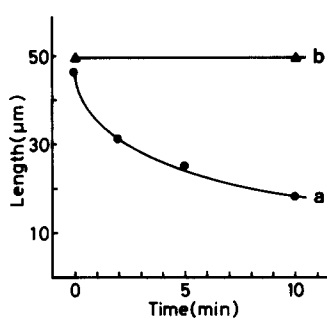

FIGURE 4 Characterization of contraction of the CMB. (*a*) An example of the measurement of the rate and the degree of shortening of the CMB. In this case, the total length of the CMB (the right one in Fig. 3) during the shortening was traced and measured. The initial rate of the shortening was ~10 μm/min, and the degree of shortening was 60%. (*b*) Effect of NEM-S1 on shortening of a CMB. No shortening was observed.

FIGURE 3 An example of contraction of isolated CMBs. Phase-contrast photomicrographs were taken successively at 0 (*a*), 2 (*b*), 5 (*c*), and 10 min (*d*) after perfusion of Mg-ATP. Each CMB showed clear contraction. The contractile profile of the right one, which shortened more rapidly and extensively than the other two CMBs, has been analyzed in Fig. 4. Bar, 10 μm. × 1,120.
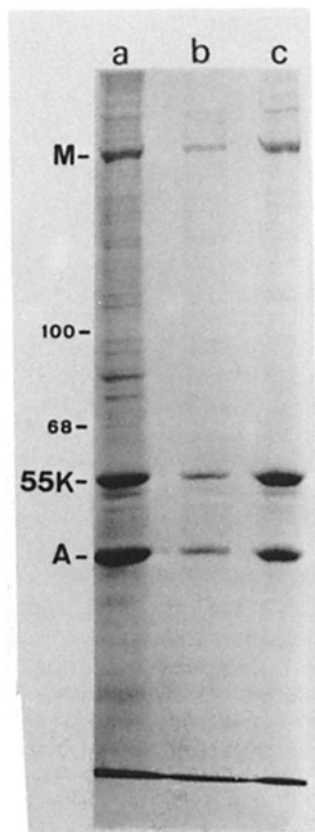

FIGURE 5 SDS gel electrophoretic patterns of pigmented epithelial cells treated with glycerol and Triton (*a*) and the purified CMB fraction (*b*, *c*). Polypeptide molecular weights were estimated using standards; M (myosin heavy chain, 200,000), phosphorylase a (100,000), bovine serum albumin (68,000), A (actin, 42,000).
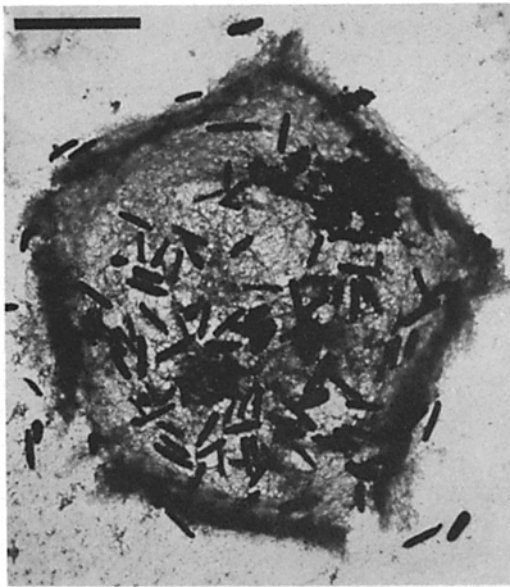
FIGURE 6 An overall view of a CMB showing thick bundles and central meshwork in a low-magnification electron micrograph. The sample was stained with 1% uranyl acetate. Bar, 3 μm. × 5,600.

patterns. Molecular weights of the major components were estimated to be 200, 55, and 42 kdaltons. By coelectrophoresis with muscle proteins, 200 and 42 kdalton proteins showed the same mobilities as myosin heavy chain and actin, respectively. The 55-kdalton protein probably corresponds to the subunit of intermediate filaments observed by electron microscopy in the CMB (see below) or to an actin-binding protein.

Ultrastructure of CMBs

In an electron micrograph of low magnification (Fig. 6), the isolated CMB was found to be composed of a polygon of bundles of filaments and a meshwork of filaments in the polygon, in other words the CMB was the polygonal plate. In a partially broken region of the polygonal plate, the bundle and the meshwork were observed separately. It is not clear whether the periphery of the meshwork is connected with the bundle. In micrographs at higher magnification, the filaments constituting the bundle were identified as actin microfilaments because the filaments have a diameter of ~6 nm and show the double-stranded helical morphology of actin filaments (Fig. 7). On the other hand, the filaments constituting the meshwork were found to be primarily intermediate filaments having a diameter of ~10 nm (Fig. 8). However, there were also some
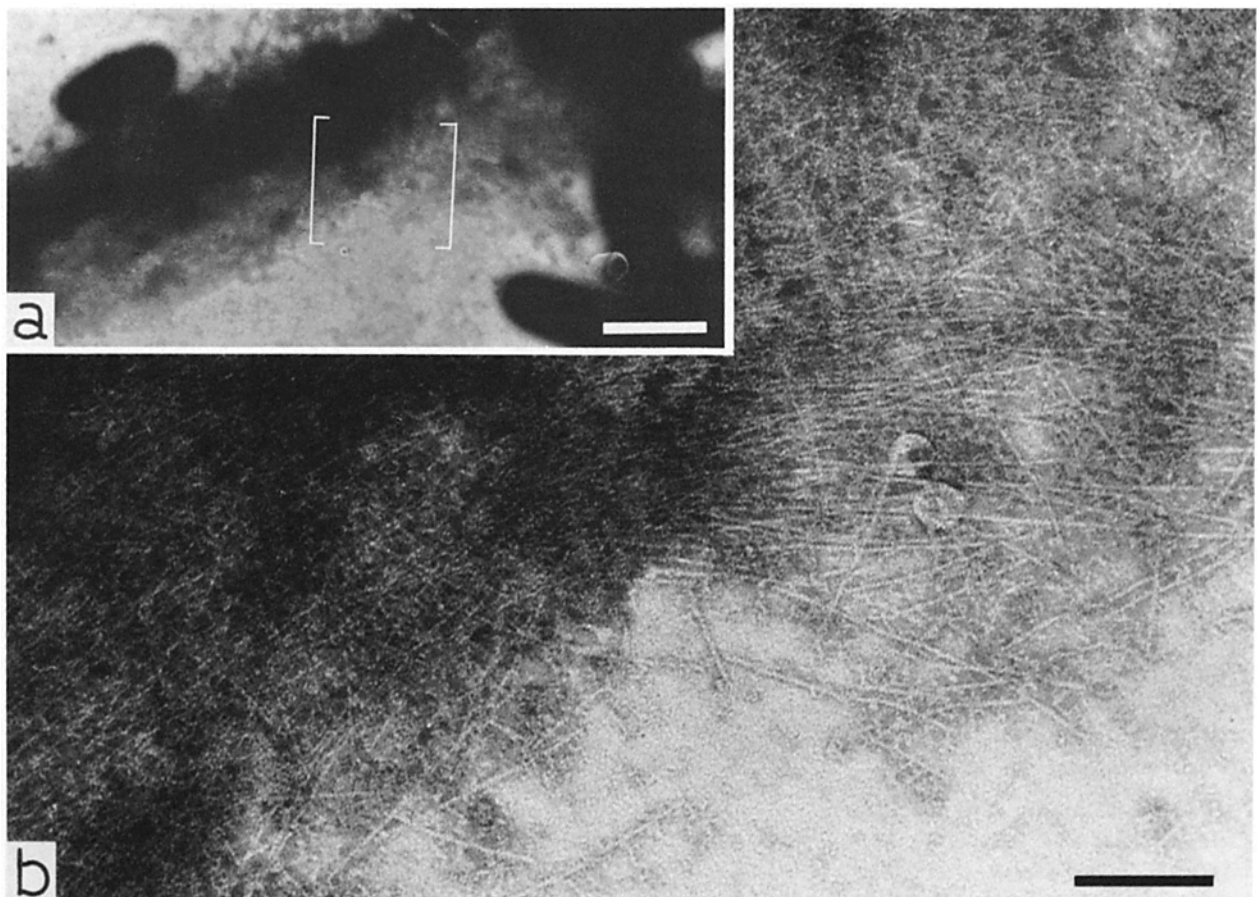

FIGURE 7 Electron micrographs of the bundle region of a CMB. The sample was stained with 2% potassium phosphotungstate. This reagent is more suitable than uranyl acetate to observe the bundle region. In this CMB depleted of meshwork, the peripheral region of a thick bundle is loosened and microfilaments are well-recognized (a). Bar, 1 μm. × 13,500. In the high-magnification photograph (b) of the peripheral region of the bundle bracketed in a, each filament shows a beaded, double-stranded structure that is typical in polymeric actin. Bar, 0.2 μm. × 91,000.
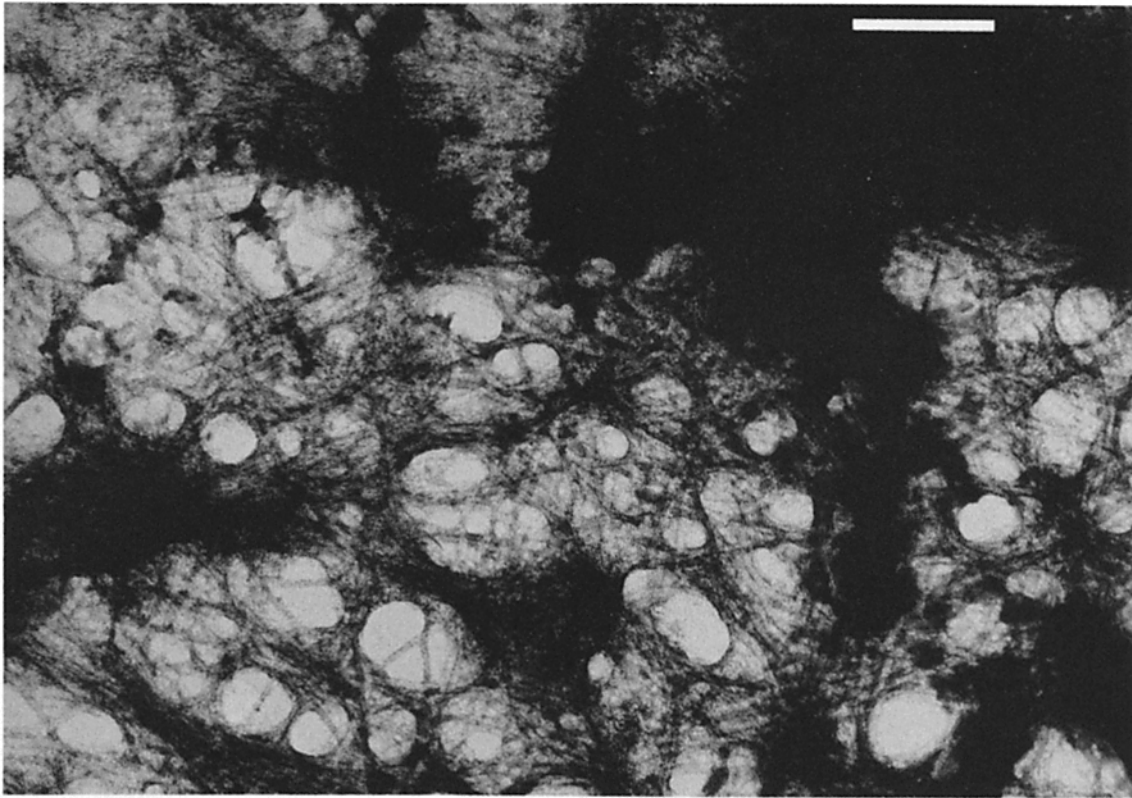
FIGURE 8 An electron micrograph of meshwork region of a CMB. The sample was fixed with 0.1% glutaraldehyde and stained with 1% uranyl acetate. This region is abundant in filaments with a diameters of ~10 nm. Bar, 0.2 μm. X 91,000.

microfilaments similar to actin filaments in the meshwork region.

DISCUSSION

We have succeeded in isolating the apical polygonal plates composed of circumferential microfilament bundles and intermediate filament meshworks from retinal pigmented epithelial cells. The microfilament bundles retain a polygonal shape and show contractility upon addition of Mg-ATP. In various nonmuscle cells, two types of microfilament bundles have been found. One is a closely packed bundle of microfilaments with unidirectional polarity. The other is a bundle of more loosely packed microfilaments having random polarity. Examples of the former type have been found in microvilli of intestinal epithelial cells (26), stereocilia of inner ear (14, 36), microvilli of fertilized sea urchin eggs (6), filopodia of sea urchin coelomyocytes (10), filopodia of platelets (27, 38), acrosomal filaments of sperm (35), and cytoplasmic fibers of Nitella (21, 30). Those of the latter type are stress fibers in cultured cells (1, 33), microfilament bundles associated with the zonula adherens in intestinal epithelial cells (19), and contractile rings in dividing cells (33). Bundles of this type are contractile: e.g. Isenberg et al. (20) showed contraction of stress fibers isolated from glycerinated cultured cells by laser microbeam dissection; Rodewald et al. (32) showed contraction of microfilaments associated with the zonula adherens in isolated brush borders from the intestinal epithelium; and Cande (7) showed cleavage in dividing cultured cells lysed with a detergent. The bundle in the epithelial cell obtained here belongs apparently to the latter type. Some of the bundles of the former type have been isolated and their structures have been studied extensively (2, 4, 5, 8,

11, 15–17, 23, 25, 34). On the other hand, isolation of the microfilament bundles of the latter type has been difficult because of their loose and labile structure.

Pigmented epithelial cells are suitable for isolation of microfilament bundles because their structure is rather stable. The isolated CMB was composed of a polygonal microfilament bundle and a meshwork extended in the polygon. Most of the filaments constituting the meshwork were intermediate filaments. Therefore, some interaction between microfilaments and intermediate filaments may stabilize the whole structure. The isolation was made possible by treatment with Triton after glycerination. Without glycerination, the integrity of the CMBs was markedly reduced. Glycerination was useful for isolation of microfilament bundles and for preservation of the samples. Although the CMB can be isolated from cultured pigmented epithelial cells, their CMBs are more irregular than from cells in situ.

The isolated CMB contracted in the presence of Mg-ATP. The contraction is probably due to the actin-myosin interaction, because the SDS-gel electrophoretic pattern gave bands with the mobilities of the subunits of these proteins and the contraction was inhibited by NEM-S1. As described previously (29), Ca^{2+} was not required for the contraction. When the CMBs were exposed to Mg-ATP in suspension, electron-dense bodies containing pigmented granules, most of which were 3–6 μm in diameter, were observed by electron microscopy. The fine structure of these CMBs was obscured because of the pigment granules. However, part of meshworks of intermediate filaments was often observed at the peripheries of the dense bodies. Each dense body probably corresponds to a contracted CMB. The intermediate filaments seemed to be excluded by the contraction of the CMBs. The shortening of the CMBs took

place, but their original shape was maintained under phase-contrast microscopy. It is very likely that the CMBs have some additional structure to make the corners of the polygon. Ultra-structural studies on the packing, polarity, and connection of microfilaments in the CMBs are needed for further understanding of the process of shortening. The same process may work in the contraction of stress fibers and cleavage furrow fibers.

Chicken retinal pigmented epithelial cells have junctional complexes in their apical region. The junctional complex in the cells is composed of gap junction, tight junction, and zonula adherens (9, 18). Desmosomes, which have been described in the junctional complexes of many other epithelia (13, 19), are not found. Crawford et al. (9) reported that epithelial cells have apical webs of microfilaments extended across the apices of the cells and bundles of microfilaments associated with the cell membranes in the region of the zonula adherens. Hudspeth and Yee (18) reported that many 10-nm filaments run circumferentially around each cell at the level of zonula adherens, and that these filaments are largely confined to the cellular periphery, without forming terminal webs.

Although there are some differences among our observation on the CMBs and their electron microscopic observations, it is probable that the CMBs are the bundles associated with zonula adherens, as first described by Hull and Staehelin (19) in intestinal epithelial cells. Therefore, further studies on the CMBs at the molecular level are helpful for an understanding of the constitution and maintenance of epithelial structure.

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