TWO ACTIONS OF CYCLIC AMP ON MELANOSOME MOVEMENT IN FROG SKIN

Dissection by Cytochalasin B

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ABSTRACT

Photomicrography and reflectance microphotometry were used to monitor melanosome movement in frog skin melanocytes in vitro in response to hormonal stimulation and cytochalasin B (CB). Melanocyte-stimulating hormone (MSH), theophylline, and dibutyryl cyclic AMP (DiBcAMP) induced melanosome dispersion (darkening) which was promptly arrested by cytochalasin B in concentrations of 5–20 μg/ml. Melanosome aggregation (skin lightening) occurred only after removal of the darkening agent (MSH, theophylline, or DiBcAMP) and proceeded in the presence or absence of CB. When CB was added to darkened skins, they did not lighten and melanosomes remained in the dispersed state. Use of CB has permitted the dissection of cyclic AMP-mediated melanosome dispersion into two distinct events. The first, induction of melanosome dispersion, is CB sensitive. The second action of intracellular cyclic AMP involves an uncoupling of the centripetal motive force, and is CB insensitive. In the latter process, production of cyclic AMP appears to produce the same result as application of microtubule-disrupting agents.

INTRODUCTION

The frog skin melanocyte has long been a model for demonstrating the in vitro effects of various agents on intracellular motility. Rapid changes in melanosome dispersion (darkening) and melanosome aggregation (lightening) are easily monitored photometrically in an in vitro assay system (17). Melanocyte-stimulating hormone (MSH) induces melanosome dispersion, while aggregation occurs in the absence of the hormone. MSH-induced melanosome movement has been shown to be a special case of the second messenger hypothesis, advanced by Sutherland and his associates (16), whereby darkening is induced by intracellular accumulation of adenosine 3′:5′-monophosphate (cyclic AMP). The latter compound is produced by presumed activation of adenyl cyclase in frog skin (1). When applied separately, MSH, cyclic AMP, and the phosphodiesterase inhibitor theophylline all induce an intracellular dispersion of melanosomes in frog skin melanocytes (1, 2, 14, 15).

An approach to the understanding of the motile processes involved has been made by the use of compounds which directionally inhibit melanosome motility. Colchicine and other microtubule-disrupting agents have been shown to inhibit melanosome aggregation, but not dispersion (11, 12, 20). Cytochalasin B (CB), a disrupter of microfilaments and various types of cellular motile functions (19), has been reported to inhibit melanosome dispersion in lightened skins (12) and to induce melanosome aggregation in dark-

ened skins (8, 9). The present investigation was undertaken in order to dissect, by means of CB, the cyclic AMP-mediated processes involved in melanosome movement. The results of this investigation allow conclusions to be made concerning the involvement of cyclic AMP in the bidirectional system of melanosome movement.

MATERIALS AND METHODS

Frogs (*Rana pipiens*) were obtained from Mogul-Ed (Oshkosh, Wis.), from January through May, 1972, and kept no longer than 3 wk.

Animals were killed by decapitation, after which four skins were removed: two leg and two thigh skins per frog. In most experiments four frogs were used. One skin from each of four different frogs was subjected to the same experimental procedure. Further details are provided in the figure legends.

After soaking in Ringer's solution for 1 h, skins were mounted on plastic rings and inserted into incubation chambers (Fig. 1). A total of four incubation chambers was used, each containing a skin and approximately 1 ml of solution. The chambers were open at the top to permit gas exchange, and were keyed to fit into a plastic receptacle on the microscope stage (Figs. 1, 2). By this arrangement, each of four incubation chambers was inserted suc-

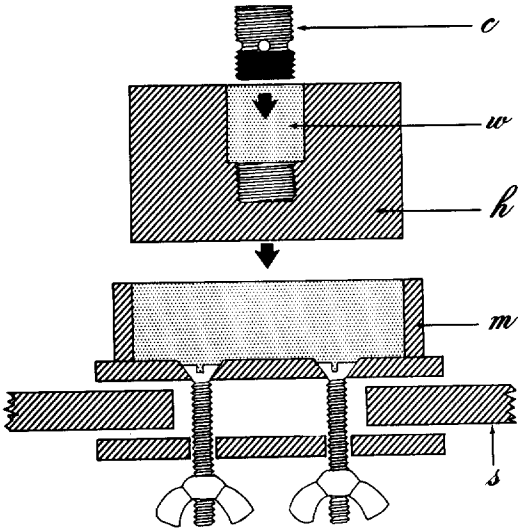

FIGURE 1 Incubation apparatus for frog skin (actual size). Skin is stretched over top of threaded cylinder (*c*) and secured with thread. Cylinder is then screwed into holder (*h*) after well (*w*) has been filled with incubation medium. The holder is inserted into the microscope mount (*m*) which has been secured to microscope stage (*s*) by nuts and blots. All parts shown are plexiglass except nuts, bolts, and stage.

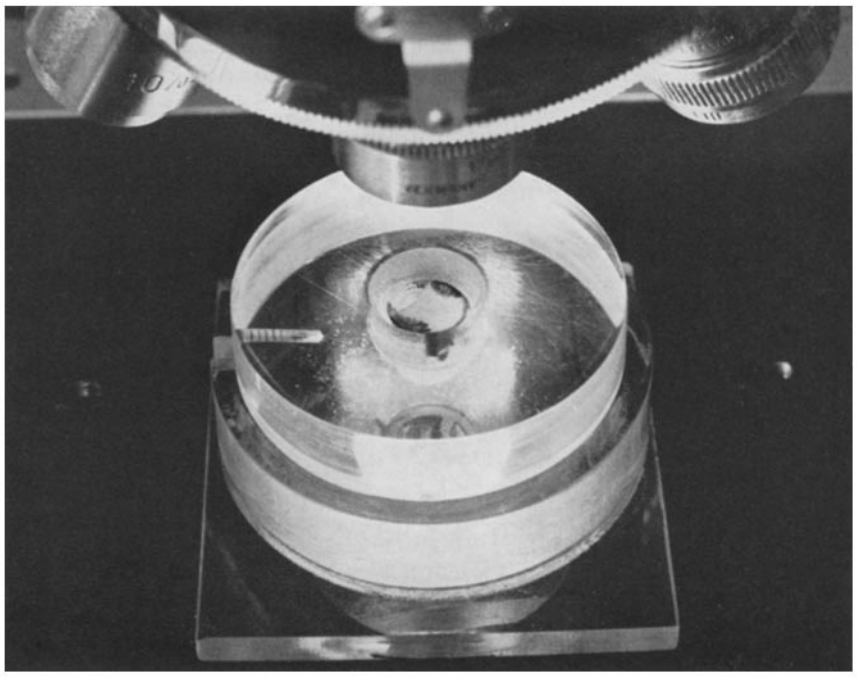

FIGURE 2 Incubation apparatus with frog skin *in situ*. Interspot region of frog skin was used for all experiments. A small hole has been made at edge of skin to prevent entrapment of air bubbles and to permit circulation of incubation medium.

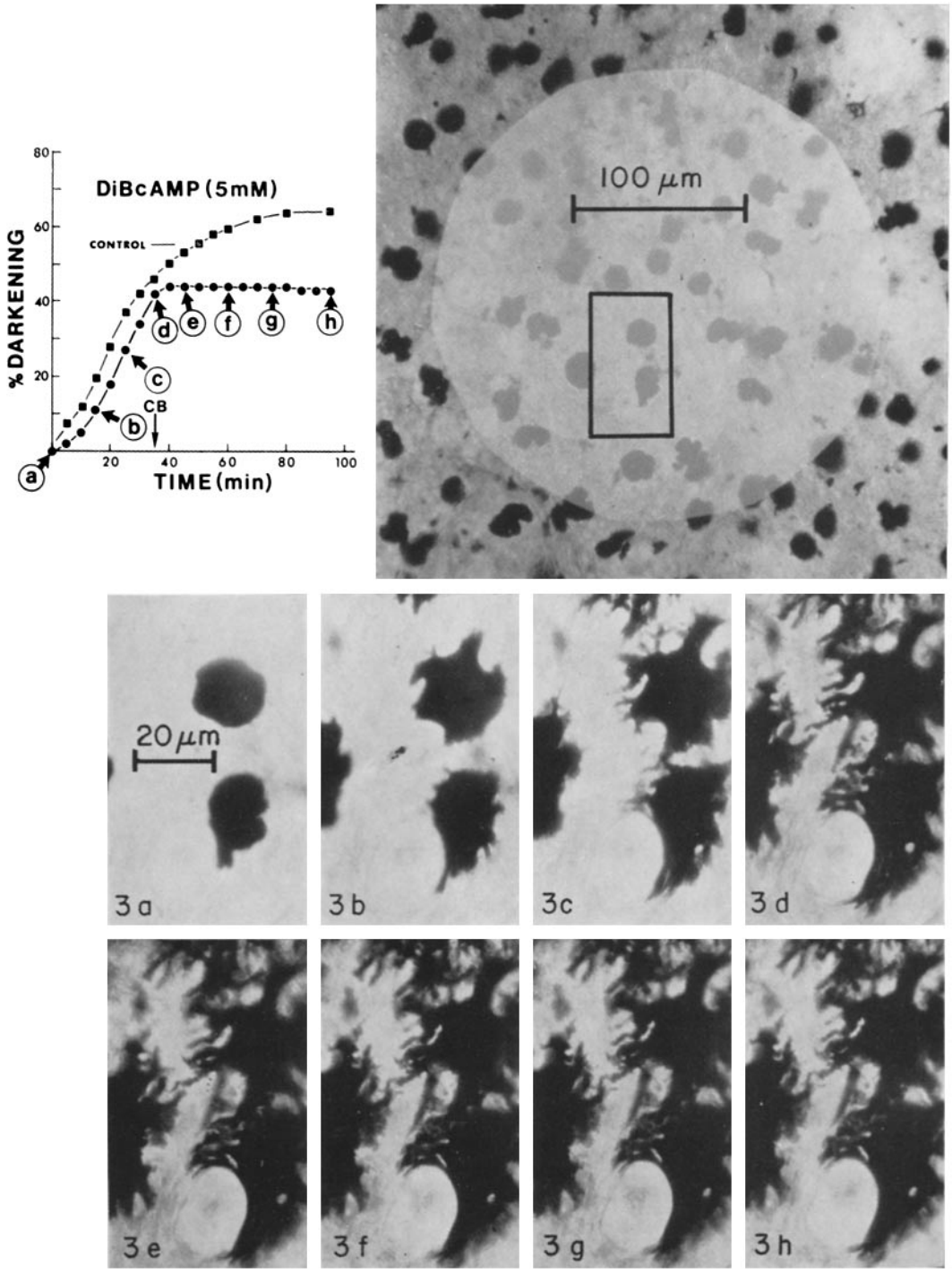

FIGURE 3. Effect of CB on darkening induced by 5 mM DiBcAMP. At 35 min, solution was replaced by fresh DiBcAMP containing 10 μg/ml CB. Control (squares) represents the average values of four skins from one frog incubated in 5 mM DiBcAMP. Letters *a–h* in graph correspond to photomicrographs (bottom) taken at 0, 15, 25, 35, 45, 60, 75, and 95 min, respectively. Photomicrographic field (upper right), × 180. Sequential melanocyte photographs (bottom), × 450.

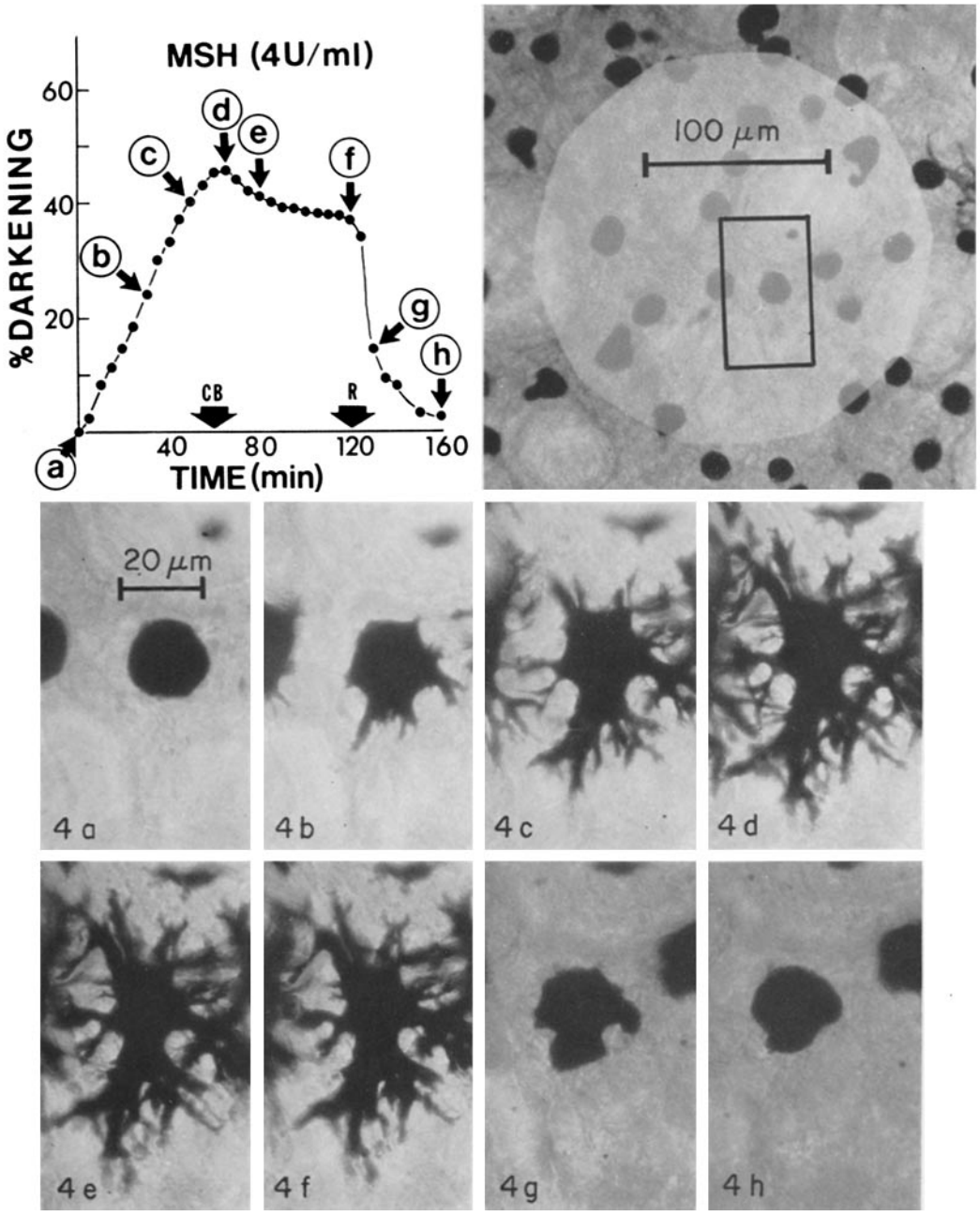

FIGURE 4 Effect of CB on darkening induced by 4 U/ml MSH. At 60 min solution was replaced by fresh MSH containing 10 μg/ml CB. Letters *a–h* in graph correspond to photomicrographs (bottom) taken at 0, 30, 50, 65, 80, 120, 130, and 160 min, respectively. Photomicrographic field (upper right), × 180. Sequential melanocyte photographs (bottom), × 450.

cessively into the stage receptacle in order to retrieve the same microscopic field for each specimen.

A Leitz MPV microphotometer was used to record melanosome dispersion both photometrically and photographically. A standard fluorescent lamp (American Optical Corp., Scientific Instrument Div., Buffalo, N. Y.). provided epi-illumination for reflectance measurements through a 3.5 × objective. The measuring diaphragm was adjusted to circumscribe an area of skin approximately 1 mm^2. Transmitted light via substage illumination (150 watt xenon) was used with a 25 × objective for photographic recording of individual cells during the course of each experiment.

Ringer's solution (17) was the diluent for all experiments except those involving MSH, in which case 0.01% bovine serum albumin in Ringer's (BSA-Ringer's) was used. BSA was necessary to prevent loss of MSH by adsorption to the walls of the plastic incubation chamber. BSA alone had no effect on melanosome movement.

β-MSH was kindly provided by Drs. S. Lande and A. B. Lerner. Theophylline and the disodium salt of dibutyryl 3′:5′-adenosine monophosphoric acid (DiBcAMP) were obtained from Sigma Chemical Co., St. Louis, Mo. Cytochalasin B (CB), (Imperial Chemical Industries, Amersham, England) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/ml. Working solutions of CB were prepared from this stock.

RESULTS

In order to determine the effect of CB on melanosome movements, experiments were performed using MSH, theophylline, and DiBcAMP as darkening agents. In separate experiments, CB was introduced before addition of the darkening agent, in midcourse of darkening, or after maximal dispersion had been obtained. Changes in photometric reflectance are primarily determined by melanosome movement in dermal melanocytes.

CB Addition in Midcourse of Darkening

Photographic and photometric results are compared when CB was added during the DiBcAMP- and MSH-induced darkening (Figs. 3 and 4). A low magnification view of the skin (Figs. 3 and 4, upper right) shows both the area measured through the microphotometer diaphragm (pale circle) and the field photographed under higher magnification (rectangle). Dermal melanocytes photographed at different time intervals correspond to letters in the graph (upper left). With

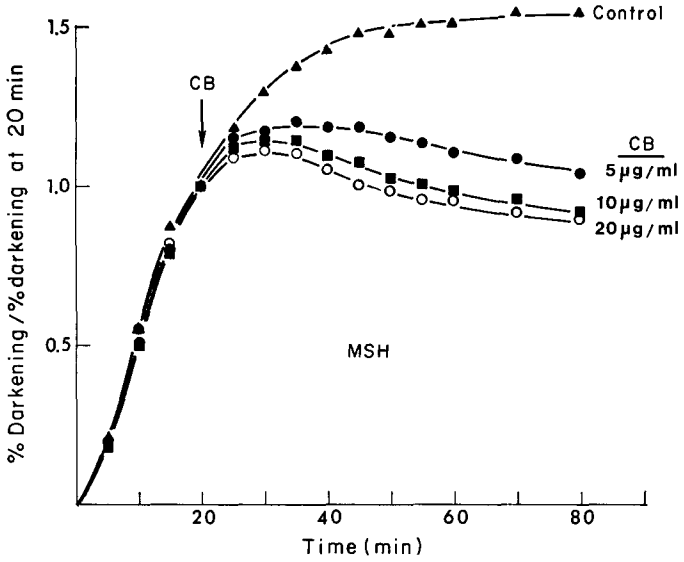

FIGURE 5 Effect of CB concentration on frog skin darkening in MSH. 20 min after induction of darkening by MSH (4 U/ml), solutions were replaced by fresh ones containing 5 (filled circles), 10 (squares), or 20 (open circles) μg/ml CB. In each case total DMSO concentration was 20 μl/ml. At 20 min control solutions (triangles) were replaced by fresh MSH containing 20 μl/ml DMSO. Each point represents the average value from four skins. Four frogs were used, each frog contributing one skin to each of the four groups. Degree of darkening has been expressed as % darkening/% darkening at 20 min in order to set values equal at the time of CB addition. By doing this, interpretation of results has been facilitated.

DiBcAMP as darkening agent (Fig. 3) progressive darkening (determined photometrically) was accompanied by melanosome dispersion in which melanosomes moved from an aggregated or central position in the undarkened skins (Fig. 3 a) to a dispersed position within numerous dendritic processes (Figs. 3 b–d). After addition of fresh DiBcAMP containing CB, melanosome dispersion ceased (Figs. 3 e–h) and was accompanied by stable photometric values. Neither melanosome aggregation nor lightening (as determined photometrically) occurred over the 1 h period after replacement by fresh MSH containing CB (Figs. 4 a–d). The slight photometric lightening which subsequently occurred paralleled a slight centripetal movement of melanosomes (Figs. 4 d–f). After replacement of the medium by Ringer's solution at 2 h, there occurred a rapid aggregation of melanosomes (Figs. 4 g, h). Results similar to those in Figs. 3 and 4 were obtained using theophylline (10 mM) as darkening agent.

The effect of increasing CB concentrations is shown in Fig. 5. CB, from 5 to 20 μg/ml, was added at 20 min to skins darkening in MSH. All CB concentrations arrested darkening and in addition caused some lightening. The time interval between CB addition and cessation of darkening was shorter with 20 μg/ml CB than with 10 μg/ml CB or 5 μg/ml CB. The rate of lightening that occurred after CB addition did not appear to be dependent on CB concentration.

Does CB affect lightening? Two groups of skins were darkened in MSH for 20 min, and followed by fresh MSH containing CB (10 μg/ml) for an additional hour (Fig. 6). One group was then lightened in Ringer's, the other in CB-Ringer's

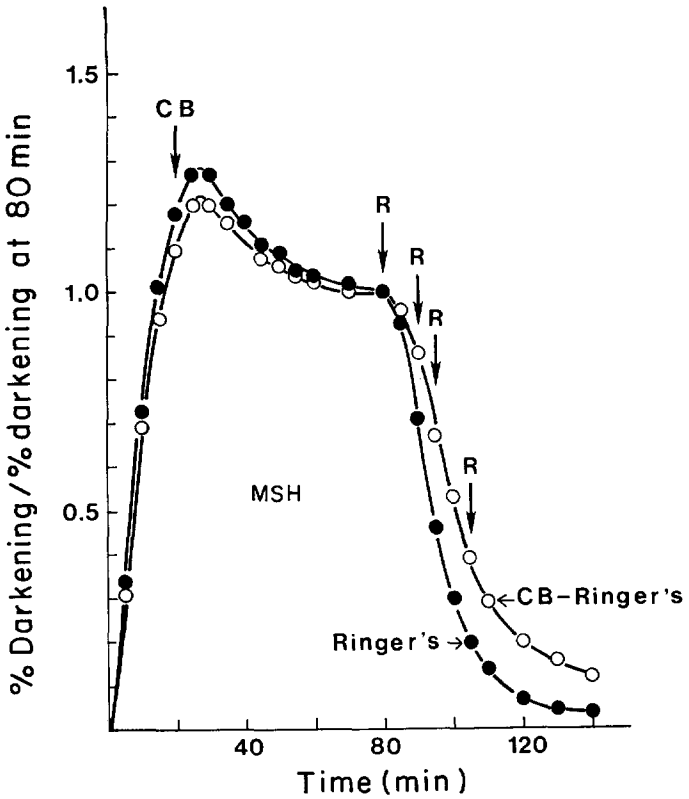

FIGURE 6 Effect of CB on ability of skins to lighten. Both groups of skins were incubated in MSH (4 U/ml) in BSA-Ringer's for 20 min, at which time solutions were replaced by fresh ones containing 10 μg/ml CB. 60 min later one group was rinsed with BSA-Ringer's containing 10 μg/ml CB at 80 min, 90 min, 95 min, and 105 min (open circles). At identical times the control group was rinsed with BSA-Ringer's containing 10 μl/ml DMSO (filled circles). Each point represents the average value from four skins. A total of two frogs was used, each frog contributing two skins to each of the two groups. Degree of darkening has been expressed as % darkening/% darkening at 80 min in order to set relative values equal at 80 min. Comparison of the two lightening rates has thereby been facilitated.

The presence of CB slowed the rate of lightening, but did not prevent complete lightening. 2 h after removal of MSH, both sets of skins had lightened equally. It should be noted, importantly, that removal of MSH alone from the skins in CB-Ringer's (filled circles) was sufficient to permit lightening of skins. It is the presence of the darkening agent which therefore inhibited lightening in CB-treated skins.

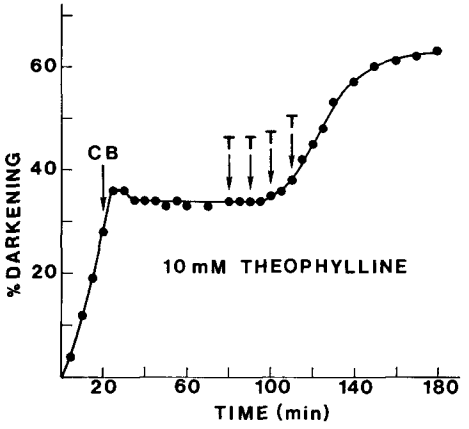

FIGURE 7 Reversibility of CB action. Skins were exposed to 10 mM theophylline for 20 min, at which time solutions were replaced by fresh ones containing 10 μg/ml CB. 60 min later (at 80 min) solutions were replaced by fresh CB-free theophylline. Additional changes were made at 90 min, 100 min, and 110 min. Each point represents the average value from four skins of one frog.

Reversibility of CB action was demonstrated by repeated rinsing of skins in fresh theophylline after a 1-h exposure to theophylline containing CB (Fig. 7). 20 min after the first of four successive 10-min rinses of fresh theophylline, skins resumed darkening. Similar results were obtained using MSH (4 U/ml) or DiBcAMP (5 mM) as darkening agent.

CB Addition after Completion of Darkening

Fresh solutions of CB-containing darkening agent were added to skins after near-maximal darkening in MSH, theophylline, and DiBcAMP (Fig. 8). Over a 4-h interval, skins lightened slightly; MSH-treated skins lightening more than those in theophylline or DiBcAMP. At the end of that time interval photometric readings had reached stable values, i.e., the rate of lightening had become negligible. After removal of the darkening agent, MSH- and theophylline-treated skins promptly lightened. Before lightening ensued,

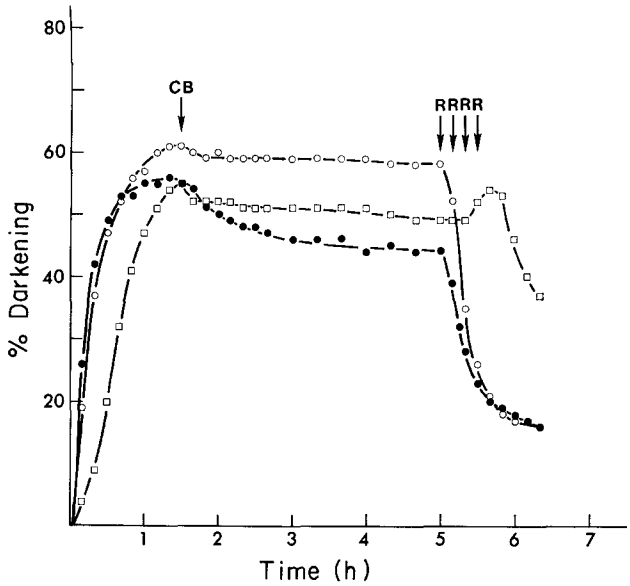

FIGURE 8 Effect of CB after near-maximal darkening. Skins were darkened for 80 min in 4 U/ml MSH (filled circles), 10 mM theophylline (open circles), or 5 mM DiBcAMP (squares). DiBcAMP and theophylline were dissolved in Ringer's; MSH was dissolved in BSA-Ringer's. At 80 min solutions were replaced by fresh ones containing 10 μg/ml CB. At 5 h CB-theophylline and CB-DiBcAMP were replaced by fresh Ringer's, and CB-MSH was replaced by fresh BSA-Ringer's. Three more changes were made at 10-minute intervals. Each point represents the average value from four skins of one frog.

skins treated with DiBcAMP underwent a transitory darkening, indicative perhaps of the time interval between unbinding of CB and hydrolysis of residual DiBcAMP by phosphodiesterase. It appears that removal of darkening agent is a prerequisite for lightening, even in the presence of CB.

In the preceding experiment, photomicrographic results paralleled the photometry (Fig. 9). Addition of DiBcAMP to a lightened skin (Fig. 9 a) resulted in extensive melanosome dispersion at 80 min (Fig. 9 b), at which time CB was added. The degree of dispersion did not appear to change 1 or 2 h later (Figs. 9 c, d). Similar results were recorded photographically when theophylline (10 mM) or MSH (4 U/ml) were used as darkening agents.

The effects of different concentrations of darkening agents on CB-induced lightening are shown in Fig. 10. Skins were darkened in graded concentrations of MSH (Fig. 10 a) or theophylline (Fig. 10 b) to near-maximal values. At that time (80 min) all were treated with fresh CB-containing darkening agent for an additional 80 min before lightening in Ringer's. The results indicate that CB induced slightly more lightening in MSH-darkened skins than those darkened in theophylline. Skins treated with concentrations of theophylline from 1–10 mM lightened an equal amount.

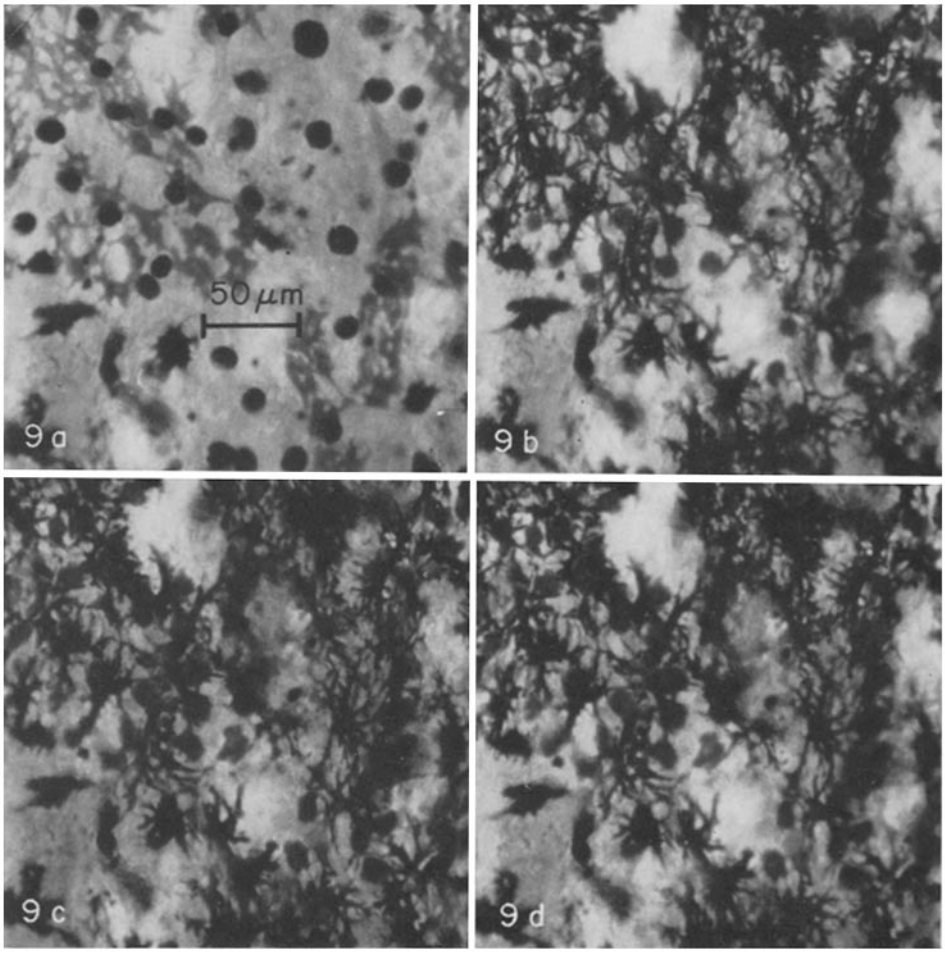

FIGURE 9 Effect of CB on skin previously darkened in DiBcAMP. 5 mM DiBcAMP was added to lightened skin at 0 time (Fig. 9 a). At 80 min (Fig. 9 b), 10 μg/ml CB in fresh 5 mM DiBcAMP was added. Dermal melanocytes were again photographed at 140 and 200 min (Figs. 9 c, d). × 180.

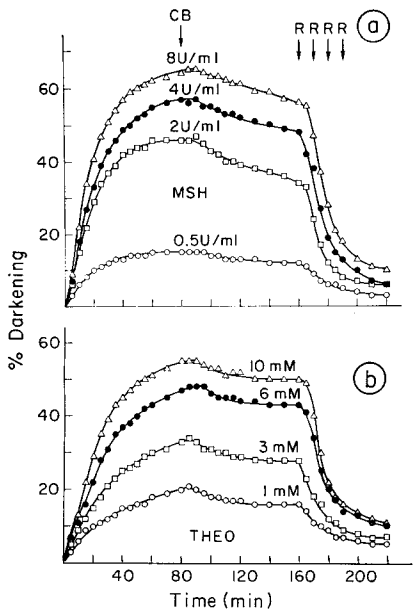

FIGURE 10 Effect of CB on skins previously darkened in varying concentrations of MSH (a) or theophylline (b). Skins were first darkened by MSH or theophylline for 80 min, at which time solutions were replaced by fresh ones containing 10 μg/ml CB. At 160 min skins were rinsed in BSA-Ringer's (a) or Ringer's (b), and rinsed three additional times at 10-min intervals. Each point represents the average value from four skins, one skin from each of four frogs.

Preincubation in CB

Although CB was able to completely arrest melanosome dispersion when added in the course of darkening, preincubation in CB did not achieve the same result. Skins which had been preincubated for 30 min in Ringer's containing CB (10 μg/ml) were treated with fresh CB-Ringer's containing MSH (Fig. 11). In the presence of CB, skins darkened to approximately half the photometric reflectance values of those without CB. The degree of darkening produced by MSH after CB preincubation appeared to be dosage dependent, although the differences observed were quantitatively small. Similar results were obtained using theophylline or DiBcAMP as darkening agent.

Photographic evidence supported the photometric data that preincubation in CB did not arrest melanosome dispersion (Fig. 12). 20 min after CB had been added to a lightened skin, dermal melanocytes maintained their punctate appearance (Figs. 12 a, b). After addition of MSH, melanosome dispersion was observed 40 and 60 min later (Figs. 12 c, d).

Since addition of CB at 20 min halts darkening, but pretreatment in CB does not, a series of experiments were performed in which CB was added at intermediate times with DiBcAMP, MSH, and theophylline as darkening agents (Fig. 13). CB was added at: (*a*) −20 min (pretreated in CB for 20 min), (*b*) 0 min, (*c*) 10 min, and (*d*) 20 min after addition of darkening agent. Preincubation in CB and addition of CB at 0 min were unable to halt dispersion. Addition of CB at 20 min arrested dispersion with 5 min, while addition of CB at 10 min slowed dispersion considerably within 5 min.

Epidermal Melanocytes

In Fig. 14, an epidermal melanocyte is shown in punctate form at the time of theophylline (10 mM) addition (Fig. 14 a). After replacement of the solution by fresh theophylline containing 10 μg/ml CB at 20 min (Fig. 14 b), dispersion was arrested and maintained in that position for 40 min (Figs. 14 c, d). At 80 min (Fig. 15 e) aggregation became marked and at 120 min epidermal melanocytes had become almost punctate (Fig. 14 f). Similar results were obtained with MSH (4 U/ml) as darkening agent.

In contrast to results obtained with MSH or theophylline, when skins were darkened with DiBcAMP, epidermal melanocytes showed little or no melanosome aggregation induced by CB (Fig. 15).

DISCUSSION

In this study quantitative photometric data were combined with continuous morphological data from photomicrography. These complementary methods were employed in order to confirm photometric changes as real changes in melanosome distribution. (Iridophores also contribute to reflectance changes).

Whenever feasible, three different darkening agents were employed in each experiment: β-MSH, theophylline, and DiBcAMP. Although each of these compounds acts at a different chemical site (1, 2, 15) all have in common the ability to effect a melanosome dispersion. Since CB acts at some locus between cyclic AMP production and melanosome movement (8, 9), CB action

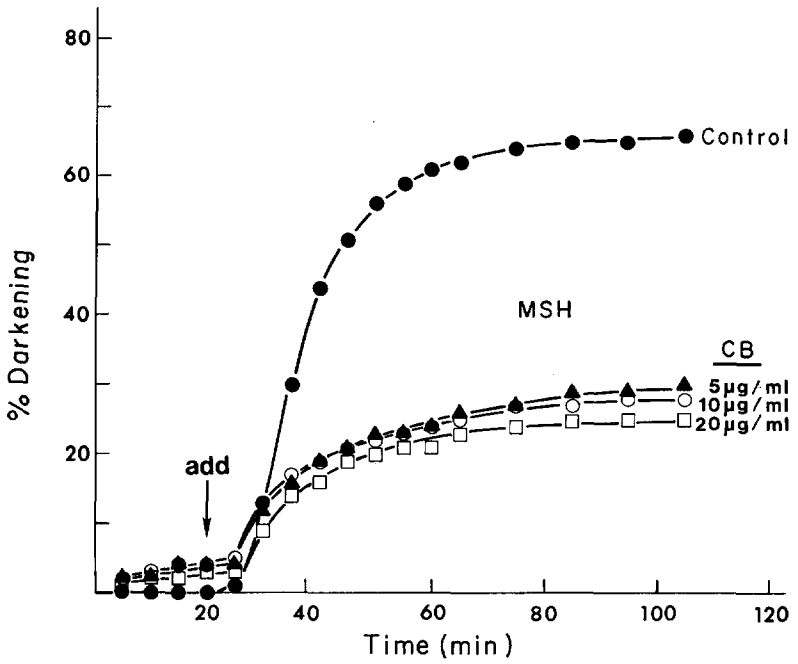

FIGURE 11 Effect of CB preincubation on MSH-induced darkening. Skins were incubated for 20 min in BSA-Ringer's containing CB (5, 10, or 20 μg/ml) or 20 μl/ml DMSO (control). At that time solutions were replaced by fresh ones containing 4 U/ml MSH (in addition to CB or DMSO). The total DMSO concentration for each group was 20 μl/ml. Each point represents the average value for four skins, one from each of four frogs.

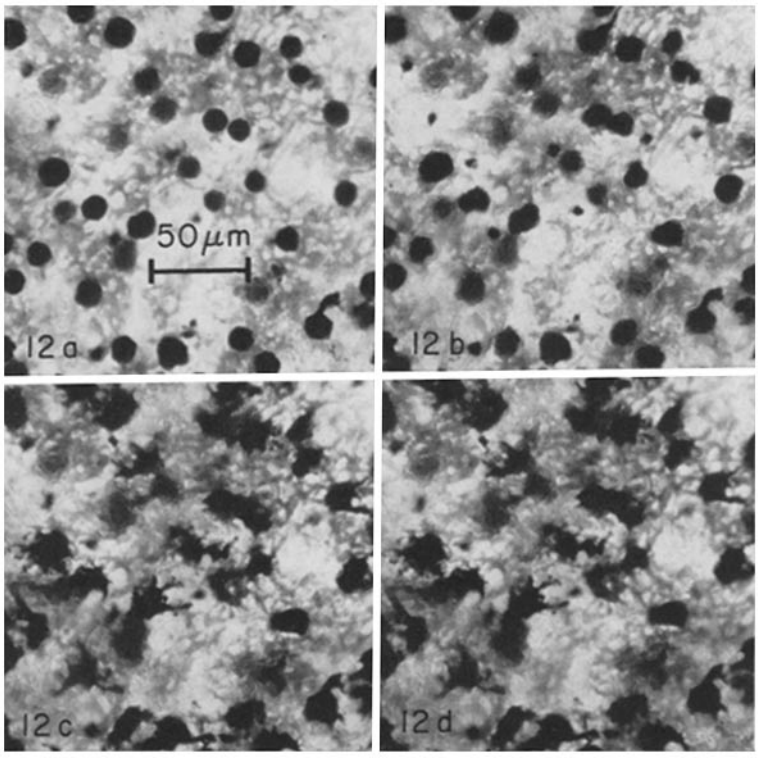

FIGURE 12 Effect of CB preincubation on MSH-induced melanosome dispersion. Skins were preincubated in Ringer's containing 10 μg/ml CB for 20 min, at which time the medium was replaced by a fresh one containing 4 U/ml MSH in addition to CB. Photographs were taken at 0 min (Fig. 12 a), 20 min (Fig. 12 b, time of MSH addition), 60 min (Fig. 12 c), and 80 min (Fig. 12 d) × 180.

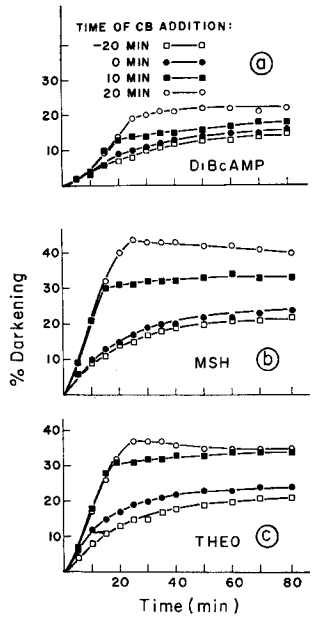

FIGURE 13 Effect of CB (10 μg/ml) when added at varying times. Darkening agents were 5 mM DiBcAMP (a), 4 U/ml MSH (b), and 10 mM theophylline (c). See text for details.

should be independent of the method of darkening. The use of three different means of darkening reduces the possibility of misinterpreting extraneous CB effects as direct effects on the "motile system."

DiBcAMP was much more effective than the naturally-occurring cyclic AMP in producing melanosome dispersion. The potency of DiBcAMP in mimicking cyclic AMP-mediated responses has been explained by resistance of the synthetic compound to phosphodiesterase inactivation (4). The transitory darkening which occurs when DiBcAMP is washed from CB-treated skins (Fig. 8) appears to corroborate the resistance of DiBcAMP to breakdown.

When added during the course of darkening, CB (5–20 μg/ml) caused complete cessation of melanosome dispersion within minutes. The ability of CB to stop dispersion was independent of the agent used to promote dispersion, confirming the participation of CB at some point in time after intracellular production of cyclic AMP (8, 9).

The action of CB in stopping darkening appears to be reversible, confirming the observation of Malawista (13). After repeated rinses in fresh CB-free darkening agents, skins quickly resumed darkening.

The dosage-dependent effects of CB in halting melanosome dispersion were related to the time interval between CB addition and cessation of dispersion. Since increasing concentrations of CB did not increase the rate of lightening, it was concluded that the slight lightening induced by CB was not dosage dependent.

Because CB-induced lightening was much more pronounced with MSH or theophylline than with DiBcAMP, it may represent an action of CB only indirectly related to its effect on the motile apparatus. This explanation is supported by the inability to demonstrate dosage dependency between CB concentration and lightening.

When CB was added at completion of darkening, some lightening followed; more with MSH than with theophylline or DiBcAMP. The rate of lightening decreased quickly, becoming insignificant after 1 h. Melanosome aggregation was not, therefore, a continuous process.

At tested concentrations of theophylline and MSH, the slight lightening always occurred and did not seem to be related to concentration of darkening agent. Complete lightening always occurred shortly after removal of darkening agent, either in the presence or in the absence of CB.

McGuire et al. (8, 9) reported that CB was able to reverse the dispersion induced by either α-MSH (1 U/ml) or DiBcAMP-theophylline (5 mM/1 mM). The experimental evidence of the present investigation strongly indicates that this action of CB appears to be a secondary one: (*a*) the CB-induced lightening was much greater with MSH than with DiBcAMP, (*b*) the CB-induced lightening was not dependent on CB dosage, and (*c*) the CB-induced lightening was not a continuous process. From this evidence we suggest that the lightening effect of CB is secondary to its primary effect of preventing darkening.

Neither preincubation in CB nor addition of CB and darkening agent simultaneously were able to prevent melanosome dispersion. The rate of darkening, however, was markedly reduced and the final degree of darkening was much less. The inhibition of darkening by CB preincubation appeared to be dosage dependent, but the differences were small in the concentration range 5–20 μg/ml.

The basis for the inability of CB to completely arrest dispersion when added to undarkened skins

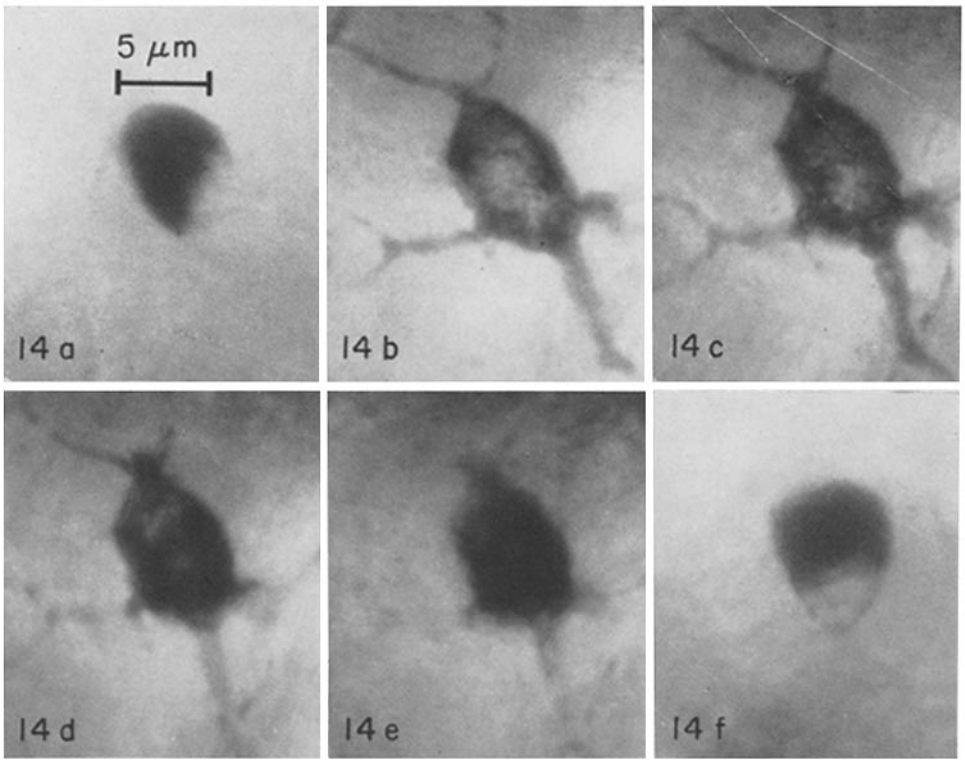

FIGURE 14 Effect of CB on theophylline-induced melanosome dispersion in an epidermal melanocyte. See text for details. × 2,100.

is unknown. Since the rate of dispersion is decreased in the CB-preincubated skins, it is suggested that the dispersion process is at least partially CB sensitive in initial stages of granule movement. At later times (10 min and thereafter) the dispersive process becomes totally sensitive to CB.

The epidermal melanocytes were similar to dermal melanocytes in their CB sensitivity. Epidermal melanocytes showed much more CB-induced lightening with MSH and theophylline, but not with DiBcAMP. As in the case of the dermal melanocyte, action of CB in promoting lightening in these cells is interpreted as a secondary effect.

The use of CB has permitted the dissection of those cyclic AMP-dependent events in melanosome dispersion. The cyclic AMP-induced process of centrifugal melanosome movement has previously been shown to be CB-sensitive and probably involves the "microfilament" motile system (8, 9, 13). It has been shown in the present study that addition of CB during melanosome dispersion does not result in melanosome aggregation as long as cyclic AMP is present. It appears, therefore, that cyclic AMP is acting to prevent the centripetal flow of melanosomes to the perinuclear aggregated state. Upon removal of cyclic AMP, aggregation immediately begins, even in the presence of CB.

Microtubule-disrupting agents are also capable of preventing melanosome aggregation. Wright (20) and Malawista (11) showed that preincubation of frog skins in colchicine did not affect subsequent MSH-induced darkening, but did prevent skins from lightening. The study was extended to include other microtubule-disrupting agents such as colcemid, vinblastine, vincristine, and griseofulvin (12).

Microtubule-disrupting agents also interrupt other nonmitotic cellular motile phenomena such as antigen-induced release of histamine (3), hydrolytic enzyme release from phagocytes (18), and emiocytosis of insulin-containing granules (6). Cyclic AMP appears to be involved in the mediation of some of these microtubule-associated phenomena. Insulin secretion is stimulated

(10), while hydrolytic enzyme release (18) and histamine release (7) appear to be inhibited by cyclic AMP.

Malawista has proposed that microfilaments provide the motive force for dispersion, while microtubules are important in aggregation (12). It appears that in the frog skin melanocyte, production of intracellular cyclic AMP acts as a switch, inactivating the microtubule-associated aggregating system while simultaneously activating the CB-sensitive microfilament system (Fig. 16). Decreasing the cyclic AMP concentration

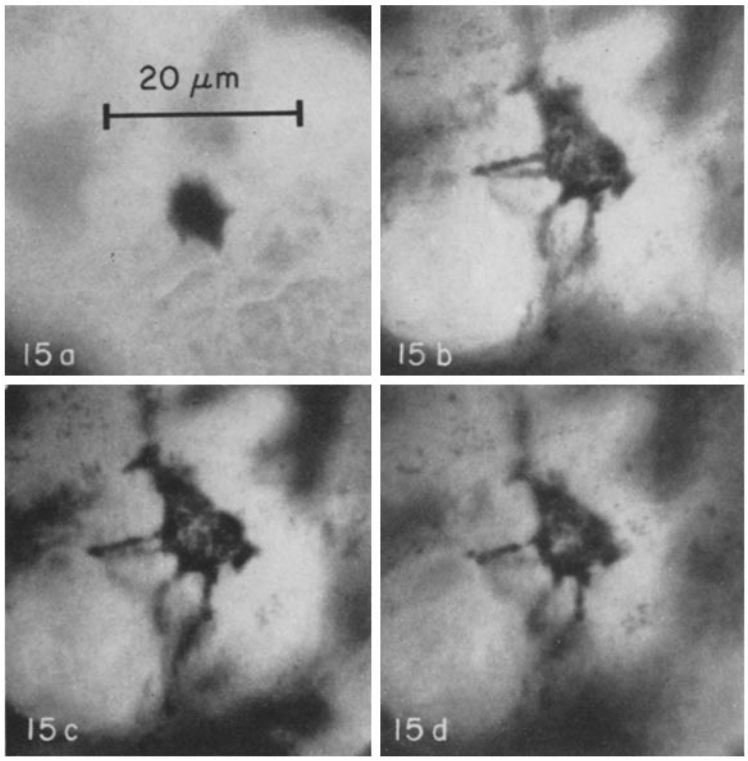

FIGURE 15 Effect of CB on DiBcAMP-induced melanosome dispersion in an epidermal melanocyte. 5 mM DiBcAMP was added to a lightened skin (Fig. 15 a). At 80 min the medium was replaced by a fresh one containing 10 μg/ml in DiBcAMP (Fig. 15 b). The same cell is seen 60 and 120 min later (Figs. 15 c, d). × 1,200.

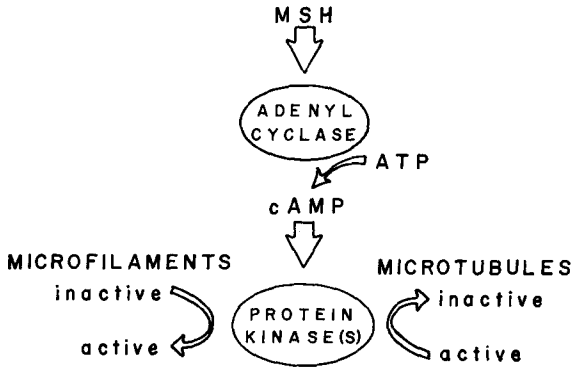

FIGURE 16 Involvement of cyclic AMP in melanosome movement. See text for explanation.

reverses the effect. Although it has been hypothesized that all physiological effects of cyclic AMP are due to activation of protein kinases (5), the chemical events transpiring between cyclic AMP production and melanosome movement remain to be elucidated.

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