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The Involvement of Microtubules in the Light Response of Medaka Melanophores

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ABSTRACT—The light response of isolated medaka melanophores is accompanied by remarkable changes in cell shape; the peripheral region is extended in light and retracted in the dark. This change in the peripheral region of the cytoplasm can be prevented by the antimitotic agents, colcemid and nocodazole, and by cytochalasin B. The dark-induced aggregation of melanosomes is scarcely inhibited by antimitotic agents or cytochalasin B, while the pigment dispersion induced by irradiation is prevented almost completely by antimitotic agents and partially by cytochalasin B. When melanophores are treated with colcemid or nocodazole in the dark, their radially-arrayed microtubules disappear and the cell outline becomes irregular. However, the distribution of microtubules is preserved in the light-dispersed cells even after treatment with antimitotic agents. These findings suggest that microtubules of the normal number and distribution, and not microfilaments, are required for the centrifugal pigment migration of melanophores responding to light and for the retainment of cell shape.

INTRODUCTION

Color changes of the integument, which are widely observed in lower vertebrates, occur due to pigment translocation in the chromatophores [1]. The force-generating mechanism in transporting pigments has been studied in conjunction with other non-muscle cell motility. However, the mechanism causing pigment migration has been too complex to be elucidated [2]. For example, for the microtubules, two studies have given contradictory results. In one, the number of microtubules in angelfish melanophores with aggregated pigments decreased to 40% in the cells with dispersed pigments [3], while in another study on *Fundulus* melanophores, the number of microtubules in the dendritic processes remained unchanged before and after pigment withdrawal [4]. In general, microtubules are thought to be indispensable for the fast transport of pigments within chromatophores in some teleost species [4–6] and to be required for the development of normal cell shape and pigment distribution [7].

In *Oryzias* melanophores, pigment aggregation at the cell center occurs in response to α-adrenergic agonists [8], melatonin [9], melanophore-concentrating hormone (MCH) [10, 11] and changes in illumination [12]. The reactions to the former three agents are completed within 2 min to punctate state in cultured melanophores [11], whereas 30 min is required to complete the aggregation in the dark [12]. The difference in the velocity between these reactions raises the question of whether the same motive force is used for both reactions, i.e., the microtubules may not be involved in the slow reaction, the dark-induced melanosome aggregation.

The present experiment was undertaken to elucidate the role of microtubules in light-dependent pigment displacement by using antimitotic agents, colcemid and nocodazole, and cytochalasin B, a drug that disrupts cytoplasmic microfilaments. A specific immunological probe for microtubules was used to study the changes in their distribution in melanophores responding to illumination.

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MATERIALS AND METHODS

Melanophores were isolated from scales of the wild type medaka, *Oryzias latipes* (BBRR) [13], and cultured in L-15 medium supplemented with 5% FCS for 2–6 days at 25°C before use. Melanosome translocation, which was induced by changes in the illumination, was observed using an inverted microscope (Nikon Diaphoto). The intensity of the incident light was adjusted to 600 lux with a halogen lamp (12 V, 50 W) equipped with neutral density filters. The responses of melanophores were recorded with an image analyzer, Planimex 25 (Nireco). The full-aggregated state attained by epinephrine was usually corresponding to about 15% of the full dispersion in the culture medium. For indirect immunofluorescence microscopy, melanophores cultured on a cover glass were permeabilized with Brij 58 and polyethylene glycol for 1 min [11]. After fixation with 3.7% formaldehyde in phosphate-buffered saline (PBS) for 6 min at room temperature, the cell preparations were rinsed with PBS. Next, a 1:20 dilution of anti-tubulin antibody (Miles Yeda) was applied to the specimen for 1 hr at 37°C. After washing with PBS, the specimen was incubated with FITC labeled IgG (Miles Yeda) for 1 hr at 37°C followed by washing with PBS, and enclosed in 90% (vol/vol) glycerol/PBS on a slide glass. The fluorescence was observed with a Nikon fluorescence microscope. All drugs, colcemid (Sigma) and epinephrine (Sigma) except cytochalasin B (Aldrich) were directly dissolved in the saline solution (127 mM NaCl/2.7 mM KCl/1.8 mM $CaCl_2$/5 mM Tris-HCl buffer, pH 7.3/5.5 mM glucose). Cytochalasin B was diluted from a stock solution dissolved in dimethyl sulfoxide.

RESULTS

When melanophores of *Oryzias* (Fig. 1a) were moved to the dark, centripetal translocation of the melanosomes occurred within 30 min at the velocity of 0.6 ± 0.02 μm/min, accompanied by retraction of the cytoplasm in the marginal area (Fig. 1b). Subsequent illumination brought about extension of the cytoplasm in the peripheral region (Fig. 1c), and then the pigment granules moved centrifugally within 5 min at the rate of 4.9 ± 0.5 μm/min (Fig. 1d). These alterations in the cell outline occurred with all changes in illumination, though the dark-induced alteration in the cell shape was more remarkable than the light-induced one. There was little shape alteration in the cells when pigment was aggregated with epinephrine (10 μM) within 2 min at the rate of 20 ± 1.2 μm/min.

Effect of colcemid

A melanophore, when incubated with 5 μM colcemid in darkness for 60 min, showed centripetal migration of its pigment and decreased in cell size by about 35% (Fig. 2b). Subsequent illumination for 20 min did not cause dispersion of the melanosomes (Fig. 3), and the cytoplasm in the marginal area did not extend but retracted as shown in Figure 2c.

Effect of nocodazole

Nocodazole, like colcemid, had an inhibitory effect on the melanophores (Fig. 4). When the melanophores were exposed to 1 μM nocodazole for 20 min in the dark, the melanosomes migrated to the cell body. However, the cell shape changed remarkably, as shown in Figure 4b. The cell processes were almost retracted and the cell size became exceedingly small. Centrifugal migration of melanosomes scarcely occurred with subsequent irradiation, while slight extension of the cytoplasm perceived (Fig. 4c). As the time period of nocodazole treatment was prolonged to 30 min, melanophores whose outlines became irregular were incapable of pigment dispersion (data not shown).

Effect of cytochalasin B

When melanophores were exposed to cytochalasin B (10 μg/ml) in the dark for 60 min, melanosomes aggregated more or less incompletely as shown in Figure 5b, and the retraction in the peripheral region of cytoplasm occurred, though its extent was insufficient when compared to the untreated melanophores. Subsequent irradiation for 10 min in the drug caused pigment dispersion, whereas the extent of dispersion was inhibited by 20% (Fig. 3). The marginal area of cytoplasm scarcely expanded by this irradiation (Fig. 5c).

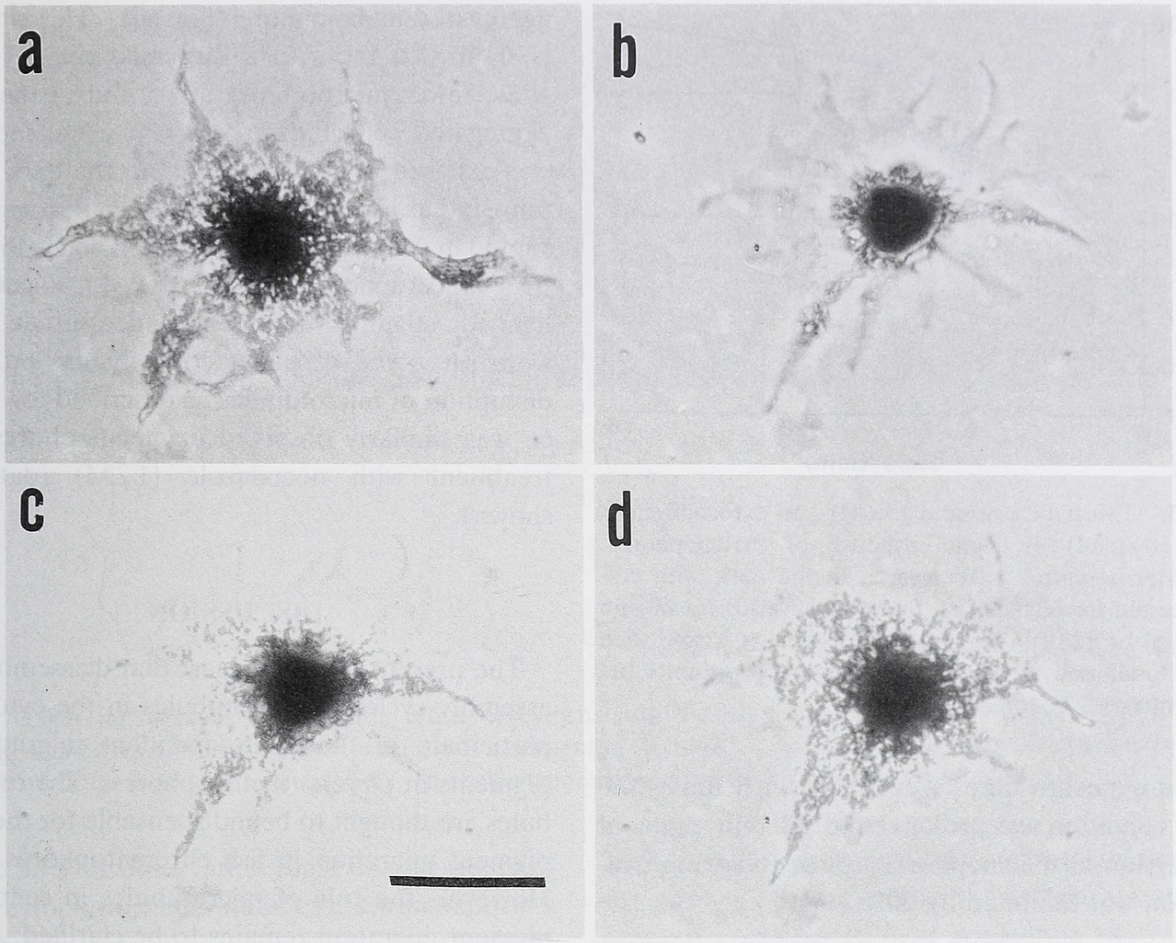

FIG. 1. Melanophore response to illumination. a. Dispersed state in light. b. Aggregated state after 60 min in the dark. c, d. Re-dispersed state after 2 min and 5 min illumination. (In all figures, the bar = 30 μm)

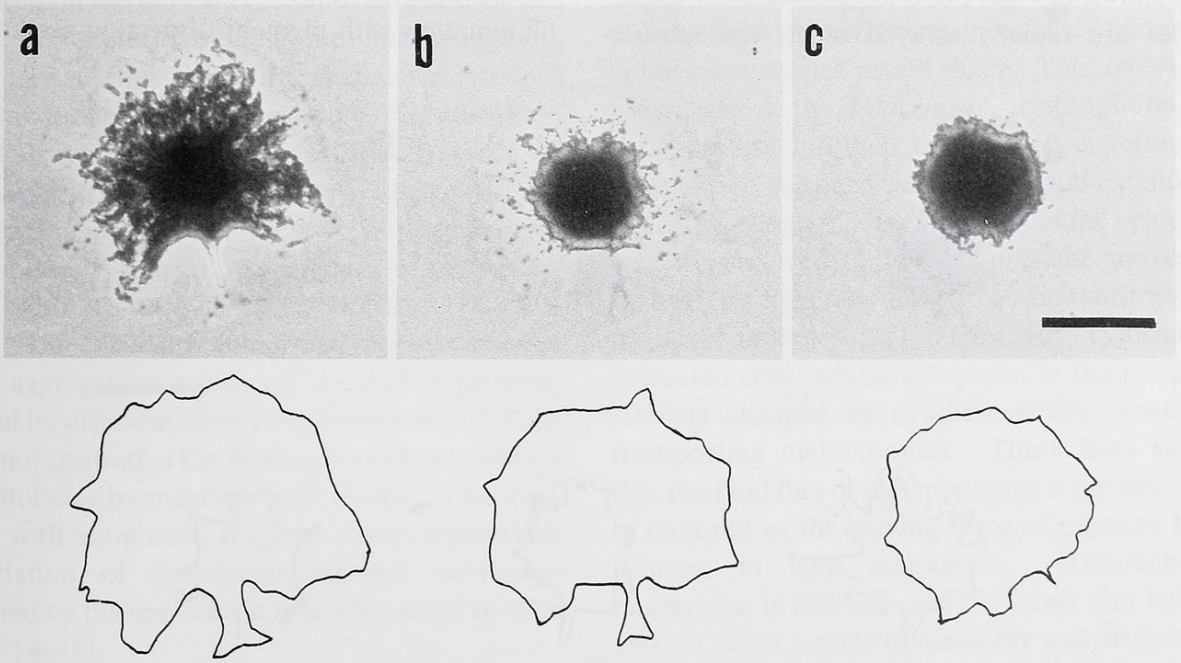

FIG. 2. Light micrographs showing the effect of colcemid (5 μM). a. Control, dispersed state. b. Aggregated state after 60 min colcemid treatment in the dark. c. Aggregated state after 20 min illumination followed the same treatment as in b. The cell shape is drawn below the micrograph of each cell.

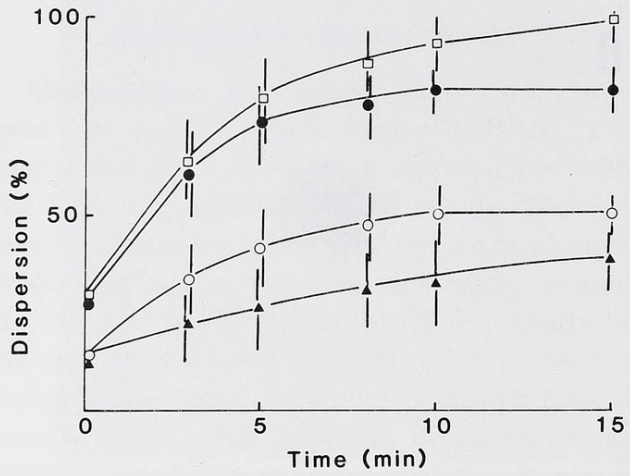

FIG. 3. Effect of colcemid (5 μM) and cytochalasin B (10 μg/ml) on light response of melanophores. Melanophores were treated in the dark with colcemid for 60 min (▲), or cytochalasin B for 60 min (●) or 120 min (○), or in the saline (□), and then illuminated at 600 lux. Each point represents the mean ± S.E. for 23–30 cells.

When exposure time to cytochalasin B under the dark condition was prolonged to 120 min, pigment dispersion in melanophores by the following irradiation was inhibited by 60% (Fig. 3).

Immunofluorescence staining of cytoplasmic microtubules

As shown in Figure 6, a large number of microtubules are radially arrayed in an epinephrine-aggregated melanophore (Fig. 6a). This situation is similar to those in a dispersed cell (Fig. 6b), while fewer microtubules are found in the dark-aggregated cell (Fig. 6c). When a melanophore was exposed to 5 μM colcemid in the dark for 60 min, radial alignments of microtubules were disrupted in most areas except the cell body (Fig. 6e). In a melanophore exposed to 5 μM colcemid in light for 60 min, a number of microtubule arrays were preserved as indicated in Figure 6d. The disruption of microtubules as described for Figure 6e was similarly observed in melanophores after treatment with nocodazole (1 μM) (data not shown).

DISCUSSION

The present results suggest that diassembly and assembly cycles of microtubules in the cytoplasm participate in the light-dependent migration of pigments in *Oryzias* melanophores. The microtubules are thought to be indispensable for the rapid pigment migration in fish chromatophores [4–6]. However, the role of microtubules in centrifugal pigment migration remains to be clarified. In the present study, colcemid-treated melanophores, where radial alignments of microtubules almost disappeared (Fig. 6e), responded no longer to illumination with pigment dispersion as shown in

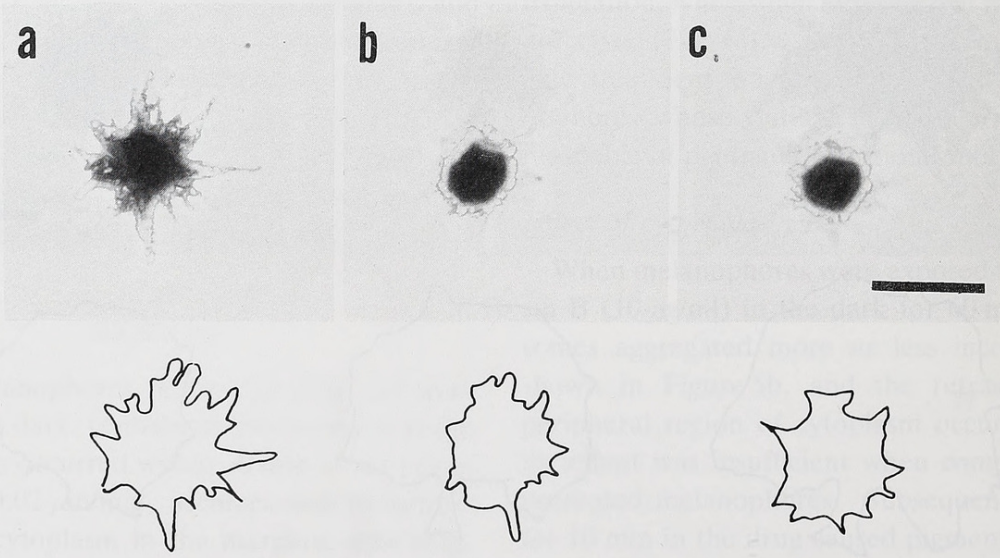

FIG. 4. Light micrographs showing the effect of nocodazole (1 μM). a. Control, dispersed state. b. Aggregated state after 20 min nocodazole treatment in the dark. c. Aggregated state after 10 min illumination followed the same treatment as in b. The cell shape is drawn below the micrograph of each cell.

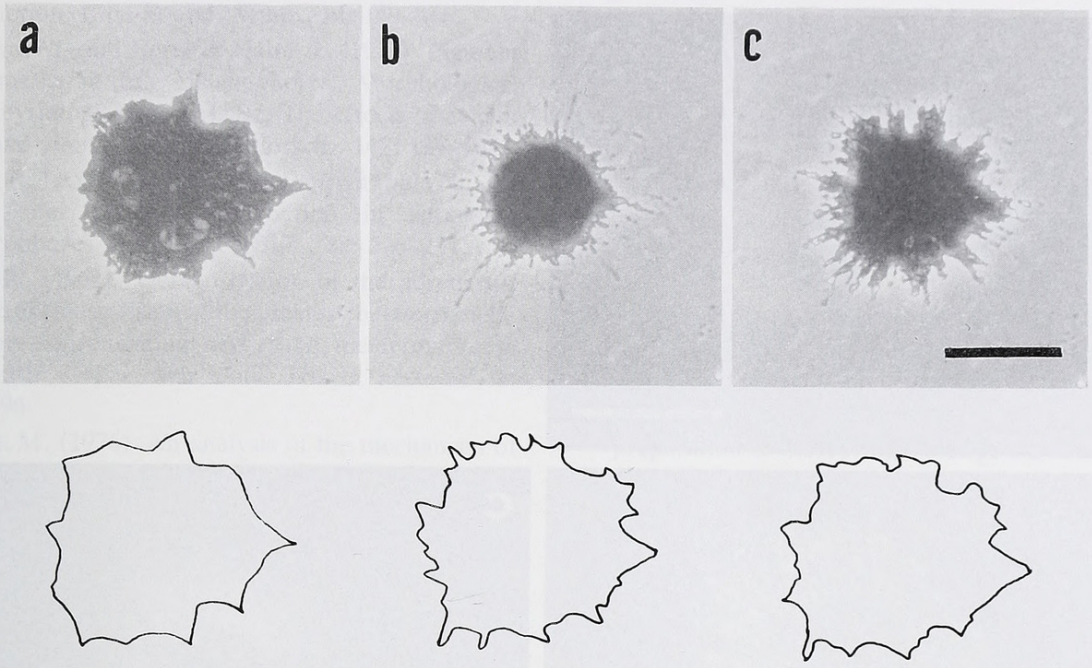

FIG. 5. Light micrographs showing the effect of cytochalasin B (10 μg/ml). a. Control, dispersed state. b. Aggregated state after 60 min cytochalasin B treatment in the dark. c. Dispersed state after 10 min illumination followed the same treatment as in b. The cell shape is drawn below the micrograph of each cell.

Figure 3. This observation suggests that assembly of the cytoplasmic microtubules plays an important role in the centrifugal pigment transport induced by light.

The fact that the rate of centripetal melanosome migration in the dark was 20 times slower than that in the epinephrine-treated one, may suggest a difference in the transport mechanism between both kinds of pigment aggregation. The number of radial arrays of microtubules is distinctly less in the dark-aggregated melanophores than in the epinephrine-aggregated ones, suggesting that the dark-induced pigment aggregation is closely related with disassembly of cytoplasmic microtubules. The concept that the slow speed of melanosome aggregation induced in the dark is probably caused by disassembly of cytoplasmic microtubules does not contradict the findings that disassembly of microtubules by means of cold treatment or treatment with antimitotic reagents causes remarkable retardation of centripetal pigment movement attained by the application of α-adrenergic agonists [3–6, 14–16].

In the melanophores, whose outlines were not changed by the treatment with cytochalasin B, pigment migration occurred in both directions. This observation suggests that microfilaments are not always important as the motile system of pigment transport in light adaptation of *Oryzias* melanophores. The cell shape of melanophores changed greatly with the colcemid treatment, suggesting that the microtubules, which run from the cell center to the periphery, most likely participate in the maintenance of cell shape. This agrees with the results with *Holocentrus* erythrophores [7]. Melanophores, in their light response, retract the cytoplasmic periphery in the dark and extend it in light, suggesting that the fluid flux in the cytoplasm may be involved in the melanosome movement derived by light on and off as demonstrated in *Xenopus* tadpoles [17]. However, cytochalasin B-treated cells, whose cytoplasm in the periphery scarcely changed by irradiation, are capable of transporting melanosomes. These facts suggest that the fluid flux of melanophores is not necessarily required as the driving force of pigment translocation in light adaptation. Although the machanism is still not clear, it seems that light on and off starts a cycle of assembly and disassembly of microtubules, which is involved in pigment displacement of melanophores, and results in the alteration of cell shape in response to light. Thus,

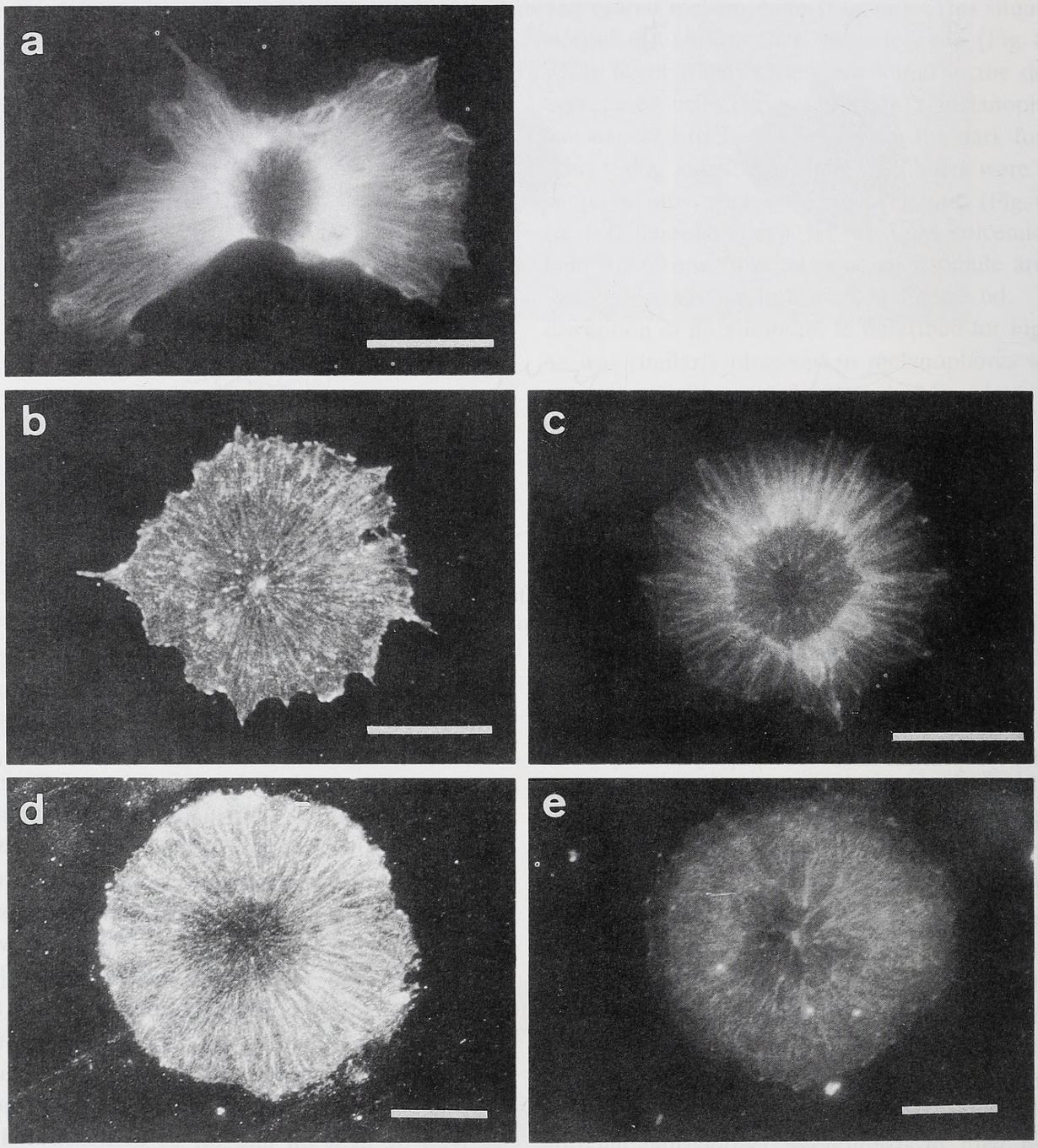

FIG. 6. Localization of microtubules in melanophores revealed by immunofluorescence with tubulin antibody. a. Melanophore aggregated by epinephrine. b. The cell under light. c. The dark aggregated cell. d, e. Colcemid-treated cells in light (d) and darkness (e) for 60 min.

normal number and distribution of microtubules appears to be required to support cell shape.

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