Demonstration of Contractility of Circumferential Actin Bundles and Its Morphogenetic Significance in Pigmented Epithelium In Vitro and In Vivo

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ABSTRACT Each pigmented epithelial cell bears circumferential actin bundles at its apical level when the pigmented epithelium is established in eyes *in situ* or in culture in vitro. Well-differentiated pigmented epithelia in culture were treated with a 50% glycerol solution containing 0.1 M KCl, 5 mM EDTA, and 10 mM sodium phosphate buffer, pH 7.2, for 24 h or more at 4° C. When the glycerinated epithelium was transferred to the ATP solution, each cell constituting the epithelium began to contract. The epithelium was cleaved into many cell groups as a result of contraction of each cell. The periphery of each cell group was lifted to form a cup or vesicle and eventually detached from the substratum. However, those cells that had not adhered tightly and not formed a monolayer epithelium with typical polygonal cellular pattern contracted independently as observed in the glycerinated fibroblasts.

Contraction of the glycerinated pigmented epithelial cells was inhibited by N-ethylmaleimide but not by cytochalasin B. ITP and UTP also effected the contraction of the glycerinated cells, but GTP and ADP did not. Ca^{2+} was not required. This contractile model of pigmented epithelium provides a useful experimental system for analyzing the function of actin in cellular morphogenesis.

Eukaryotic cells contain contractile proteins similar to muscle proteins. Their function in cellular motility has been suggested by a number of studies (2, 8, 16, 21). However, there have been only a few studies directly demonstrating how the characteristic motile behavior of nonmuscle cells is realized by the function of contractile proteins. Organization of the contractile proteins in nonmuscle cells might be much more complicated and dynamic than that in muscle cells.

Glycerinated muscle fibers seem to be a useful model system for analyzing relationships between the molecular organization of the contractile proteins and cell motility. Since Hoffmann-Berling (10) first demonstrated in 1954 that ATP induces contraction of glycerinated cells, several workers have applied glycerin models of cells to analyze the contractility in nonmuscle cells (13, 23). However, most of these studies have been conducted with cells of indistinctive morphology, such as fibroblasts, which do not form any characteristic tissue structure by themselves. In comparison with such fibroblasts, epithelial cells in functional tissues are organized into a certain static structure characteristic of the tissue's function, and their morphology is stably maintained. Therefore, the contractile proteins in the epithelial tissue cells are expected to have much more regular organization than those in fibroblasts and free cells in the circulatory system.

We have suggested, through observations of pigmented epithelial cells in culture, that cell shape is closely related to the organization of actin filaments (6, 18). In the chick's well-differentiated, embryonic , pigmented epithelia in culture, hexagonal actin bundles were found exclusively in the apical region of each cell. These actin bundles are necessary to maintain the epithelial sheet because destruction of bundles by cytochalasin B causes breakdown of the epithelial structure (5, 6, 18). This culture system of the pigmented epithelial cells may provide a useful experimental model system for analyzing the function of characteristic actin bundles in the tissue cells.

We attempted to analyze the role of the contractile proteins in the characteristic tissue cells, using glycerinated pigmented epithelia developed in vivo and in vitro. We deal here with the typical morphological changes of the tissue structure that can be mediated by the contraction of individual cells.

MATERIALS AND METHODS

Cell Culture

Pigmented epithelial cells from 8-d-old chick embryos were cultured in Eagle's

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minimum essential medium supplemented with 8% fetal calf serum, according to the method of Eguchi and Okada (7). 1×10^6 cells dissociated from pigmented epithelia were seeded in a Falcon 6-cm culture dish (No. 3002; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.). When the cells became confluent and formed an epithelium with typical hexagonal cellular pattern within 2 wk, they were transferred to successive passages of culture. Secondary or tertiary cultures, grown on small glass cover slips, were used for the following experiments.

Glycerination of Pigmented Epithelial Cells

The glycerol solutions in various salt conditions were prepared to determine the best condition for contraction of the cell sheet. Particularly, the following substances expected to influence the muscle contraction were systematically tested: Mg^{2+}, Ca^{2+}, EDTA, EGTA, and dithiothreitol (DTT). After repeated trials, we finally determined the following salt solution as the standard condition: 50% glycerol, 0.1 M KCl, 5 mM EDTA, 10 mM sodium phosphate buffer, pH 7.2.

The cultured pigmented epithelial cells were rinsed in phosphate-buffered saline (PBS), pH 7.2, then glycerinated at 4°C. After 24 h or more, the glycerinated specimens were carefully washed with ice-cold 10 mM sodium phosphate buffer (pH 7.2) containing 0.1 M KCl for ~30 min. This washing was performed as gently as possible to avoid any damage or detachment of the glycerinated cells.

Pigmented epithelia developed in *in situ* eyes were obtained from 11-d-old chick embryos. After removal of the vitreous body and neural retina, the posterior halves of eyes were treated with the glycerol solution at 4°C for 24–48 h, and stored in the fresh glycerol solution at −20°C. The pigmented epithelium was isolated from the glycerinated specimen in 10 mM sodium phosphate buffer (pH 7.2) containing 0.1 M KCl under a stereoscopic microscope, and cut into small pieces with a pair of scissors. Small pieces of the epithelium were transferred with Pasteur pipettes.

Assay of Contraction

Phase-contrast microscope observations were carefully performed to determine the contraction of glycerinated cells or epithelia. Contraction of pigmented cells was induced by the transfer of the cells into the ATP solution: 10 mM sodium phosphate buffer, pH 7.2, 0.1 M KCl, 3 mM $MgCl_2$, and 3 mM ATP at room temperature. For a more quantitative assay, the length of the cell boundary was measured on the phase-contrast photomicrographs taken between appropriate time intervals, and the degree of contraction was estimated. Effects of EGTA, *N*-ethylmaleimide (NEM), and cytochalasin B on the contraction were tested. Nucleotide specificity for the contraction was also tested.

Immunofluorescence Microscopy

The specific antibody to actin was obtained as described previously (19), with highly purified actin from *Physarum* as an antigen. Cross-reactivity of this antibody and indirect immunofluorescence have been also described in our previous paper (20). Matured pigmented epithelia grown on cover slips were treated with a Triton solution (0.2% Triton X-100, 0.1 M KCl, 10 mM Tris-HCl, pH 7.5) for 5 min at 0°C to observe actin bundles more distinctly and to avoid reduction of cell adhesiveness to the substratum by glycerination. The epithelia were then fixed with 10% formalin in PBS, and were washed with PBS and distilled water. The specimens were treated with acetone for 5 min at −5°C, air-dried, and treated with actin antibody for 60 min at 37°C. After washing, the epithelia were stained for 60 min at 37°C with fluorescein-conjugated goat antibody to rabbit IgG. Finally, the coverslips were washed and mounted on microscope slides and sealed with paraffin.

Transmission and Scanning Electron Microscopy

Cultured pigmented epithelium was glycerinated and, with or without an incubation for 10 min in the ATP solution, it was fixed with 2.5% glutaraldehyde for 1 h at 4°C, postfixed with 2% OsO_4 for 1 h at 4°C, and dehydrated in a series of ethanol solutions. For transmission electron microscopy, it was embedded in Epon, sectioned with a diamond knife, and observed with the JEM-100C transmission electron microscope. For scanning electron microscopy, it was transferred to amyl acetate, critical-point-dried with liquid CO_2, coated with gold, and observed with the JSM-F7 scanning electron microscope.

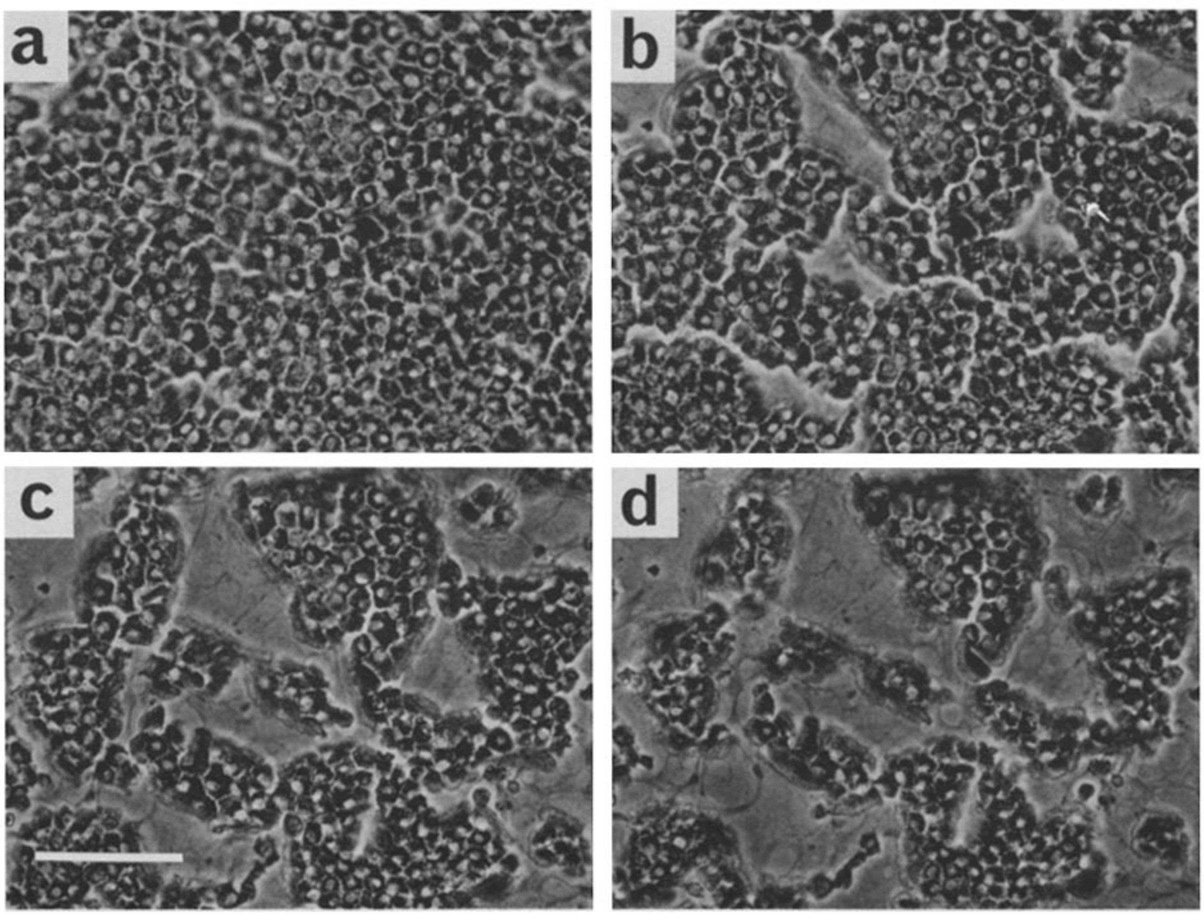


FIGURE 1 A series of phase-contrast photomicrographs showing the contraction of glycerinated pigmented epithelium. Photographs were taken successively at 0.25 min (*a*), 2.5 min (*b*), 6 min (*c*), and 10 min (*d*) after transfer to the ATP solution. Bar, 50 μm. × 400.

RESULTS

Contraction of Pigmented Epithelial Cells

Well-differentiated epithelia in the confluent culture of pigmented epithelial cells were glycerinated and transferred to the ATP solution. They began to contract immediately. The process of contraction is shown in Fig. 1. When the epithelium was glycerinated, it shrank a little, and very narrow clefts were seen from place to place. As the contraction of individual cells advanced in the ATP solution, the clefts became wider until they divided the epithelium into cell groups consisting of 20–30 cells (Fig. 1 *a*). Then the periphery of each cell group was lifted up and in shape appeared like a concave watchglass (Fig. 1 *b* and *c*). Eventually, these cell groups changed their shape to a cup or vesicle (Fig. 1 *d*) and completely detached from the substratum. These shapes were much more obvious in the scanning electron micrograph (Fig. 2).

There were some variations in the velocity and the degree of contraction among cell groups. However, most of the cell groups changed into round shapes within 10 min after exposure to the ATP solution, and further contraction of them was difficult to detect. This movement was not reversible even if the contracted cells were washed with 10 mM sodium phosphate buffer (pH 7.2) containing 0.1 M KCl, or Ca^{2+}/Mg^{2+}–free ATP solution (see *Divalent Cations*).

When these glycerinated cells had been further treated with a Triton solution (0.5% Triton X-100, 10 mM sodium phosphate buffer, pH 7.2, 0.1 M KCl), they contracted similarly in the ATP solution.

Fig. 3 shows the immunofluorescent pattern of the pigmented epithelial cells stained with actin antibody. The actin bundles underneath the cell membrane at the apical level of each epithelial cells are visualized by focusing of the fluorescent microscope. The non-immune IgG fraction used as control showed no fluorescent polygon of epithelial cells (Fig. 3 *b*).

To show the relationship between the contraction of the epithelium and these actin bundles, glycerinated cells before and after the contraction were carefully sectioned as illustrated in Fig. 4 *a* and *b*, and were observed with a transmission electron microscope (Fig. 4). When sectioned at plane *A*, both samples showed the hexagonal pattern of microfilament bundles (Fig. 4 *c* and *d*), but the contraction caused the following changes in their ultrastructure (Fig. 4 *e* and *f*). (*a*) The dense bodies in the microfilament bundles became denser and more conspicuous. (*b*) Cells tended to dissociate at vertices, giving triangular space there, so that the cells became more round. Several cleavages seen in the sample before contraction did not have such tendency, and cells remained polygonal. When the contracted sample was sectioned at plane *B*, conspicuous condensation of the filamentous material was observed just under the apical surface of the cells (Fig. 4 *g*).

These observations seem to show that Mg-ATP caused contraction of microfilament bundles and that its degree was higher in the part that detached from the substratum.

To compare the length of the microfilament bundles before and after contraction in the same cell, phase-contrast photomicrographs of the glycerinated cells were taken and the length of cell boundary was measured on photographs. The center of the white line between adjacent cells is considered to be the

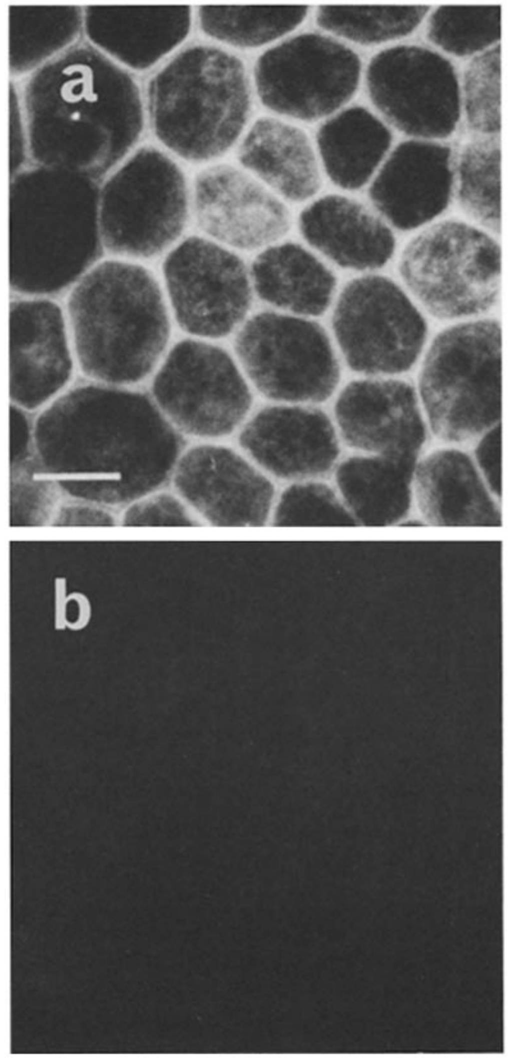


FIGURE 3 Indirect immunofluorescence of pigmented epithelia (*a*) stained with actin antibody and (*b*) stained with nonimmune IgG fraction. The epithelia were treated with a Triton solution (0.2% Triton X-100, 0.1 M KCl, 10 mM Tris-HCl, pH 7.5) before immunofluorescent staining. Actin bundles appear as bright polygons at the apical region of cell periphery in *a*. Note that bright bands are intersected by dark lines showing cell boundaries. The polygons exhibit almost no fluorescence in *b*. Bar, 10 μm. × 1,200.

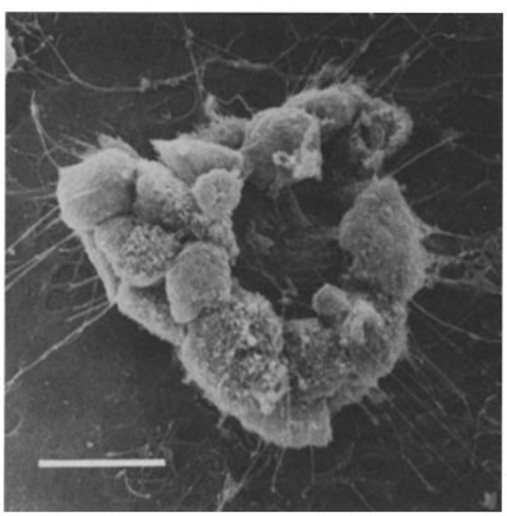
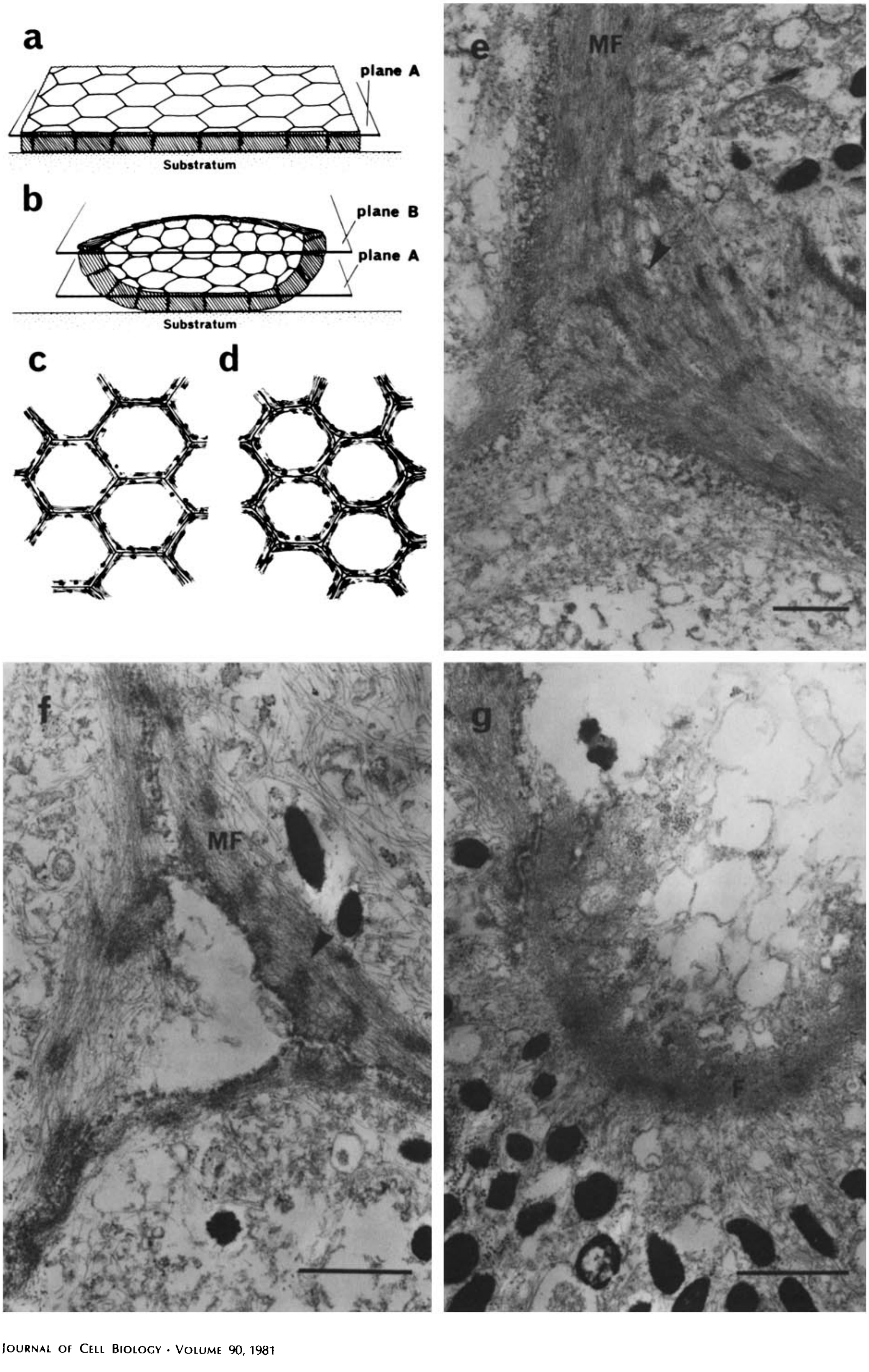


FIGURE 2 An example of scanning electron micrographs of contracted pieces derived from glycerinated pigmented epithelia when treated with the ATP solution. Bar, 20 μm. × 850.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cell boundary, and the total boundary length of about ten cells adhering to one another was measured (instead of individual cells) to avoid errors in measurement. Although this measurement can not give the actual value of the contraction of actin bundles, it might be regarded as the best approximation at the present stage, because the bundles are located just underneath the opposed membranes which adhere tightly to one another. With this method, the boundary length of only those cells that remain attached to the substratum during the contraction can be measured.

Fig. 5 shows an example of phase-contrast photomicrographs of cell groups before and after the contraction. In this case, a cell group consisting of seven cells was chosen, and the cell boundary was traced as shown and measured. Measurements on three such cell groups showed that, after a 10-min incubation in the ATP solution, the boundary length decreased 12.5, 8.4, and 14.9% from the original value. Boundary length reached the minimum value in about 10 min. The decrease of boundary length of each cell was more varied, but every cell in a cell group shown in Fig. 5 contracted. The degree of contraction in those cells that detached from the substratum could not be measured with this method, but more vigorous contraction was expected from the observation of the ultrastructure (see Fig. 4).

During morphogenesis of monolayer epithelia, pigmented epithelial cells take various morphologies, depending on cell density (18). When pigmented epithelial cells were glycerinated at preconfluent stages of culture, they did not show any organized movement as was mentioned above. Individual cells contracted at their original positions, respectively.

Epithelia isolated from *in situ* eyes and glycerinated were trimmed into pieces as small as possible (a few millimeters square). These pieces, however, were still much larger than the cell groups seen after contraction of glycerinated pigmented epithelia cultured in vitro. Contraction of these pieces was apparently different from that of the epithelia in culture. They were not cleaved into small cell groups and did not form cuplike structures as did the glycerinated epithelia in vitro. They showed, however, waving or folding and sometimes formed tubular structures as a whole, probably as the result of contraction of individual cells, which adhered tightly to each other and were freed from the substratum.

Nucleotide Specificity

Effects of the following nucleotides on the contraction of glycerinated epithelia were tested: UTP, ITP, GTP, and ADP. UTP and ITP resulted in contraction of the epithelia in the same way as ATP, but the speed of the contraction was rather reduced. GTP and ADP showed little or no effect on the glycerinated epithelia (Fig. 6*a* and *b* for GTP; and *e* and *f* for ADP). GTP seemed to induce a few narrow clefts when epithelium was exposed to it for >70 min, but ADP did not. They could not substitute for ATP. Transfer of the epithelia from GTP or ADP solution to the ATP solution resulted in contraction of the epithelia (Fig. 6*c* and *d* for GTP-ATP; and *g* and *h* for ADP-ATP).

Divalent Cations

When glycerinated epithelia were placed in a Ca^{2+}/Mg^{2+}-free ATP solution (3 mM ATP, 5 mM EDTA, 10 mM sodium phosphate buffer, pH 7.2, 0.1 M KCl), no contraction was observed. When such epithelia were further transferred to the ATP solution, they contracted. This contraction obviously required divalent cations. To determine whether Ca^{2+} was

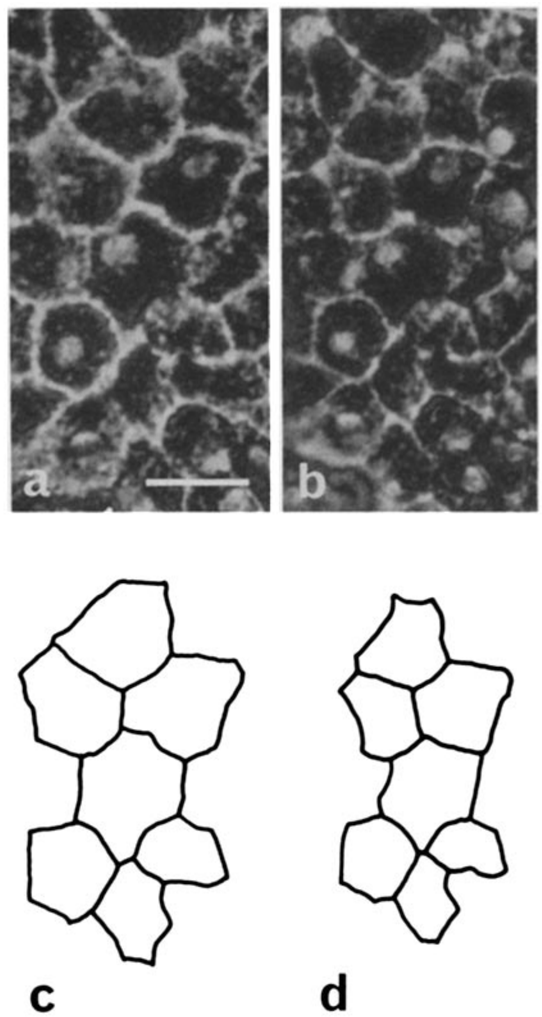

FIGURE 5 An example of phase-contrast photomicrographs of a part of pigmented epithelia used for the measurement of total cell boundary length. Phase-contrast photomicrographs were obtained immediately (*a*) and 10 min (*b*) after transfer to the ATP solution, and their partial line drawings of cell boundaries are given in *c* and *d*, respectively. Bar, 20 μm. × 700.

FIGURE 4 Transmission electron microscopic images of microfilament bundles of glycerinated epithelia before and after the ATP treatment. Glycerinated epithelia before (*a*) and after (*b*) the ATP treatment were sectioned through plane *A* or *B*. *c* and *d* schematically represent overall cellular pattern and circumferential microfilament bundles in a section through plane *A* before (*a*) and after (*b*) the ATP treatment, respectively. Dense bodies (arrowhead) in microfilament bundles (*MF*), seen in a section through plane *A* of an epithelium before the ATP treatment (*e*), became much more conspicuous after the ATP treatment (*f*). Condensed filamentous materials (*F*) just under the apical surface of contracted cells are clearly shown in a section through plane B of an epithelium after the ATP treatment (*g*). The diameter of the microfilaments in these figures is ~6 nm, and 10-nm filaments are also seen. Bar, 1 μm. *e*, × 14,000; *f* and *g*, × 21,000.

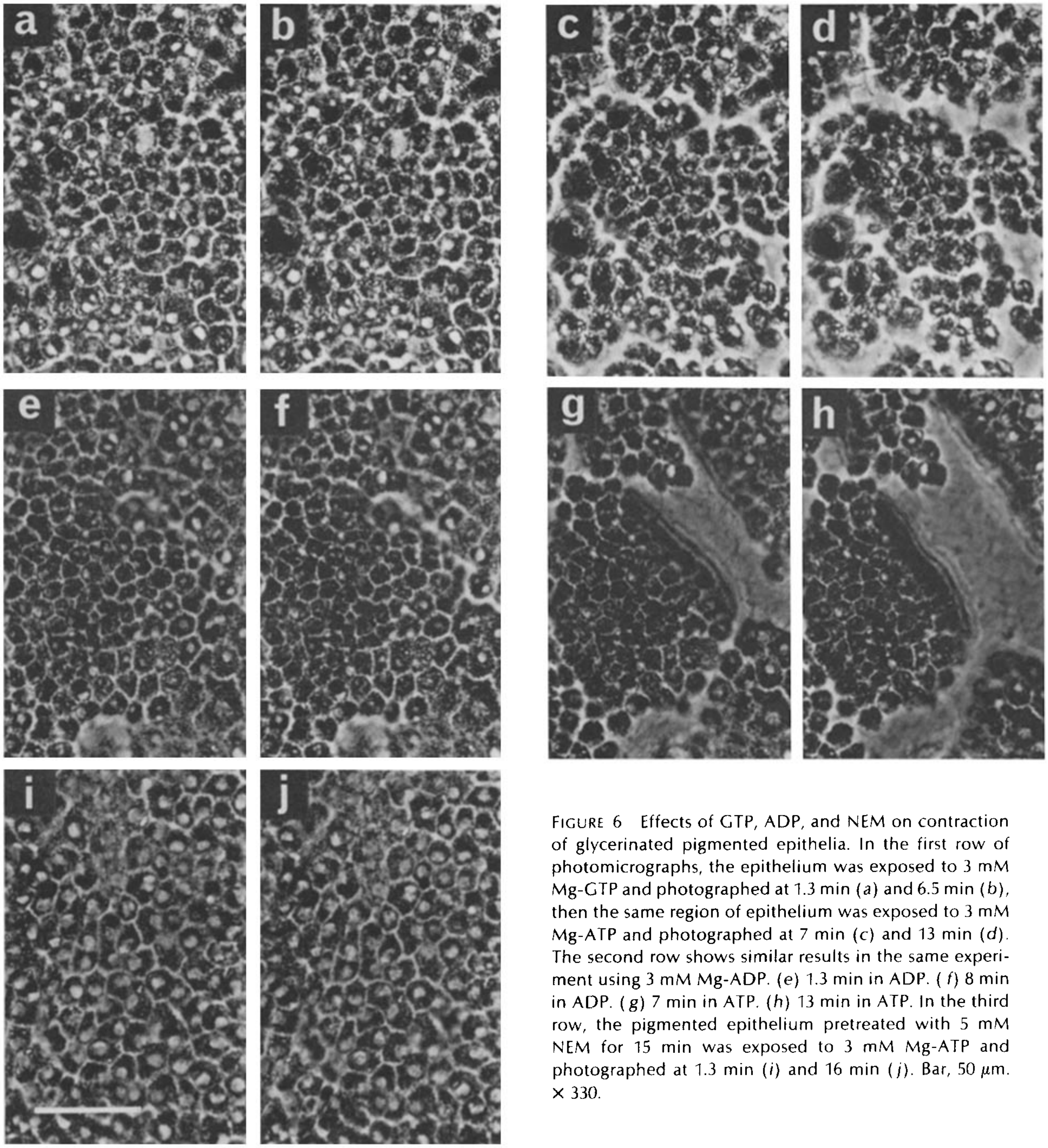

FIGURE 6 Effects of GTP, ADP, and NEM on contraction of glycerinated pigmented epithelia. In the first row of photomicrographs, the epithelium was exposed to 3 mM Mg-GTP and photographed at 1.3 min (*a*) and 6.5 min (*b*), then the same region of epithelium was exposed to 3 mM Mg-ATP and photographed at 7 min (*c*) and 13 min (*d*). The second row shows similar results in the same experiment using 3 mM Mg-ADP. (*e*) 1.3 min in ADP. (*f*) 8 min in ADP. (*g*) 7 min in ATP. (*h*) 13 min in ATP. In the third row, the pigmented epithelium pretreated with 5 mM NEM for 15 min was exposed to 3 mM Mg-ATP and photographed at 1.3 min (*i*) and 16 min (*j*). Bar, 50 μm. × 330.

necessary for the contraction, epithelia were placed in a Ca^{2+}-free ATP solution containing 2 mM EGTA. The epithelia contracted as usual, indicating that Ca^{2+} was not an obligatory requirement.

Effect of NEM on Contraction

Solutions of 5 mM and 10 mM NEM in 10 mM sodium phosphate buffer, pH 7.2, containing 0.1 M KCl were used. The cells treated with these solutions were rinsed in the solution without NEM, then transferred to the ATP solution. Treatment of glycerinated pigmented epithelia with either solution for 15 min at room temperature resulted in complete inhibition of contraction. The effect of the duration of the treatment with 5 mM NEM solution was observed as follows. After a 2-min treatment of an epithelium, slight inhibition was shown. The lifting of the periphery of each cell group was reduced to some extent. After a 4-min treatment, the clefts were produced in the epithelium and extended a little. The cleft formation was barely evident after a 6-min treatment, and no sign of contraction was observed after a 12-min treatment (Fig. 6 *i* and *j*).

Effect of Cytochalasin B on Contraction

The effect of cytochalasin B solution (10 mM sodium phosphate buffer, pH 7.2, 0.1 M KCl, 10 μg/ml cytochalasin B, 1% dimethylsulfoxide) was tested. Treatment with cytochalasin B for 30 min or more at room temperature did not result in any morphological change under the light microscope. Cytochalasin B showed no inhibition of cell contraction. The epithelia treated with this reagent contracted as usual.

DISCUSSION

Glycerinated pigmented epithelial cells dramatically contracted in the presence of ATP and Mg^{2+}. Several aspects of this contraction were studied to clarify the mechanism involved in the cellular movement.

NEM, a sulfhydryl-group modifying reagent, inhibits irreversibly actin-activated myosin ATPase but does not affect actin polymerization (24, 27). Contraction of the glycerin model of pigmented epithelial cells was inhibited by NEM treatment. The nucleotide specificity of the contraction coincided well with that of muscle myosin (26). These observations obviously suggest that myosin actively participates in the contraction of our system. To elucidate the molecular basis of mechanisms involved in this contraction, immunohistochemical analyses, and ultrastructural studies with heavy meromyosin decoration will be needed.

Cytochalasin B is known as a reagent which breaks down the microfilament bundles in various nonmuscle cells, including pigmented epithelial cells (see review in reference 30). Even in the presence of cytochalasin B, glycerinated pigmented epithelial cells do not exhibit any morphological changes and can contract just as in the absence of the reagent. This result is consistent with the observation by Weber et al. (28) on glycerinated fibroblasts. Effects of cytochalasin B on actin bundles may occur only in living cells.

Several authors have reported the Ca^{2+}-requirement for motility in nonmuscle cells. Taylor et al. showed that contractility of isolated cytoplasm from single amoeba was controlled by Ca^{2+} and ATP (25). Hsu and Becker (12) reported that the volume decrease in glycerinated polymorphonuclear leucocytes was induced by ATP and Ca^{2+}. Izzard and Izzard (14) also reported that fresh and naked cytoplasm of fibroblasts showed Ca^{2+}-dependent contraction. In the contraction of brush borders of intestinal epithelium, two different results on the Ca^{2+}-requirement have been reported. Rodewald et al. (22) have observed that contraction of the brush borders, untreated with detergent, is induced by ATP and Mg^{2+} or Ca^{2+}, and is characterized by a pinching in of the plasma membrane at the zonula adherens. On the other hand, Mooseker (17) showed that microvillar contraction in Triton-treated brush borders required 10^{-6} M Ca^{2+}. The regulatory system in nonmuscle cells must be highly delicate or unstable (see review, reference 9). We have failed to show the Ca^{2+}-requirement, because the contraction of our glycerin model occurred in the presence of EGTA. However, we are unable to reach any conclusion about Ca^{2+}-requirement for contraction at the present stage of our study. Further studies on Ca^{2+}-requirement in motility are now in progress, including use of other detergents.

Immunofluorescence microscopy revealed the presence of circumferential actin bundles at the apical level of each cell in the pigmented epithelia, and thick circumferential microfilament bundles were observed in the same region of pigmented cells with an electron microscope. Observation with a phase-contrast microscope and a scanning electron microscope obviously revealed that glycerinated pigmented epithelia changed their shape upon contraction. Transmission electron microscopy showed evidence for the contraction of circumferential microfilament bundles, i.e., electron-dense bodies of bundles became prominent, and clefts among microfilament bundles in neighboring cells appeared at the corners of polygonal shapes. Measurement of boundary length of the epithelial cells on phase-contrast photomicrographs showed that this length decreased obviously as the result of this contraction. Considering these observations, we concluded that characteristic movements in the glycerin model of pigmented epithelial cells were brought about by contraction of the circumferential actin bundles in the apical region of each cell.

The physiological role of such thick circumferential actin bundles as observed in the pigmented epithelial cells may be that of a rigid cytoskeleton responsible for maintenance of the cellular pattern of monolayer epithelia. Recently, Crawford (3, 4) described three kinds of movements observed in clonal cultures of chick pigmented epithelia in vitro. They are focal contraction, extension and retraction of apical protrusions, and undulations of lateral membranes. This result suggests that there may be such cell movements also in the stable epithelium in vivo. If this is possible, focal contraction must be due to the contractile function of actin bundles. Honda and Eguchi (11) suggested theoretically by computer simulation that the pigmented epithelium in vivo can attain a stable regular hexagonal pattern by shortening the boundary length without a change in the surface area of each cell. We assume that the circumferential actin bundles might participate, in part, in the formation of a stable cellular pattern of epithelium as active contractile machinery and, in part, in the maintenance of the formed pattern as a cytoskeleton.

The formation of cup or vesicular structures by the contraction of circumferential actin bundles can be thought of as an example of three-dimensional morphogenesis from two-dimensional cell sheets. Tubular and vesicular structure formations from epithelia are essential processes of organogenesis during development. In neurulation in an amphibian embryo, Baker and Schroeder (1) found microfilament bundles at the outer apical ends of neural cells, and presumed that their purse-string-like contraction was a motive force in neural tube formation. Similar observations have been obtained in other glandular tissues (15, 29, 30, 31, 32). The glycerinated monolayered cell sheet of pigmented epithelial cells was divided into cell groups which finally formed cups or vesicles when immersed in Mg-ATP solution. These changes were found to be mediated by the contractile function of circumferential actin bundles which were present in each pigmented epithelial cell. We emphasize, on the basis of our study, that the glycerinated model of the pigmented epithelium can be a useful tool for studying the function of actin bundles in tissue morphogenesis as well as in essential cell movements during organogenesis.

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