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Switching between Microtubule- and Actin-Based Transport Systems in Melanophores Is Controlled by cAMP Levels

Vladimir Rodionov,[1] Julie Yi,[2] Anna Kashina,[1] Abiola Oladipo,[1] and Steven P. Gross[2,*]
[1]University of Connecticut Health Center
Department of Physiology and
Center for Biomedical Imaging Technology
263 Farmington Avenue
MC150 Farmington, Connecticut 06032-1507
[2]Department of Developmental and Cell Biology
2222 Nat. Sci. I
University of California, Irvine
Irvine, California 92697

Summary

Background: Intracellular transport involves the movement of organelles along microtubules (MTs) or actin filaments (AFs) by means of opposite-polarity MT motors or actin-dependent motors of the myosin family. The correct delivery of organelles to their different destinations involves a precise coordination of the two transport systems. Such coordination could occur through regulation of the densities of the two cytoskeletal systems or through regulation of the activities of the cytoskeletal motors by signaling mechanisms.
Results: To investigate the mechanisms of switching between MT and AF-dependent transport, we examine the influence of the densities of the MT and AF network on pigment transport in fish melanophores. We also change signaling by using activators and inhibitors of Protein Kinase A (PKA). We find that the key parameters characterizing pigment granule transport along MTs do not depend on MT density and are not significantly altered by complete disruption of AFs. In contrast, the kinetics of changes in these parameters correlate with the kinetics of changes in the intracellular levels of cAMP and are affected by the inhibitors of PKA, suggesting the regulation of MT- and AF-dependent motors by cAMP-induced signaling. Furthermore, perturbation of cAMP levels prevents the transfer of pigment granules from MTs onto AFs.
Conclusions: We conclude that the switching of pigment granules between the two major cytoskeletal systems is independent of the densities of MT or AF but is tightly controlled by signaling events.

Introduction

Intracellular transport of organelles is essential for the functioning of living cells and has been shown to underlie such fundamental biological processes as secretion [1], neuronal signaling [2], organization of endomembranes [3], and cell division [4, 5]. The driving force for intracellular transport is provided by molecular motors bound to the surface of cargo organelles.

*Correspondence: sgross@uci.edu

Molecular motors interact with cytoskeletal structures, microtubules (MTs), or actin filaments (AFs), which serve as "rails" for the movement of cargo organelles. AF-dependent motors, myosins, generally move the cargo to the plus ("barbed") ends of AFs [6, 7, 8], although a minus-end directed variant of myosin has been also reported [9]. MT-dependent motors include kinesins, which generally support transport to the MT plus ends [2, 10], and dyneins, which are exclusively minus-end directed [11, 12]. It is generally believed that MT motors support long-distance movement of organelles, whereas myosins are responsible for local transport [13, 14, 15].

The two transport systems work together to deliver organelles to various cellular destinations (see [16] for a review). The same organelles often bear both MT-dependent and AF-dependent motors [17, 18, 19], and genetic manipulations also indicate interconnection between MT-based and AF-based transport [20, reviewed in 16]. Thus, intracellular transport is a complex process, driven by at least three types of motors involved in bidirectional MT transport and transport along AFs. The question of how these types of transport are coordinated has been a focus of many recent studies; however, the mechanisms of this coordination are still largely unknown. In this work, we address the question of the mechanisms that control the switching between MT- and AF-based organelle transport.

Recent work indicated that the switching between the two types of cytoskeletal tracks is based on a continuous tug-of-war between the transport systems [21], wherein MT and AF motors are simultaneously active on the same organelle and the choice of tracks is determined by competition between these motors. Furthermore, the dynein-dependent but not the kinesin-dependent component of MT transport was shown to be sensitive to AF-based interference [21]. It is still unknown, however, how cells control the competition and therefore determine the contribution of each system to the transport.

Here we investigate how the interaction between the two systems is controlled by examining the movement of pigment granules in fish melanophores. These large cells function by rapidly redistributing thousands of membrane-bounded pigment granules to allow chromatic adaptation of fish to the environment [22, 23]. This pigment transport involves aggregation or dispersion of pigment granules along radial MTs mediated by cytoplasmic dynein [24] and kinesin motors [25, 26]. During dispersion, rapid transport of pigment granules along MTs toward the cell periphery is followed by homogeneous distribution of pigment granules in the cytoplasm along the randomly arranged AFs [27]; this latter distribution is mediated by an AF-dependent motor, myosin V [18]. During aggregation, pigment granules leave AF tracks and move to the cell center along MTs. Therefore, redistribution of pigment granules in melanophores requires a remarkable degree of functional interaction between the two transport systems. However, it remains

unknown how the coordination between these transport systems is controlled.

Conceivably, there are two likely mechanisms that could control the relative activity of each transport system and influence the competition between them. The first mechanism concerns the physical availability of MT and AF "rails" in the vicinity of the moving granule. Because the density of MTs is higher at the cell center, whereas AFs are distributed more or less uniformly with a bias to the cell periphery [27, 28], the frequency of interactions of MT motors with cytoskeletal substrate is potentially enhanced in the middle of a cell, whereas myosin motors could contact AFs more often at the margin. Thus, during dispersion, variations in the densities of cytoskeletal elements might play a significant role in determining when granules leave MTs and bind to AFs, and the outcome of the tug-of-war between the transport systems might be determined by the frequencies of interactions of pigment granules with each type of cytoskeletal substrate.

The other possible mechanism for controling the balance between MT- and AF-dependent transport is through a signaling cascade. In melanophores, global regulation of pigment transport involves changes in cytoplasmic levels of the second messenger, cAMP ([30, 31, 32]; reviewed in [23]). High cAMP levels induce pigment dispersion, whereas low levels cause aggregation. In some pigment cells, aggregation could be also triggered by a transient increase in intracellular concentration of Ca^{2+} ions [32, 34]. The level of the second messenger then controls the activities of protein kinases or phosphatases, which presumably change the activities of the relevant molecular motors ([33, 34] reviewed in [23]). Therefore, signaling cascades might independently regulate MT and AF-based motors and thus control their relative contributions at different times during dispersion or aggregation.

To investigate the mechanisms that control the balance between different transport systems during pigment redistribution in fish melanophores, we quantified the physical parameters of MT-dependent and AF-dependent movement of pigment granules in the course of pigment aggregation or dispersion. We find that during dispersion the key parameters characterizing pigment granule transport along MTs did not depend on MT density and were not significantly altered by complete disruption of AFs. In contrast, the changes in MT- and AF-dependent transport correlated with the levels of the second messenger cAMP and were affected by an inhibitor of Protein Kinase A (PKA). Furthermore, perturbation of cAMP levels prevented the transfer of pigment granules from MTs onto AFs. We conclude that the switching of pigment granules between the two major cytoskeletal systems is independent of the densities of MT or AF but is tightly controlled by signaling events.

Results

Pigment Granule Movement in Fish Melanophores Employs Dynein, Myosin, and Kinesin Family Members

Cultured black tetra melanophores rapidly aggregate pigment granules in the cell center after the stimulation with adrenalin or more slowly redisperse granules after adrenalin withdrawal (see reference [25] and Figure 1A). Redistribution of pigment granules in melanophores generally requires the activities of opposite-polarity MT motors and myosin V (reviewed in [23]): the MT motors move along radial MT tracks (Figure 1C, left), whereas myosin V moves along more uniformly distributed AFs (Figure 1C, right). The apparent high density of AFs at the cell center (Figure 1C, right) simply reflects variations in the cell's thickness, as discussed in (ii), below. To examine the composition of molecular motors involved in pigment transport in black tetra melanophores, we have probed the preparations of pigment granules with antibodies against the major motor protein types (Figure 1B). In agreement with previous observations [18, 25, 29], antibodies against the intermediate chain of cytoplasmic dynein (74.1; [35]) and the heavy chain of myosin V (DIL-2; [36]) recognized the polypeptides with expected molecular masses of approximately 80 kDa (Figure 1B, left lane) and approximately 200 kDa (Figure 1B, right lane). A pan-kinesin function-blocking antibody HD Kin2 that inhibits pigment dispersion in black tetra mela-

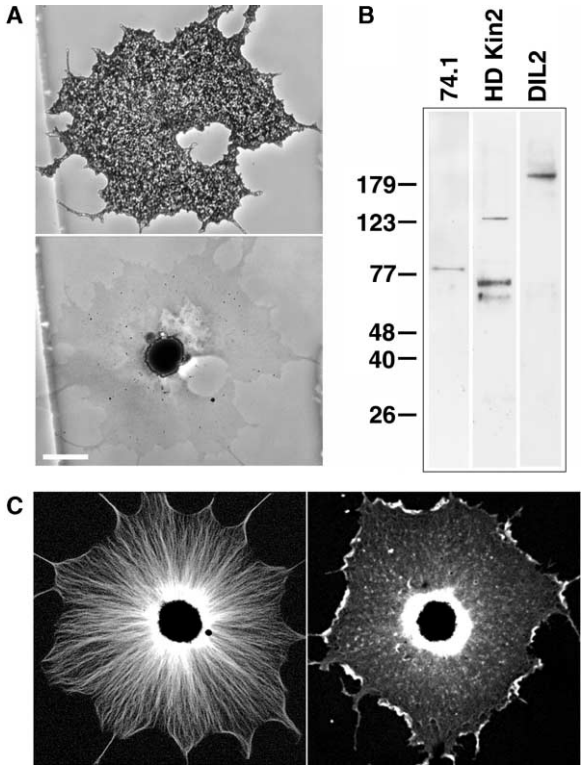

Figure 1. Pigment Granules in Fish Melanophores Are Transported by Means of MT and AF Motor Proteins

(A) Black tetra melanophore before (top) or 5 min after (bottom) the stimulation with adrenalin (0.5 μM)

(B) Immunoblotting of pigment granules with antibodies specific to major types of motor proteins. Left lane, dynein antibody 74.1; middle lane, a pan-kinesin antibody HD Kin2; right lane, myosin V antibody DIL2

(C) Fluorescence staining of fish melanophores with anti-tubulin (left) and Rhodamine-phalloidin (right) shows the intracellular distribution of MTs and AFs, respectively. The scale bar represents 30 μm.

nophores [25] crossreacted with three polypeptides of appriximately 130, 70, and 60 kDa (Figure 1B, middle lane), indicating the presence of kinesin motors in the granule preparation. Therefore, pigment granules in black tetra melanophores contain the three major motor types, cytoplasmic dynein, kinesin family member(s), and myosin V, that can transport granules along MTs and AFs.

Pigment Transport Occurs through MT and AF Transport Coordination that Involves Switching between the Two Cytoskeletal Systems

To study the mechanisms that control the balance between different transport systems during granule movement, we tracked individual pigment granules and computed the physical parameters of the movement along each type of cytoskeletal substrate [37, 38, 39]. We measured the MT component of the movement by observing the movement of granules along the MT axes to MT plus (kinesin) and minus (dynein) ends; the computed parameters included velocity and duration of each continuous granule run. We quantified AF (myosin V) motion after MT disruption with nocodazole by measuring the average granule displacement as a function of time.

The velocity of the motion of pigment granules in opposite directions along MTs did not change significantly after the stimulation of aggregation or dispersion, as expected from the known data about the mechanochemical activity of the cytoskeleton motors and in agreement with the previous measurements ([21]; our unpublished data). However, travel distances displayed distinct kinetics. During pigment dispersion, the average length of minus-end MT runs was low, and the average length of plus-end runs was high (Figures 2A and 2B, left plots). After the application of aggregation signal (10 min time points on Figures 2A and 2B), the length of continuous minus-end runs rapidly increased approximately 15-fold (Figure 2A), whereas the length of plus-end runs decreased (Figure 2B). At the same time, the relative amount of runs in each direction remained constant (unpublished data). Taken together, these results indicate that the movement of pigment granules along MTs is regulated through concurrent changes in the length of plus- and minus- end runs.

Remarkably, an initial rapid raise in the length of plus-end runs during dispersion was followed by a slow 2.4-fold decrease in plus-end run length as the granules approached the cell margin (Figure 2B, left plot). The decline in the length of plus-end runs is consistent with the predominance of MT transport early during dispersion, and AF-dependent transport at later stages. To check whether this decrease in MT plus-end runs correlated with an increase in AF-based motion, we examined the actin-based motion of granules in cells lacking MTs; such cells were produced by treatment with the MT inhibitor nocodazole. The average displacement of granules in the absence of MTs rapidly increased at the onset of pigment dispersion and reached the highest level at later stages, when the MT plus-end runs decreased (Figure 2C, left).

Such a simultaneous decrease of MT-based and increase of AF-based transport strongly suggests the existence of a switching mechanism that controls the change from MT-based onto AF-based transport during later stages of dispersion. This switching could be explained by the physical competition of the two cytoskel-

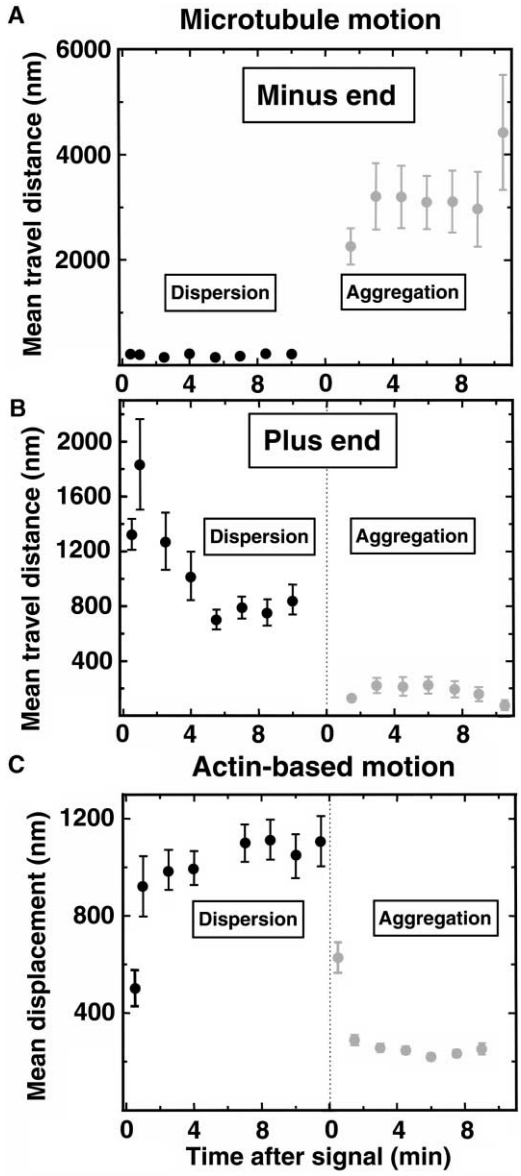

Figure 2. Lengths of Pigment Granule Runs along MTs and AFs Show Distinct Kinetics

(A and B) MT-based transport: changes in the lengths of minus-end runs (A) and plus-end runs (B) along MTs calculated by measurement of continuous displacements of granules to the cell center or to the periphery along the MT axes as determined from the movement trajectories.

(C) Displacement along AFs was measured in MT-disrupted cells produced by combined treatment with cold and nocodazole. AF-dependent movement was quantified by measurement of the distances between the initial and the final positions (r(t)) during the 14 s period of observation. The error bars are standard error of mean. Note the rapid abrupt changes in the lengths of minus-end runs and AF-based motion during transitions between aggregated and dispersed states and the transient increase in the length of plus-end runs during dispersion.

etal systems for binding to the moving granules or by the existence of a regulatory mechanism that controls the relative activities of MT- and AF-based motors.

Granule Movement during Dispersion Is Independent of the Density of AFs and MTs

Because the competition-based switching mechanism would involve concurrent interaction of pigment granules with both types of cytoskeletal substrates, such switching could be sensitive to the densities of MTs and AFs in the cytoplasm. We therefore asked if the length of runs along MTs was affected by the changes in cytoplasmic densities of MTs or AFs.

To examine the role of MT density, we compared the movement of pigment granules during dispersion in areas of the cell with different densities of MTs. To do this, we stimulated melanophores with adrenalin either for 5 min to allow for the complete aggregation of pigment granules or for only 2 min to allow for the movement of the granules about halfway to the cell center. A dispersion stimulus was then applied by perfusion of adrenalin-free medium, and the parameters of MT-dependent transport were measured 1 min thereafter. During 1 min the dispersing granules traveled the distance about 1/4 (H, Figure 3A) or 3/4 (L, Figure 3A) of the radius from the cell center. To estimate the actual difference in MT density at 1/4 and 3/4 cell radius, we have taken into account the following three parameters:

(1) Geometrically, MT density is inversely proportional to the distance from the cell center. Therefore, MTs at 1/4 distance should be three times more dense than at 3/4.

(2) Cell thickness decreases from the cell center to the periphery. Our measurements with injection of fluorescent dextran showed that at 1/4 of the radius the cell thickness is 1.6 times higher than at 3/4 of the radius.

(3) Distribution of MT plus ends examined in our previous work [29] indicates that at 1/4 of the cell radius the amount of MT plus ends is 2.5 times higher than at 3/4. Because the vast majority of cytoplasmic MTs in melanophores are connected to the centrosome at their minus ends, the number of plus ends in a given area of the cytoplasm is an accurate representation of the population of MTs that grow from the centrosome and have reached the area in question.

Taken together, these parameters allowed us to calculate that the pigment granules that traveled the distance of 1/4 of the cell radius at the time of recording were exposed to an MT density approximately five times (i.e., $3 \times 2.5/1.6$) higher than that encountered by those that traveled 3/4 of the cell radius. Despite the significant differences in the MT densities, the movement statistics of MT-dependent transport remained very similar (Figure 3A, lower panel), indicating that the granule movement during dispersion is independent of the density of MTs.

Next, we examined the kinetics of changes in the parameters of MT-dependent transport in cells lacking AFs; such cells were produced by treatment with Latrunculin A, an inhibitor of AF assembly [27]. We reasoned that the inability of pigment granules to interact with AFs might enhance their movement along MTs and therefore prevent the decrease in the length of plus-end runs that is commonly observed during pigment dispersion in intact cells (Figure 2B, left). We found that Latrunculin treatment not only did not enhance the movement along

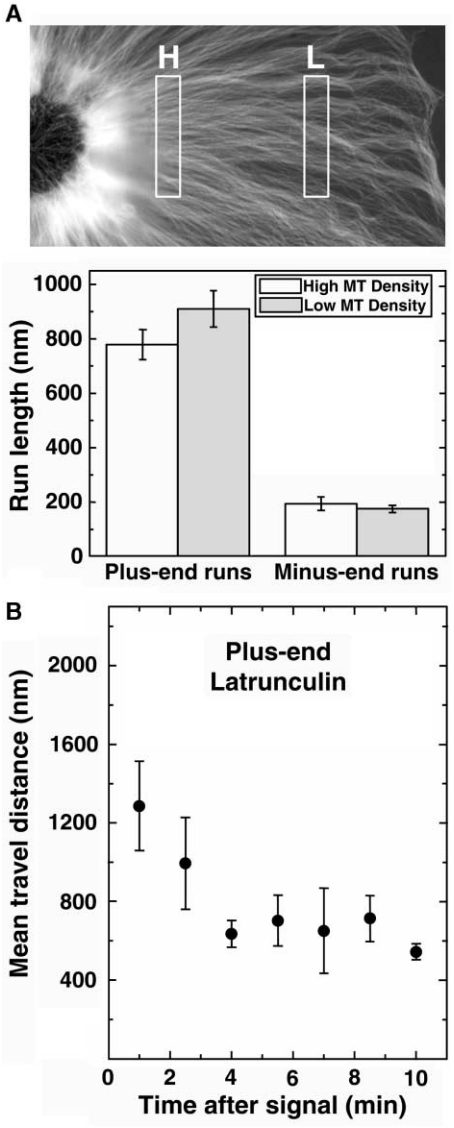

Figure 3. Length of Pigment Granule Runs along MTs Is Independent of the Densities of MTs and AFs

(A) Top, fluorescent staining of MTs in an aggregated cell showing the areas of high (H) and low (L) density of MTs at 1/4 and 3/4 cell radius, respectively. Bottom, the lengths of plus-end (left pair of bars) and minus-end (right pair of bars) runs along MTs were calculated for dispersing pigment granules, which moved through regions of cytoplasm with a high (white bars) or low (gray bars) MT density. For the quantification of the movement statistics at a high or low density of MTs, cells were stimulated with adrenalin for 5 min for the complete aggregation of pigment granules, or for 2 min for a partial aggregation, and immediately treated with dispersion stimulus. Parameters of MT-dependent transport of pigment granules were similar at different MT densities.

(B) Kinetics of changes in the length of plus-end runs in the cells lacking AFs. For the complete depolymerization of AFs, melanophores were treated with an AF-disrupting drug, Latrunculin A (5 μM). Plus-end motion along MTs was not affected significantly by the disruption of AFs (compare bottom panel with Figure 1D). Error bars are SEM.

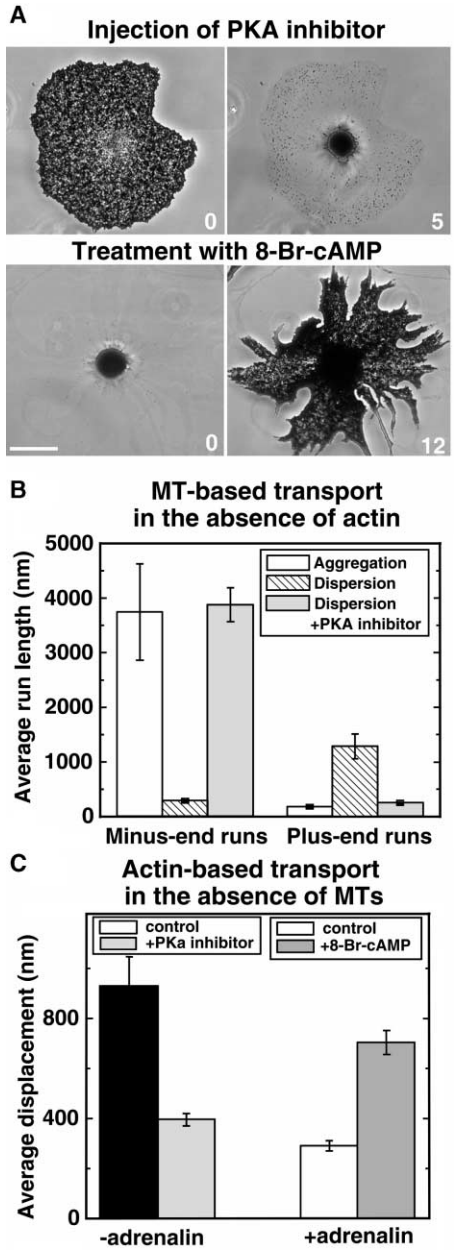

Figure 4. Changes in PKA Activity Induce Changes in Pigment Granule Transport that Are Similar to Aggregation and Dispersion

(A) Images of a cell with dispersed pigment granules before (top left) or after (top right) injection of a peptide PKA inhibitor, as well as a cell with aggregated pigment granules before (bottom left) or after (bottom right) application of PKA activator, membrane-permeable cAMP analog 8-Br-cAMP (1 mM). The scale bar represents 30 μm. Injection of PKA inhibitor triggered pigment aggregation, whereas treatment with 8-Br-cAMP stimulated dispersion.

(B and C). Pigment granule motion parameters quantified separately for MT (B) and AF (C) in cells treated with PKA activator or inhibitor are consistent with those characteristic for pigment aggregation and dispersion. (B) MT transport in the absence of AFs. Cells were treated with Latrunculin A to disrupt AFs, and the granule movement along MTs was examined under the conditions for aggregation (adrenalin treatment), dispersion (adrenalin removal), or PKA inhibitor injection into dispersed (adrenalin-free) cells 10 min after each stimulus. Control (white and striped) bars show the average length of minus-end (left) and plus-end (right) runs during aggregation (white) and dispersion (striped). Filled bars show the length of plus-(right) and minus (left)-end runs in cells injected with PKA inhibitor. Injection of PKA inhibitor into dispersed cells results in run lengths characteristic for pigment aggregation, thus demonstrating quantitatively the result shown in panel (A), top. (C) AF transport in the absence of MTs in cells with MTs disrupted by a combination of cold and nocodazole treatment. Left: PKA inhibition induces a change in the AF-based transport characteristic of the transition from pigment dispersion to aggregation—from the large average displacement at 14 s typical of dispersing cells (black bar) to the smaller average displacement in the PKA inhibitor-treated cells (gray bar). Right: PKA induction by BrcAMP treatment induces a change in the AF-based average displacement characteristic of the transition from pigment aggregation to dispersion—from smaller displacement in aggregated cells (white bar) to larger displacement in cells treated with BrcAMP to stimulate PKA activity (gray bar). The average displacement was calculated as the distance traveled by granules within a 14 s time interval, 10 min after the stimulation. Error bars are SEM.

MTs but also slightly lowered the initial length of plus-end runs at the onset of dispersion, possibly as a result of a slight flattening of cells in the absence of an actin cytoskeleton. At the later stages of dispersion, the decline in the length of plus-end runs seen in cells with intact AFs in Figure 1D (left) was still observed in the cells lacking AFs (Figure 3B). Therefore, the granule movement along MTs during dispersion is independent of the presence of AFs.

Thus, the decrease in the length of plus-end runs during later stages of dispersion is not a simple result of the physical availability of the cytoskeletal tracks that come in contact with the moving granules. These results strongly suggest that the switch between MT- and AF-based transport should occur through independent regulation of the corresponding motors by a signaling cascade.

Pigment Transport in Melanophores Is Regulated by PKA

Regulation of global pigment transport involves changes in the levels of the second messenger, cAMP [23]. High cAMP levels during dispersion activate protein kinase A (PKA), whereas a decrease in cAMP levels during aggregation decreases its activity. Past work showed that PKA is involved in the regulation of pigment granule transport [34]. To confirm the involvement of PKA signaling pathway in the regulation of pigment transport in black tetra melanophores, we studied the role of PKA in pigment granule movement by measuring the lengths of plus and minus end-directed runs in cells either injected with a PKA inhibitor or treated with 8-Br-cAMP, which can serve as a PKA activator.

Injection of dispersed cells with a specific PKA-inhibitory peptide (PKA Inhibitor 6-22 Amide, [40]) induced pigment aggregation mimicking that induced by adrenalin (Figure 4A, top); moreover, granule movement statistics after PKA inhibition were quantitatively the same as those in cells treated with adrenalin (Figures 4B and 4C). Thus, inhibition of PKA activity alone is sufficient to induce pigment aggregation by increasing the average length of minus-end runs, similar to the situation in aggregating cells. At the same time, treating aggregated cells with a cell-permeable cAMP 8-Br-cAMP analog that causes activation of PKA induced pigment disper-

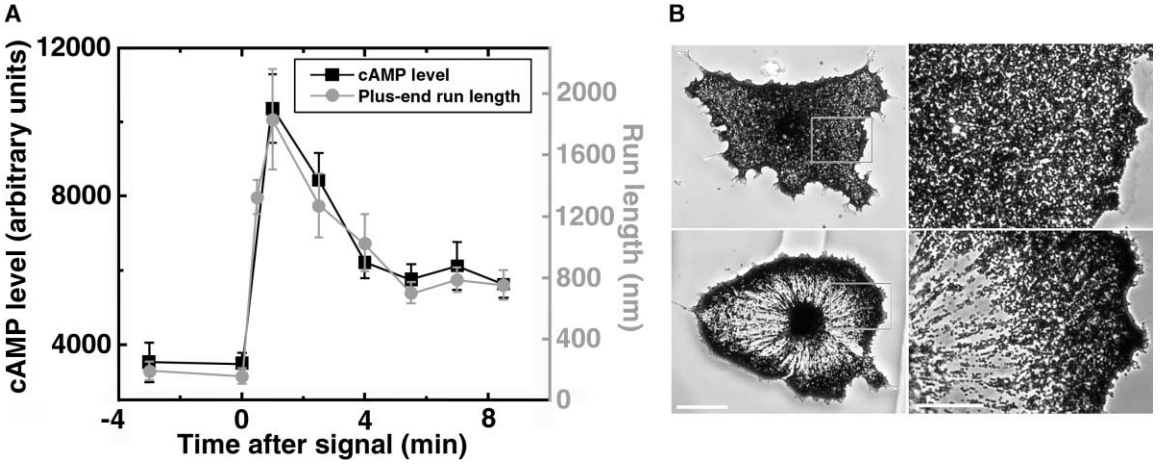

Figure 5. Changes in the Levels of cAMP Control the Switching of Pigment Granules from MTs onto AFs during Dispersion

(A) Changes in cAMP levels during dispersion. For the quantification of cAMP levels, cells were fixed with acrolein at increasing time intervals after the stimulation of dispersion and immunostained with an antibody specific to cAMP-acrolein complex. cAMP fluorescence was measured within the cell outlines and plotted as a function of time (black line). Note that the length of plus-end runs shown on the same plot for comparison (gray line) closely follows the kinetics of cAMP levels.

(B) Distribution of pigment granules in a control cell (top panels) stimulated to disperse pigment granules by withdrawal of adrenalin. Bottom panels: a cell stimulated with a combined treatment of the inhibitor of cAMP phosphodiesterase, IBMX (5 mM), and a membrane-permeable stable cAMP analog 8-Br-cAMP (1 mM). Together, this treatment increases cytoplasmic cAMP levels. Right panels are enlarged images of boxed regions indicated on the left panels. Scale bars represent 30 μm (left panels) or 10 μm (right panels). Note that in the IBMX/8-Br-cAMP-treated cell pigment granules accumulate at the cell margin and are arranged in radial strings consistent with radially arranged microtubules. This pigment distribution is similar to the one observed during the suppression of AF-dependent transport. Error bars are SEM.

sion (Figure 4A, bottom); quantitative measurements of granule movements indicated that 8-Br-cAMP independently activated AF-based motion (Figure 4C), increased plus-end motion, and decreased minus-end motion similar to results in dispersing cells (not shown).

These results indicate that MT- and AF- based pigment transport is controlled through the PKA signaling pathway and that both classes of transport are independently regulated by PKA activity.

Switching between MTs and AFs Is Controlled through Changes in the Levels of Intracellular cAMP

Given that PKA regulates each class of transport independently, the kinetics of changes in granule movement observed during dispersion (an increase in AF runs correlated with a decrease in MT runs, Figures 2B and 2C, left plots) could result from a corresponding temporal change in the levels of the second messengers that control these transport systems. Because PKA activity is known to be controlled by the second messenger cAMP, we next looked at the changes in cAMP levels in the course of dispersion. To measure cAMP levels, we fixed melanophores with acrolein at increasing time intervals after the application of dispersion signal and immunostained them with cAMP antibody specific to cAMP-acrolein covalent complex. Quantification of fluorescence in the cytoplasmic regions free from pigment granules indicated an initial increase in the levels of cAMP at about 1.0 min and a subsequent decrease of approximately 2-fold by 4 min (Figure 5A). The dynamics of cAMP levels were strikingly similar to the length of plus-end runs along MTs (Figure 2B, left; same plot also shown in gray in Figure 4A for direct comparison), which strongly suggested that intracellular cAMP levels control pigment granule movement during dispersion.

Because the levels of cAMP so precisely correspond to the MT-dependent granule movement throughout dispersion, it seems likely that cAMP-induced signaling is also responsible for the switching to AF in the later stages of dispersion. To test this possibility, we studied the behavior of granules in cells where the decline of cAMP level after the onset of pigment dispersion was prevented by addition of a combination of isobutylmethylxantine (IBMX), a potent inhibitor of cAMP phosphodiesterase, and 8-Bromo-cAMP. This combined treatment acted both to prevent the hydrolysis of endogenous cAMP and to supply an external source of it, maintaining the constitutively high level of intracellular cAMP and of plus-end directed runs of granules along MTs that is characteristic for earlier stages of dispersion. Independent experiments showed that this treatment did not affect the overall distribution of AFs and MTs. Furthermore, the density of MT and AF polymers, determined by fluorescence measurements in cells injected with fluorescent tubulin or stained with Rhodamine-phalloidin, was not significantly affected by this treatment.

We found that combined IBMX/8-Bromo-cAMP treatment resulted in a strikingly different pigment granule distribution compared to that in control cells stimulated for pigment dispersion by the withdrawal of adrenalin. Instead of the homogeneous distribution observed in control cells (Figure 5B, top panels), pigment granules in the cells treated to elevate cAMP levels accumulated at the cell margin (Figure 5B, bottom panels). Granules left behind at the center were organized into radial lines as if they were continuing to move along MTs. This striking distribution of pigment granules bears similari-

ties to the granule distribution in cells with AFs disrupted by Latrunculin A [27]; in these cells, pigment granules accumulate at the cell rim during dispersion and fail to distribute evenly in the cytoplasm. We believe that in both cases transfer of pigment granules from MTs onto AFs is inhibited but that in the in IBMX/Br-cAMP-treated cells some residual transfer onto AFs may occur because its inhibition is less complete than in the case of complete AF disruption. These results indicate that transfer from MTs onto AFs during dispersion is indeed regulated by the PKA signaling pathway through the changes in the levels of intracellular cAMP.

Switch between MT and AF Transport during Dispersion Correlates with the Increase in the Average Length of Minus-End Runs

It was previously suggested that in *Xenopus* melanophores the switching between MT- and AF-dependent transport occurs during the granule movement in the minus-end direction [21]. Therefore, in principle, an increase in the number of minus-end runs would allow increased opportunities for the microtubule-to-actin switch. To study whether the switching control involves any changes in the minus-end runs, we quantified the percentage of time the granules spent moving toward the MT minus ends during the progression of pigment dispersion (Figure 6A). Upon application of the dispersion signal, all granules start moving towards the MT plus ends, and thus the initial percentage of minus end-directed runs is low (around 4% within the first minute of dispersion). However, as dispersion progresses, the average length of plus-end runs decreases, and the percentage of time granules spend moving toward the minus ends increases nonlinearly; it reaches the level of 10% by 4 min and approximately 12% by 7 min of dispersion (a 3-fold increase). This timing coincides with the timing of the late stages of dispersion when the observed switching between MT- and AF-based transport takes place. Because the average length of a minus-end run does not change throughout dispersion (Figure 2A), the increase in time spent moving to the minus-end is due to increased numbers of minus-end runs.

To directly test whether the transfer of pigment granules from MTs onto AFs occurs during the minus-end runs, we examined pigment dispersion in the cells injected with antibody 74.1, which has been shown to inhibit the activity of cytoplasmic dynein and prevent pigment aggregation [29]. After withdrawal of adrenalin, pigment granules in antibody-injected cells accumulated at the cell margin in a display of behavior strikingly similar to that of IBMX/Br-cAMP-treated cells (Figure 6B). Thus, the disruption of minus-end runs achieves the same effect on granule transport as the cAMP-induced increase of plus-end runs in that it apparently inhibits the switching from MT- to AF-dependent transport.

These results indicate that the switching from MT to AF indeed occurs during minus end-directed MT runs. Thus, the PKA-based control of MT motors that influences the length of plus-end runs indirectly controls the frequency of minus-end runs, which in turn determines the rate of switching of organelles from MT onto AF "rails."

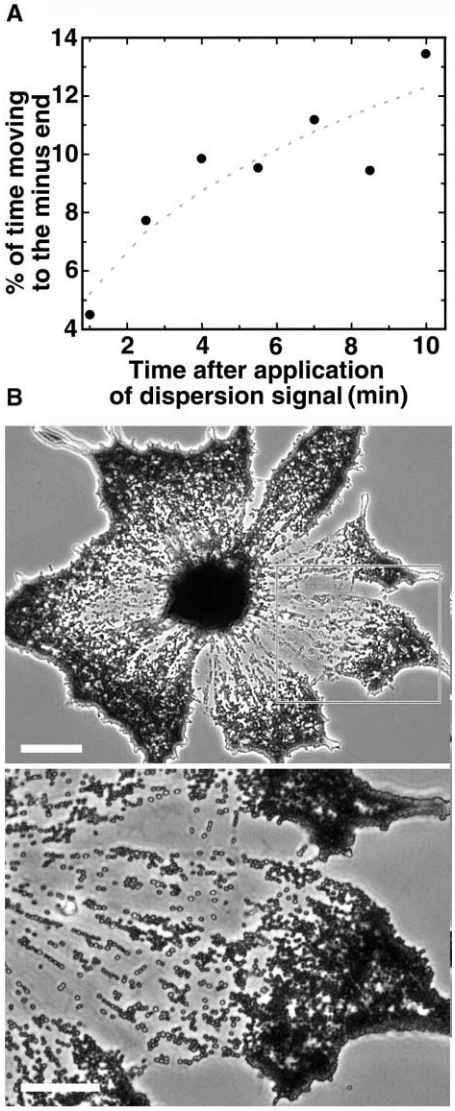

Figure 6. Increase in the Number of Minus-End Runs during Pigment Dispersion

(A) Changes of the percentage of time that pigment granules spent moving in the minus-end direction during dispersion. The average length of minus-end runs remained constant during dispersion, but the shortening of plus-end runs resulted in the elevation of the number of minus-end runs. As MT-to-AF transfers occur only during minus-end runs, this increase results in increased transfers from MTs onto AFs. The dotted line is a fit of the function $y = bx^c$; c was found to be approximately 0.1.

(B) A cell injected with function-blocking dynein antibody 74.1 (5 mg/ml) at the onset of pigment dispersion. Pigment granules accumulate at the cell periphery in a display of behavior similar to that of IBMX/8-Br-cAMP-treated cells in Figure 5. Scale bars represent 30 μm (top) or 10 μm (bottom).

Discussion

Our results indicate that balancing the activities of transport systems in melanophores involves independent regulation of MT- and AF-based transport by a signaling mechanism that involves changes in the levels of the second messenger cAMP. Our data further suggest that

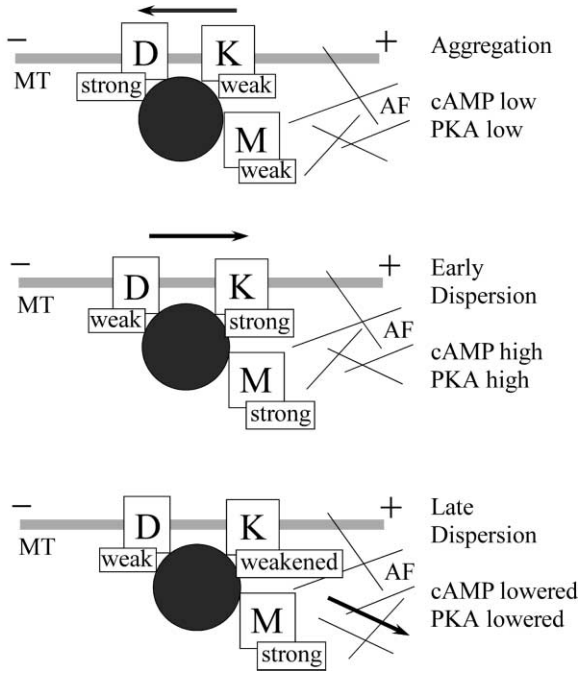

Figure 7. A Model for the Regulation of the Switching of Pigment Granules between MTs and AFs by cAMP

During aggregation (top), low cAMP levels result in downregulation of kinesin and myosin V and activation of dynein. The myosin V and kinesin are downregulated sufficiently that whenever there is a tug-of-war between the motors, dynein wins. Residual myosin V-dependent motion allows for the granules to move along AFs until they contact MT and engage in dynein-based motion. In contrast, early during dispersion (middle), the levels of cAMP go up and the activity of dynein is significantly reduced, whereas kinesin and myosin V are very active. As a result, pigment granules move toward MT plus ends. Because the switching onto AFs occurs during the minus-end runs, the probability for the granules to leave the MTs is low at this time. At late stages of dispersion (bottom), the activity of myosin V remains high, whereas the cAMP levels and the activity of kinesin decrease. This decrease in plus end-directed motion increases the frequency of minus-end runs and therefore the fraction of time that granules spend moving toward MT minus ends. Therefore, the number of opportunities for the transfer from MTs onto AFs is increased at this stage, resulting in increased AF-based transport. K, kinesin; M, myosin; D, Dynein. Pigment granules are shown as black circles.

cAMP signaling differentially regulates MT and AF motors during pigment aggregation and dispersion by a combination of all-or-nothing switches and fine-tuning of the motor activities. During pigment aggregation the lowering of cAMP levels and PKA activity apparently leads to activation of dynein-dependent minus end-directed MT transport. During pigment dispersion the situation is more complex because two motors, kinesin and myosin V, are involved in pigment granule transport. Here, elevation of cAMP levels during dispersion leads to constitutive activation of myosin (all-or-nothing switch), and the time-dependent variation of these elevated cAMP levels induces changes in the levels of kinesin activity (fine tuning).

The proposed model of such regulation is shown in Figure 7. During aggregation cAMP levels are low, suggesting low PKA activity, and the activities of myosin and kinesin are very low as compared to those of dynein; therefore, the granule movement occurs predominantly toward the minus end (Figure 7, top). During dispersion, cAMP levels are elevated, the activity of dynein is greatly reduced, and the other two motors become active; therefore, the prevalent runs occur in the plus-end direction (Figure 7, middle). As dispersion progresses, the levels of cAMP reduce, and the length of plus-end runs continuously decreases, presumably because of the decrease in the kinesin motor activity, allowing the switching to myosin V-driven AF transport (Figure 7, bottom).

Our results indicate that the injection of dynein-blocking antibody 74.1 induces hyperdispersion of pigment similar to the situation with 8-Br-cAMP/IBMX treatment and therefore is likely to inhibit the switching of granules from MT onto AF tracks. This is in agreement with the previous observations that in Xenopus melanophores the switching between MT- and AF-dependent transport occurs during the granule movement in the minus-end direction [21]. An apparent constraint for this mechanism is that during dispersion, when the switching occurs, the general direction of granule movement along MTs is toward the cell periphery, i.e., the plus ends. However, as we have shown earlier (Figure 2A), during dispersion the average length of minus-end runs is greatly reduced but is by no means close to zero. Moreover, the percentage of time granules spend moving toward the MT minus ends increases at later stages of dispersion, making the switching more likely. The present data extend our previous observations by demonstrating that PKA signaling controls the number of opportunities for the transfer from MT to AF. Interestingly, injection of the dynein function-blocking antibody 74.1 after dispersion had already occurred and granules had had sufficient time to switch to the AF did not induce accumulation of pigment granules at the cell rim (data not shown). Thus, it appears that during dispersion, once the granules leave the MTs, the majority of them stay on the AFs.

The next obvious question is how motor activities during pigment transport are regulated. The mechanism of such regulation has to be very efficient because it appears to involve regulation by only one type of messenger molecule that, directly or indirectly, affects the activities of molecular motors in a differential way. It has been shown that besides cAMP-dependent PKA, pigment transport regulation involves the activity of a protein phosphatase [34]. These two enzymes may affect motor activities either directly by phosphorylation/dephosphorylation or indirectly by modification of the activity of adaptor molecules or components of other signaling cascades. Because kinesin activity appears to have such a precise correlation with the levels of cAMP, it seems likely that kinesin is directly activated by phosphorylation, possibly by PKA, and that a protein phosphatase is closely associated with it and induces its rapid dephosphorylation. In such a case, kinesin activity would closely follow the changes in the second messenger and the corresponding kinase activity. In the case of dynein, elevated levels of cAMP appear to induce its inactivation by PKA or other kinases, providing an all (aggregation)-or-nothing (dispersion) switch of its motor activity. Myosin V, another example of an all-or-nothing switch induced by phosphorylation, has been suggested

to be regulated not through changes in its activity, but through changes in its association with pigment granules (off during aggregation or on during dispersion [21]). One of the possible ways to achieve such efficiency in regulation would be to have motor molecules form complexes with the signaling molecules involved in their regulation. The exact downstream targets of cAMP signaling involved in regulation of motor activities, and the possibility of their direct association with the motor molecules, are important questions for future studies. Therefore, our work provides a guideline for a detailed investigation of the mechanisms of coordinated regulation of molecular motors during intracellular transport.

Experimental Procedures

Melanophores were cultured from the scales of black tetra (*Gymnocorymbus ternetzii*) onto carbon-coated glass coverslips as described previously [28, 45]. Aggregation of pigment granules was induced by adrenalin (500 nM). For pigment redispersion, adrenalin was washed out via 5–6 changes of tissue culture medium. For facilitating redispersion, caffeine (5 mM) was added to the washing medium in some experiments. Rapid (10 s) reversal of the direction of pigment movement was achieved by perfusion of tissue culture medium containing or lacking adrenalin via a home-made perfusion chamber.

For the disruption of MTs, cells were incubated with nocodazole (10 μg/ml) for 30 min on ice followed by 60 min at 30^{0}C. The combined treatment with cold and nocodazole resulted in complete depolymerization of MTs in black tetra melanophores [27]. Cells lacking AFs were produced by Latrunculin A treatment as described before [27]. PKA activity was inhibited by injection of PKA inhibitor 6-22 Amide (Calbiochem) at a needle concentration of 1 mM. The volume of injected solution was approximately 10% of cell volume, and therefore the final concentration of inhibitor in the cytoplasm was about 0.1 mM.

The movement of pigment granules was recorded with a Nikon TE300 microscope (Nikon) equipped with a 100$\times$ 1.25 NA Plan Achromat objective lens. Time-series (each 15 s long) of phase contrast images of pigment granules were acquired at a video rate (30 frames/s) with a Watec 902B CCD video camera (Watec Co) via stream acquisition option of Metamorph image acquisition and analysis software (Universal Imaging Corp.). So that the spatial resolution of images would be increased, an additional projection lens (2$\times$) was placed in front of the camera chip.

The measurement of the movement statistics was performed as described [21, 38, 39]. Pigment granules were tracked with Isee image processing software (Inovision Corp.). Tracking was performed at the edge of the aggregating or dispersing pigment mass, where individual granules could be observed. Measurements of the behavior of granules at different distances from the cell center confirmed that this behavior was position independent.

For examination of the MT component of the motion, the trajectory of a granule was fit by a straight line to determine the MT axis. The movement of a pigment granule along the MT axis was then analyzed by breaking the displacement into periods of continuous runs to the MT minus end (to the cell center), to the plus end (to the cell periphery), and pauses with a custom-written software as described in [38].

For determining the average plus-end or minus-end run length, the mean value of all plus-end or minus-end runs, respectively, was taken. Past work has shown that the distribution of run lengths is skewed and is described by the sum of two decaying exponentials [39], so one might be concerned that the average run length was sensitive to this skewness and was not a robust characterization of the motion. However, even for skewed distributions, the central limit theorem applies as long as the population has a finite standard deviation σ, and the sampling distribution of the sample mean $\bar{x}$ is approximately normal as long as the sample size n is large enough [46]. In practice, for a skewed distribution such as a decaying exponential, n typically must be larger than 25 [46]. When this is the case, the standard error associated with the mean is a statistically well-defined measure of the uncertainty of the estimate of the mean value of the distribution [46]. In our experiments, n is always larger than 60, and in most cases it is more than 200.

As a direct experimental check, we confirmed that $n = 60$ was large enough by generating synthetic run lengths drawn from a distribution of known mean that was described by sum of two decaying exponentials. In this artificial data, the sampling distribution was indeed approximately normal, and the difference between the actual mean of the distribution and the experimentally determined sample mean was less than the calculated standard error of the mean in approximately 68% of the trials, and it was within two standard errors of the mean in approximately 95% of the trials. Thus, from both a theoretical and experimental point of view, in our case the mean and standard error of the mean are reasonable characterizations of the cargo's motion when moving in a linear fashion along microtubules.

The AF-dependent component of the motion, which involved the periods of random non-directed travel of pigment granules, was examined by the measurement of $r(t)$ statistics, indicating how far a granule traveled from the initial position [21]. Each data point represents the average of at least 200 measurements derived from tracking granules in five or more different cells.

The levels of cAMP were measured by immunostaining of melanophores fixed with acrolein (Aldrich) with antibodies specific to cAMP-acrolein covalent complex (a gift from Dr. Arthur McMorris, Wistar Institute and Mahoney Institute of Neuroscience, Philadelphia, PA) as described in [47]. For immunostaining, cells were incubated overnight at 4°C with primary antibody against cAMP-acrolein complex. Incubations with biotinylated goat anti-rabbit IgG (1:200, Vector Laboratories) and tetramethyl rodamine-coupled avidin (1:100, Sigma) followed. Images of immunostained cells were taken with a 10 s exposure time with a CH350 back-illuminated cooled CCD camera (Photometrics) and a 40$\times$ (1.4 NA) Plan-Apochromat objective lens. Fluorescence was quantitated with Metamorph image acquisition and analysis software (Universal Imaging Corp.). Background fluorescence measured by the placement of 50 $\times$ 50 pixel squares outside the cell outlines was subtracted from cell fluorescence measured by placement of squares of the same size over the areas free from pigment granules inside the cells. About ten measurements were performed for each cell. Average intensities for each time point after the stimulation of pigment dispersion or aggregation were then calculated and plotted as a function of time.

Immunoblotting was performed as described previously [29]. Pigment granules were isolated by a procedure modified from Rogers et al. [48]. Melanophores dissociated from collagenase-treated cells by vigorous shaking were collected by centrifugation for 5 min at 200 $\times$ g, washed in Ringer solution containing protease inhibitor, and lysed on ice into BRB 80 buffer [48] by passing though a 22 syringe needle. Intact cells and cell nuclei were removed from the lysates by centrifugation at 200 $\times$ g for 5 min at 4°C, and pigment granules were collected by centrifugation at 5,000 $\times$ g for 15 min at 4°C. Rabbit antibodies specific to myosin Va (DIL-2, a gift from Dr. John Hammer, National Institutes of Health) and to a motor domain of kinesin heavy chain (HD Kin2, [25]) and mouse monoclonal antibodies against 74 kDa subunit of cytoplasmic dynein ([35]; Chemicon) were used for the detection of motor proteins in preparations of pigment granules. Immunoblotting was performed as described previously [29]. Reactive protein bands were revealed with SuperSignal West Femto detection system (Pierce).

Dynein antibody 74.1 was purified from ascites fluid (Covance) by Protein A chromatography and injected into cells with aggregated pigment granules at the concentration of 5 mg/ml just prior to the withdrawal of adrenalin.

Cell thickness measurements were performed by microinjection of a solution of FITC-labeled dextran (MW 10,000, Molecular Probes) into cells and measurement of the levels of fluorescence in different areas of the cytoplasm with Metamorph imaging software.

Levels of MT and AF polymers in control and 8-Bromo-cAMP/IBMX-treated cells were calculated as follows: for MTs, cells were injected with Cy3-labeled tubulin, and the polymer level was calculated with Metamorph imaging software as the difference between the total brightness measured in a given area of the cytoplasm and the background brightness inside the smaller areas of the cytoplasm

devoid of MTs; for AFs, cells were fixed with 4% formaldehyde in PBS and stained with Rhodamine-phalloidin; polymer level was measured by Metamorph as total cytoplasmic brightness.

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