

Intracellular Cyclic AMP, Not Calcium, Determines the Direction of Vesicle Movement in Melanophores: Direct Measurement by Fluorescence Ratio Imaging

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Abstract. Intracellular movement of vesiculated pigment granules in angelfish melanophores is regulated by a signalling pathway that triggers kinesin and dynein-like microtubule motor proteins. We have tested the relative importance of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) vs cAMP ($[\text{cAMP}]_i$) in the control of such motility by adrenergic agonists, using fluorescence ratio imaging and many ways to artificially stimulate or suppress signals in these pathways. Fura-2 imaging reported a $[\text{Ca}^{2+}]_i$ elevation accompanying pigment aggregation, but this increase was not essential since movement was not induced with the calcium ionophore, ionomycin, nor was movement blocked when the increases were suppressed by withdrawal of extracellular Ca^{2+} or loading of intracellular BAPTA. The phosphatase inhibitor, okadaic acid, blocked aggregation and induced dispersion at concentrations that suggested that the protein phosphatase PP-1 or PP-2A was continuously turning phosphate

over during intracellular motility. cAMP was monitored dynamically in single living cells by microinjecting cAMP-dependent kinase in which the catalytic and regulatory subunits were labeled with fluorescein and rhodamine respectively (Adams et al., 1991. *Nature (Lond.)*. 349:694–697). Ratio imaging of FICRhR showed that the α_2 -adrenergic receptor-mediated aggregation was accompanied by a dose-dependent decrease in $[\text{cAMP}]_i$. The decrease in $[\text{cAMP}]_i$ was both necessary and sufficient for aggregation, since cAMP analogs or microinjected free catalytic subunit of A kinase-blocked aggregation or caused dispersal, whereas the cAMP antagonist $R_p\text{cAMPS}$ or the microinjection of the specific kinase inhibitor PKI_{5-24} amide induced aggregation. Our conclusion that cAMP, not calcium, controls bidirectional microtubule dependent motility in melanophores might be relevant to other instances of non-muscle cell motility.

INTRACELLULAR movement of vesicles and organelles along microtubules is an important component of mitosis, regulated secretion, endocytosis, and fast axonal transport (Allen et al., 1982; Rivas and Moore, 1989; Hyman and Mitchison, 1991; Bomsel et al., 1991) and the spatial organization of Golgi, lysosomes, and ER (Terasaki et al., 1990; Cooper et al., 1990; Hollenbeck and Swanson, 1990). In vitro models of cytoskeletal based movement have been successfully reconstituted with microtubules and dynein (Paschal et al., 1987), or kinesin (Vale et al., 1985). Reconstituted transport with a single motor protein is constant and seems unregulated, but in vivo, the direction of movement and the selective attachment of vesicles to the cytoskeleton must be under cellular control. To address the question of the regulation underlying directional organelle transport, we have chosen to study fish chromatophores.

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Among vertebrate tissues that are specialized for intracellular transport of membrane-bound organelles, the dermal chromatophores are among the most dramatic. These cells exhibit a tightly regulated stimulus-response coupling that directs a concerted movement of pigment granules inward into a tightly aggregated central mass, or outward, dispersing pigment throughout the cell. Isolated chromatophores are a unique model system for the study of the regulation of vesicle transport since activation of movement can be reversibly triggered at any of several steps in the signal transduction pathway. Cell behavior can be monitored at the molecular level since the movement of pigment vesicles reflects the activity of motor proteins. We have chosen to study the regulation of motility in the angelfish, *Pterophyllum scalare*, since movement is microtubule based (Schliwa, 1975), while the control of motility has remained largely unexplored. It has been shown in *Gymnocorymbus ternetzi* melanophores that pigment granule (melanosome) dispersion is dependent on kinesin (Rodionov, 1991). In melanophores from *Xiphophorus* and *Fundulus* (Clark and Rosenbaum, 1982; Ogawa

et al., 1987) and *Holocentrus erythrophores* (Beckerle and Porter, 1982), dynein is probably responsible for aggregation. The speed of movement in melanophores from *Pterophyllum*, *Tilapia*, *Gymnocorymbus*, and *Fundulus* is 1–2 $\mu\text{m/s}$ (Schliwa, 1973; Rozdzial and Haimo, 1986; Murphy 1974), similar to the rates of dynein- and kinesin-dependent motility in vitro. It is likely that movement in *Pterophyllum* melanophores is dependent on these motor proteins.

Transduction of a stimulus for movement requires receptor activation at the membrane, change in the concentration of a second messenger, and the action of a kinase or phosphatase on a substrate. α adrenergic receptors are responsible for aggregation and β adrenergic receptors are responsible for dispersion in the angelfish *Pterophyllum eimeki* (Reed and Finnin, 1972) and other teleost chromatophores as well (Scheline, 1963; Fujii and Miyashita, 1975; Miyashita and Fujii, 1975; Luby-Phelps and Porter, 1982; for review see Fujii and Oshima, 1986). The neurotransmitter that is responsible for α -adrenergic-mediated aggregation in melanophores is probably norepinephrine (Kumazawa and Fujii, 1984). The second messenger responsible for movement in chromatophores could be either intracellular calcium ($[\text{Ca}^{2+}]_i$), intracellular cAMP ($[\text{cAMP}]_i$), or both (see below). Evidence for the action of specific kinases and phosphatases in chromatophore motility is incomplete. In *Carassius xanthophores*, cAMP-dependent protein kinase is required for dispersion (Lynch et al., 1986), and in *Tilapia* melanophores, the calmodulin-activated protein phosphatase, calcineurin, promotes aggregation (Thaler and Haimo, 1990).

The second messenger controlling chromatophore pigment movement in various teleosts is either Ca^{2+} or cAMP. In *Holocentrus erythrophores* aggregation is probably caused by an elevation of $[\text{Ca}^{2+}]_i$ since calcium specific ionophores induce movement (Luby-Phelps and Porter, 1982). Direct measurement of $[\text{Ca}^{2+}]_i$ in *Xiphophorus* melanophores and erythrophores with fura-2 demonstrate an increase in $[\text{Ca}^{2+}]_i$ upon pigment aggregation (Oshima et al., 1988). In *Fundulus* melanophores and *Carassius xanthophores*, cAMP agonists induce dispersion (Novales and Fujii, 1970; Winchester et al., 1976). Aggregation and dispersion can be activated by alternately raising and lowering extracellular calcium in permeabilized *Holocentrus erythrophores* (McNiven and Ward, 1988) or by lowering or raising extracellular cAMP in permeabilized *Tilapia* melanophores (Rozdzial and Haimo, 1986) and in *Carassius xanthophores* (Palazzo et al., 1989). Although altering the concentration of cAMP in lysed cells will alter the direction of pigment movement, it is not the sole second messenger of importance in *Tilapia*. Aggregation is triggered by an elevation of $[\text{Ca}^{2+}]_i$, which activates a phosphatase that counters the cAMP-dependent kinase (Thaler and Haimo, 1990). In previous studies second messenger participation was decided by either the induction or the blockage of movement. These

studies, except Oshima et al. (1988), did not rely on the actual measurement of the dynamics of the second messenger in situ. Since multiple pathways can be involved, a three part approach that includes measuring, inducing, and blocking second messenger dynamics is essential for learning the sufficiency and necessity of the messenger.

Which second messenger is responsible for regulating microtubule motor proteins in *Pterophyllum* melanophores? In the present study, observations of the dynamics of both $[\text{Ca}^{2+}]_i$ and $[\text{cAMP}]_i$ in individual living cells are correlated with bidirectional movement of melanosomes. We demonstrate the requirement for $[\text{cAMP}]_i$, and not $[\text{Ca}^{2+}]_i$, dynamics in melanosome movement in angelfish, identify the receptor that controls $[\text{cAMP}]_i$, show that the effects of second messenger dynamics are mediated by the catalytic subunit of the cAMP-dependent protein kinase, and show that steady state concentrations of phosphate are maintained by a serine/threonine phosphatase. Central to our investigation was a new fluorescent indicator of $[\text{cAMP}]_i$ that permitted measurement of the concentration of free intracellular cAMP in single living cells since it is insensitive to nonexchangeable pools (Adams et al., 1991). Previous methods for measuring the concentrations of cAMP were limited to one time, destructive assays of thousands to millions of cells. These methods report total cAMP in the tissue and cannot distinguish cytosolic, free cAMP from biologically inactive cAMP. Our fluorescent indicator is the cAMP-dependent protein kinase, labeled with fluorescein on the catalytic subunit and rhodamine on the regulatory subunit (FICRhR, pronounced "flicker"). As increasing $[\text{cAMP}]_i$ dissociates the holoenzyme, removal of fluorescence energy transfer from fluorescein to rhodamine increases the ratio of emission from fluorescein to that from rhodamine. The 520/580-nm emission ratio image of cells injected with the labeled kinase reports the relative $[\text{cAMP}]_i$ over time. In vitro, the fluorescence ratio of FICRhR changes in parallel with its kinase activity, and the activities of both labeled and unlabeled enzyme are similar (Adams et al., 1991). A probe that maintains kinase activity is less perturbing to cells than a probe that would sequester endogenous cAMP by non-productive binding. The current study illustrates the capabilities and limitations of this new probe and demonstrates its effectiveness for measuring the requirement for dynamics of $[\text{cAMP}]_i$ for unexplored physiological processes.

Materials and Methods

Cell Culture

Melanophores from angelfish (*Pterophyllum scalare*) were isolated from scales and cultured on glass coverslips. Wild type fish were obtained from local pet stores and kept in a standard aquarium. Each fish was briefly removed from the aquarium and four to five scales were plucked with forceps and rinsed three times in Ringer's Saline (103 mM NaCl, 1.8 mM KCl, 2 mM CaCl_2 , 0.8 mM NaHCO_3 , 5 mM Tris). Scales were digested by swirling in a watchglass containing Ringer's and 1 mg/ml collagenase with an activity exceeding 500 U/mg (type 1A; Sigma Chemical Co., St. Louis, MO) for 20 min. The overlaying epidermal layer was gently blown off by pipetting a stream of fluid over scales that were immobilized with forceps. Scales were further digested for 30–60 min and then washed three times in sterile Ringer's Saline without calcium. After 10 min, melanophores were blown off with a stream of fluid and plated on coverslips (Corning Glass, Corning, NY) that were pretreated with 50 μl FBS. Cells were cultured overnight in Wolf and Quimby's Amphibian Medium (University of Califor-

1. **Abbreviations used in this paper:** AM, acetoxymethyl ester; BAPTA, 1,2-bis-(2)aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; C_{50} , catalytic subunit of the cAMP dependent protein kinase; $[\text{Ca}^{2+}]_i$, concentration of intracellular free calcium; $[\text{cAMP}]_i$, concentration of intracellular free cAMP; FICRhR, fluorescein labeled catalytic subunit, rhodamine labeled regulatory subunit; IC_{50} , inhibitory concentration resulting in 50% inhibition; PP, protein phosphatase; R^I (R^{II}), regulatory subunit of the type I (type II) cAMP-dependent protein kinase; R_p cAMPS (or S_p cAMPS), Adenosine-3',5'-cyclic monophosphorothioate, R_p (or S_p)-diastereomer.

nia, San Francisco Cell Culture Facility, San Francisco, CA) without whole egg ultrafiltrate, but supplemented with 10% FBS (Gibco Laboratories, Grand Island, NY), 20 mM Hepes, 0.2 mM L-ascorbic acid, 0.2 mM pyruvate, 0.1 mM atropine, 5 mg/ml penicillin, 5 mg/ml streptomycin, 10 mg/ml neomycin (Gibco Laboratories). Atropine was excluded from the culture medium for all pharmacology, FICRHR, and some fura-2 microinjection experiments. Without atropine, cells attached and spread to their normal dendritic morphology more slowly, in 2–3 d. Atropine was omitted to avoid possible receptor down regulation.

Measurement of $[Ca^{2+}]_i$ during Melanosome Movement

Cells were loaded with fura-2 either by direct microinjection of the pentapotassium salt or by incubation for 1 h at room temperature with 1 mM fura-2 acetoxyethyl ester (fura-2/AM) (Molecular Probes, Eugene, Oregon) that was solubilized in amphibian medium with 0.04% Pluronic F-127 (Molecular Probes). The fura-2 fluorescence from single cells in Ringer's at room temperature was observed with an imaging system based on a Dage silicon-intensified target camera (Dage-MTI Inc., Wabash, MI), a Zeiss IM-35 inverted microscope (Carl Zeiss, Oberkochen, Germany), and a Gould FD5000 image processor (Gould Inc., Cleveland, OH) as described in Tsien and Harootunian (1990). 350/385-nm ratio images were analyzed as described previously (Grynkiewicz, et al., 1985; Tsien and Harootunian, 1990). Melanin for control experiments was obtained from Sigma Chemical Co. (St. Louis, MO).

Experiments were performed on two imaging systems. In one imaging system (in the laboratory of R. Y. Tsien, University of California, San Diego, CA), illumination at 350 and 385 nm was provided by Spex Industries (Metuchen, NJ) xenon lamps and monochromators. In the other system (in the laboratory of T. E. Machen, University of California, Berkeley, CA), one xenon lamp and alternating interference filters provided the illumination. The bandwidth of the illuminating light was broader with the interference filter system and so two sets of calibrations were necessary to measure calcium ion concentrations from the fluorescence ratio images (broader bandwidths reduce the maximum 350/385 ratio and therefore the precision of calcium measurements). To permit comparison of cells observed on the two systems, calibration solutions were prepared with fura-2 pentapotassium salt in 10 mM Hepes, pH 7.2, 10 mM EGTA, 110 mM KCl, 10 mM NaCl, and varying concentrations of calcium (Tsien and Pozzan, 1989). Solutions were placed between two coverslips with a spacer made of coverslip fragments and the fluorescence intensity at 350 and 385 nm was recorded. Although the intensities were dependent on the thickness of the solution chamber, the ratios were not altered. Three calibration ratios are used for calculating $[Ca^{2+}]_i$: the minimum and maximum fluorescence ratios at 350/385 nm and the ratio at 385 nm with and without saturating calcium. The latter ratio, multiplied by 135 nM (the K_d of fura-2 at room temperature and the low ionic strength of fish saline) provided the effective value of K used to calculate $[Ca^{2+}]_i$ (Grynkiewicz et al., 1985). These ratios were obtained in aqueous solutions and their values in cells will be reduced depending on the viscosity of cytoplasm. The maximum and minimum 350/385-nm ratios were reduced by 0.85 based on estimated values of cytoplasmic viscosity (Poenie, 1990). On the monochromator based system the minimum and maximum fluorescence ratios and K were 0.85, 27.5, 3.4, respectively, as calculated previously in this laboratory. On the broader bandwidth, interference filter system, ratios were 0.89, 9.8, 1.98. $[Ca^{2+}]_i$ in melanophores calculated from each imaging system were similar with these calibration values.

We compared the in vitro measurement of the maximum and minimum fluorescence ratios to estimates of the ratios in melanophores. The ratios were estimated by treating cells with 5 μ M ionomycin (Calbiochem-Behring Corp., San Diego, CA) or 5–20 μ M digitonin in either calcium-free Ringer's or in Ringer's containing enough extracellular calcium to saturate fura-2. Cells in high extracellular calcium that were lysed in digitonin exhibited higher ratios than ionomycin-treated cells, but these ratios were variable and were still lower than the viscosity-corrected values in vitro. For example, the maximum intracellular ratio on the interference filter system was 7.4 (in response to 20 μ M digitonin) while the maximum ratio in vitro was 9.8. The difference was probably due to dye compartmentation, viscosity, and most importantly, incomplete Ca^{2+} entry during dye leakage in lysed cells. We chose to calibrate $[Ca^{2+}]_i$ measurements with the in vitro ratios because of the uncertainty of the ratios in ionomycin and digitonin treated cells. Any error introduced by our calibration would depress quantitative measurements of $[Ca^{2+}]_i$, but would not affect qualitative conclusions about $[Ca^{2+}]_i$ dynamics. Injection of fura-2 minimized this error compared to acetoxyethyl (AM) ester loading.

The necessity for $[Ca^{2+}]_i$ changes was tested by blocking the rise with

extracellular EGTA or with buffering concentrations of the calcium chelator, BAPTA. 1 μ M of the AM ester of BAPTA (Molecular Probes, Eugene, OR) and fura-2 were loaded simultaneously. The effects of inhibitors of calmodulin activation on the aggregation and dispersion of melanosomes was tested with 1–10 μ M calmidazolium (R24571; Janssen Pharmaceutica, Beerse, Belgium) and 10–30 μ M W-7 (Calbiochem, San Diego, CA). Isolated melanophores were washed free of atropine and well-dispersed cells were selected. Two min after each addition of agent, cells were scored as fully aggregated, fully dispersed, or partially dispersed. Weakly adherent cells that were lost were not scored.

Preparation and Observation of FICRHR

Recombinant catalytic subunit (C_α) and regulatory subunit (R^1) were separately labeled with fluorescein 5'-isothiocyanate and tetramethylrhodamine isothiocyanate, respectively, as described in Adams et al. (1991). The subunits, C_α and R^1 were gifts from the laboratory of Prof. Susan Taylor (Department of Chemistry, University of California, San Diego, CA). The labeled holoenzyme was stored in injection buffer (25 mM KPO_4 , pH 7.36, 1 mM EDTA, 1 mM mercaptoethanol, 5% glycerol) at 4°C until the day of use. FICRHR at concentrations between 7 and 60 μ M was spun for 40 min at 12,000 g in a microcentrifuge and microinjected into cells with a glass micropipette held in a Narishige micromanipulator (Narishige Scientific Laboratory, Tokyo, Japan). Cells injected with FICRHR were illuminated at 495 nm to excite the fluorescein-catalytic subunit and emission was monitored with a 500–530 nm bandpass (fluorescein) and a 570 nm long pass filter (rhodamine) by alternately positioning interference filters between the microscope and the low light video camera. Each pair of fluorescence images was compared to a standard shading sample made of equimolar fluorescein and rhodamine to correct for geometric variations of the camera and optics. Ratiometric measurement of fluorescence avoids the sensitivity to path length and dye content that limits single wavelength techniques. The ratio changes in vivo were similar to those in vitro but the absolute ratios were higher. The individual wavelengths that we ratioed changed coordinately upon hormone stimulation. However, increases in cell thickness meant that intensities at each wavelength increased upon aggregation. We anticipated that mixing of labeled and unlabeled subunits in cells might have reduced fluorescence energy transfer as it can in vitro, but the effects of mixing on the fluorescence ratio were comparable to noise levels. Although calibration of fluorescence ratios of intracellular FICRHR in terms of $[cAMP]_i$ is possible in other systems (Adams et al., 1991), the methods used previously would be relatively difficult to apply to melanophores because their high basal levels of $[cAMP]_i$ would require vast concentrations of $R_p cAMPS$ to inhibit kinase dissociation completely.

Receptor Pharmacology and cAMP Active Agents

The activity of several agents was tested on isolated melanophores. Adrenergic agents were a gift from Prof. Paul Insel, and muscarinic agents were a gift from Prof. Joan Brown, both in the Department of Pharmacology (University of California, San Diego, CA). All other cAMP agents were obtained from Sigma Chemical Co. except $R_p cAMPS$ and $S_p cAMPS$, which were obtained from Biolog Life Science Institute (La Jolla, CA). The peptide inhibitor of C_α , PKI_{5-24} amide, was obtained from Peninsula Labs (Belmont, CA). Okadaic acid was obtained from Moana Bioproducts (Honolulu, HI). The action of various agents on movement was determined by brightfield microscopic observations of the distribution of pigment (distinguished as either dispersed, aggregated, or partially aggregated). Agents were applied to isolated cells on glass coverslips at concentrations that did not produce (or block) movement, and then the concentrations were increased by 10-fold until movement was produced (or blocked). If agents blocked movement or failed to induce movement, the agent was always washed away and the cells were tested with hormone to ensure that melanophores were still competent to move pigment vesicles. The average number of cells per experiment was 25 ± 15 .

Results

Measurement of $[Ca^{2+}]_i$ during Pigment Vesicle Movement

Melanophores were isolated from scales and cultured on coverslips for most experiments. Cultured cells adhered within minutes and regained their dendritic morphology

within one to two days of culturing. Isolated melanophores were stimulated with epinephrine, which aggregated melanosomes into a central mass after 30–60 s, similar to melanophores in situ. When epinephrine was washed from the cells thoroughly, or after a single wash followed by the addition of atropine, melanosomes would disperse to their original distribution. Cells retained a dendritic shape and the ability to move pigment vesicles for more than 2 wk after isolation. Pigment vesicles remained dispersed in all cells cultured in either amphibian medium or fish Ringer's in the absence of hormones demonstrating that the normal, unstimulated state of the isolated cell is dispersed. Cells on scales can be found in either the dispersed or aggregated state suggesting that residual stimulation might reside in the scale after removal from the animal (see also Egner, 1971).

We first tested the requirement for $[Ca^{2+}]_i$ dynamics for bidirectional movement of pigment vesicles. Initial measurements were performed on melanophores in situ, on scales. Although $[Ca^{2+}]_i$ elevations were detected in response to epinephrine, autofluorescence from scales limited this approach. All subsequent experiments were performed on cells that were dissociated from scales and cultured on glass coverslips. Dye-loaded cells could be observed during three to four rounds of aggregation and dispersion under experimental conditions. There was no detectable autofluorescence from melanin or other cellular components in unloaded cells nor was there significant fluorescence in loaded cells in which fura-2 was quenched with 2 mM $MnSO_4$ (see Fig. 2).

Fig. 1 shows the $[Ca^{2+}]_i$ of an isolated melanophore that was microinjected with 500 μM (pipette concentration) of the pentapotassium salt of fura-2. Final concentration of fura-2 in this cell was estimated to be 50 μM based on the volume of the injectant. The rise in $[Ca^{2+}]_i$ in this and most isolated cells was transient, rising concomitantly with the

aggregation of vesicles, and spontaneously returning to basal levels while cells remained aggregated. The $[Ca^{2+}]_i$ elevation did not precede movement and the return to basal levels did not correspond to movement or geometrical changes in the cell. The rise was spatially uniform in most cells (within the limit of resolution of our imaging system), but a few AM ester loaded cells had higher $[Ca^{2+}]_i$ around the central pigment mass. The rise occurred in all cells induced to aggregate, but the duration of the $[Ca^{2+}]_i$ rise was heterogeneous, sometimes decreasing only upon atropine-induced dispersion.

Fura-2 was introduced into melanophores either by microinjection of the pentapotassium salt of fura-2 or by incubating cells with the AM ester of fura-2. Cells injected with fura-2 had basal $[Ca^{2+}]_i$ values of 69 ± 8 nM and upon stimulation with epinephrine, $[Ca^{2+}]_i$ values were transiently elevated to 369 ± 109 nM ($n = 5$). This represents a fivefold increase in $[Ca^{2+}]_i$ upon stimulation. Calcium dynamics in ester loaded cells were reduced (120 ± 30 nM basal, 168 ± 32 nM, peak, $n = 21$) (Fig. 2). Since microinjection of high concentrations (2 mM in the pipette) of fura-2 into cells eliminated the calcium transient, it is possible that dynamics were muted in AM ester loaded cells because they contained buffering concentrations of fura-2, unlike most injected cells. However, the fluorescence intensity of the two groups of cells was not grossly different. Possibly, ester-loaded cells exhibited smaller $[Ca^{2+}]_i$ dynamics because of incomplete ester hydrolysis in this tissue or compartmentalization of ester loaded dye. Dye compartmentalization was confirmed by permeabilizing AM ester-loaded cells with concentrations of digitonin that released cytosolic dye before compartmented dye. Upon gentle lysis in calcium-free Ringer's, compartmented dye revealed high $[Ca^{2+}]_i$ in compartments that could only be depleted with long term EGTA treatment (35 min) before permeabilization. In contrast, injected cells showed no evidence of compartmentation or

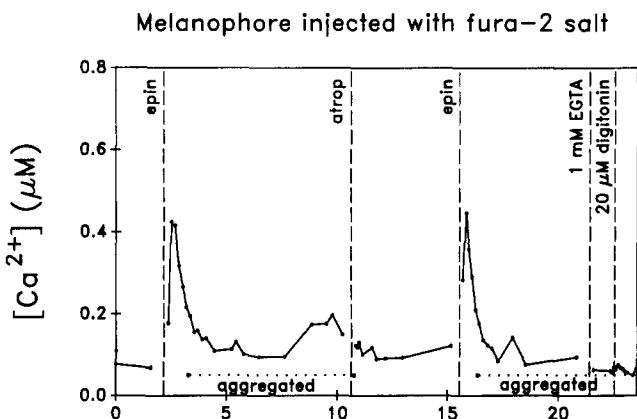


Figure 1. $[Ca^{2+}]_i$ in a melanophore microinjected with fura-2 pentapotassium salt. Melanosomes were aggregated with 50 μM epinephrine and after one rinse, dispersed with 100 μM atropine. After each addition of epinephrine, there was a $[Ca^{2+}]_i$ transient that peaked at full aggregation and spontaneously returned to basal levels. The small $[Ca^{2+}]_i$ rise at 8 min occurred spontaneously. Ringer's solution containing 1 mM EGTA and no calcium was added before digitonin in Ca^{2+} free Ringer's. Digitonin lysed cells lost fluorescence intensity uniformly while maintaining a low fluorescence ratio. The time of addition of agents is marked with vertical dashed lines and the period of complete aggregation is marked with dotted horizontal lines in this and the following graphs.

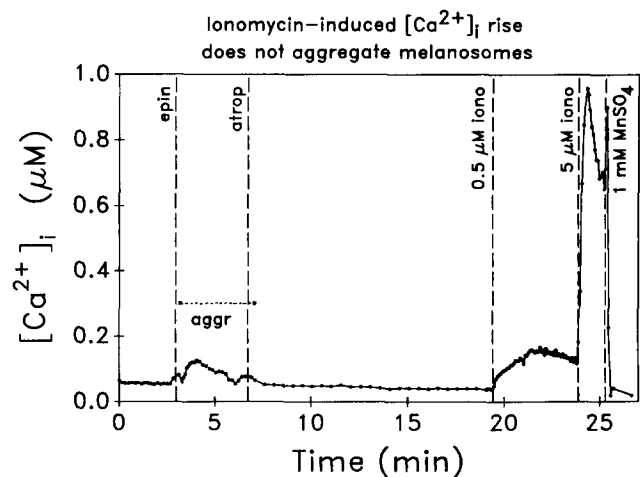


Figure 2. Ionomycin-induced $[Ca^{2+}]_i$ elevation does not aggregate melanosomes. Melanosomes were aggregated and redispersed as in Fig. 1. The epinephrine-induced $[Ca^{2+}]_i$ elevation in this fura-2/AM-loaded cell was lower than in cells injected with fura-2. 0.5 μM ionomycin produced a physiological rise in $[Ca^{2+}]_i$ and 5 μM ionomycin produced a supra-physiological rise. Neither induced melanosome aggregation. Quenching fura-2 fluorescence with $MnSO_4$ reduced intensity to background levels.

leakage of dye. Injected cells that were subsequently lysed with 20 μM digitonin in calcium-free Ringer's lost fluorescence more uniformly and monotonically than the AM ester-loaded cells (Fig. 1). Both AM ester-loading and microinjection of fura-2 were used for subsequent experiments and there was qualitative agreement between the two sets of data. However, the larger elevation measured in injected cells is probably more accurate.

The pigment in melanophores might differentially affect excitation wavelengths and it was important to measure any perturbation of fura-2 ratios. Melanosomes are 0.5 μm membrane-bound vesicles that contain melanin polymers. We tested commercial melanin for differential optical effects at 350 and 385 nm. The absorption spectra showed melanin scattered only 3% more light at 350 nm than 385 (probably Rayleigh scattering, not dispersion). Cuvette measurements of washed melanin in a Spex fluorimeter showed no fluorescent emission at 510 nm. Melanin in the excitation path (50% transmission) did not shift the emission spectra of fura-2 and produced only 1% decrease in the 350/385-nm ratio. Fluorescence ratio images of fura-2 in 5% agarose showed <1% variation of the ratio in the presence or absence of melanin granules of varying sizes (agarose was necessary to reduce Brownian motion of melanin granules). No diffraction artifacts from granule edges could be detected in ratio images under the conditions used. The predominant effect of melanosomes in cells was to eliminate fluorescence from the central mass of aggregated cells, and prevent measurements of $[\text{Ca}^{2+}]_i$ in this region. The $[\text{Ca}^{2+}]_i$ reported is a weighted average over the entire cell and so dim regions such as the central mass contribute little to the measurement. We conclude that there was no discernable effect of melanin on the fluorescence ratio from melanophores.

Inducing a Rise in $[\text{Ca}^{2+}]_i$ Does Not Aggregate Melanosomes

We tested the sufficiency of a rise in $[\text{Ca}^{2+}]_i$ for melanosome movement by artificially inducing a rise with the calcium ionophore, ionomycin. In Fig. 2, a melanophore was first aggregated and dispersed to confirm the ability to move melanosomes. Then ionomycin was added in two stages to produce physiologic, and then maximal increases in $[\text{Ca}^{2+}]_i$. This cell could not be induced to reaggregate vesicles by either of the increases in $[\text{Ca}^{2+}]_i$. In another experiment, ionomycin was added before epinephrine to demonstrate that the failure to aggregate in response to a rise in $[\text{Ca}^{2+}]_i$ was not due to a loss of the cells' ability to move vesicles. Six cells were treated with 5 μM ionomycin, which induced peak $[\text{Ca}^{2+}]_i$ of 300 to 430 nM. None of these cells aggregated pigment in response to the ionomycin and all cells subsequently aggregated in response to epinephrine.

Aggregated pigment responded to moderate and high ionomycin-induced elevations of $[\text{Ca}^{2+}]_i$ differently. First, moderate, long-term increases in $[\text{Ca}^{2+}]_i$ did not prevent melanosome dispersion. Aggregated cells in the constant presence of ionomycin maintained $[\text{Ca}^{2+}]_i > 200$ nM and were able to disperse melanosomes upon addition of atropine. This demonstrates that a return of $[\text{Ca}^{2+}]_i$ to basal levels is not required for melanosome dispersion. Second, large, rapid increases in $[\text{Ca}^{2+}]_i$ loosened the tight central mass in aggregated cells. When aggregated cells experienced a rapid, ionomycin-induced increase in $[\text{Ca}^{2+}]_i$, there was a

transient loosening of the tight central mass of melanosomes. An increase of $[\text{Ca}^{2+}]_i$ to 650 in one cell and to 2.3 μM in another produced an increase in the diameter of the tight central mass of 36 and 27%, respectively. Although we cannot exclude an indirect, transient effect of ionomycin, such as depletion of ATP as the cause of the transient movement, it is possible that some regulation of $[\text{Ca}^{2+}]_i$ is required to maintain the tightly aggregated state.

Blocking the Rise in $[\text{Ca}^{2+}]_i$ Does Not Prevent Vesicle Aggregation

We tested the necessity of the calcium rise for melanosome movement by clamping the normally observed rise in $[\text{Ca}^{2+}]_i$ with the calcium chelator, BAPTA or with calcium-free Ringer's containing EGTA. In Fig. 3, a cell was loaded with buffering concentrations of the chelator, BAPTA. Basal values were unchanged by BAPTA, but the epinephrine-induced $[\text{Ca}^{2+}]_i$ elevation was blocked without blocking movement. The rate and extent of a 5 μM ionomycin-induced $[\text{Ca}^{2+}]_i$ rise were five- and twofold lower, respectively, than in cells not loaded with BAPTA. The rate of the ionomycin-induced $[\text{Ca}^{2+}]_i$ elevation was affected by BAPTA buffering more than the extent was affected probably because the steady state levels should not be set by the buffering capacity when the plasma membrane has access to a nearly infinite Ca^{2+} reservoir (Rink and Tsien, 1983). Excessive fura-2, like BAPTA, can buffer $[\text{Ca}^{2+}]_i$ transients. Melanophores that were injected with 2 mM (pipette concentration) fura-2 produced slow, weak increases in $[\text{Ca}^{2+}]_i$ while cells that were injected with 500 μM fura-2 produced strong $[\text{Ca}^{2+}]_i$ transients. The rates of movement among all cells were similar, and were independent of the degree of the $[\text{Ca}^{2+}]_i$ elevation.

The epinephrine-induced $[\text{Ca}^{2+}]_i$ rise was also blocked by removing extracellular calcium. Calcium-free Ringer's solution containing 1 mM EGTA was added to cells for either 1 or 8.5 min before adding epinephrine. The basal levels of

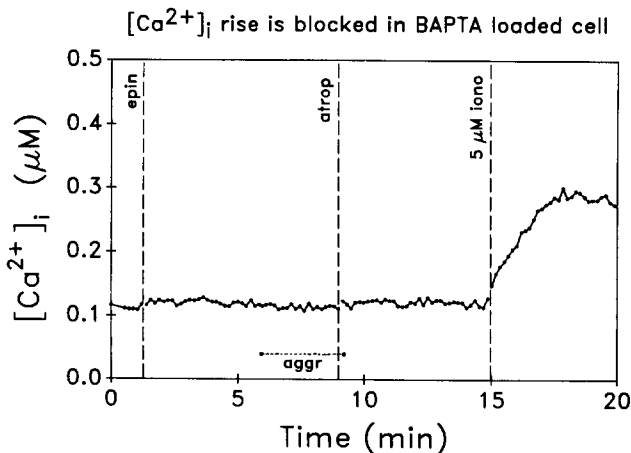


Figure 3. A $[\text{Ca}^{2+}]_i$ rise is blocked in a BAPTA/AM-loaded melanophore without blocking bidirectional movement. This cell was loaded with 1 μM fura-2/AM and 1 μM BAPTA/AM for 1 h. Melanosomes were aggregated and dispersed as in Fig. 1, but proceeded more slowly. There was no epinephrine-induced $[\text{Ca}^{2+}]_i$ increase. An ionomycin induced increase was fivefold slower and twofold lower than in cells not loaded with BAPTA/AM.

[Ca²⁺]_i decreased over several minutes presumably as internal calcium stores were gradually depleted. In cells that were treated with epinephrine early during the calcium depletion, [Ca²⁺]_i did not rise during aggregation, but instead continued to decrease (140 ± 9 to 125 ± 9 nM, *n* = 5). Despite the reversal of the normal [Ca²⁺]_i rise, these cells could aggregate and disperse melanosomes normally. Ca²⁺ depletion was reversible since incubating these same cells with normal Ringer's allowed [Ca²⁺]_i to return to pre-EGTA basal levels and promoted a [Ca²⁺]_i elevation during aggregation (155 ± 16 basal, 169 ± 27 nM peak, *n* = 5). In a second experiment, an 8.5-min incubation in calcium-free Ringer's reduced the basal levels by half. In these cells, Epinephrine produced a [Ca²⁺]_i elevation, but to levels that were less than basal levels of untreated cells (78 ± 2 basal, 118 ± 27 nM peak, *n* = 5). Bidirectional movement occurred at normal rates in these calcium-depleted cells. We conclude that movement of pigment vesicles in both directions was not affected by blocking elevations or reversing the normal [Ca²⁺]_i rise or by restricting [Ca²⁺]_i to concentrations below the normal basal levels.

Inhibitors of Calmodulin Activation Do Not Block Aggregation

To test for calmodulin participation, the inhibitors of calmodulin activation, calmidazolium, and W-7, were added to cells before addition of epinephrine or atropine. As expected, aggregation was not blocked or induced by a 3-min incubation in 1 or 5 μM calmidazolium (*n* = 8 for each) or a 2-min incubation in 10 or 20 μM W-7 (*n* = 28 for each). Surprisingly, these inhibitors induced rapid dispersion of epinephrine-aggregated melanosomes. Calmidazolium induced hyper-dispersion of melanosomes to the cell periphery in 12 of 17 cells, while W-7 partially dispersed 24 of 24 cells. Addition of atropine increased the number (in calmidazolium) or the degree (in W-7) of dispersed cells. However, the minimum dispersing concentration of inhibitor was close to the concentration where cell lysis began to occur (10 μM calmidazolium, 30 μM W-7). Although during dispersion, the effects of these inhibitors might not be specifically calmodulin mediated, we conclude that during aggregation, calmodulin does not have a role in regulating movement.

Measurement of [cAMP]_i in Melanophores

We next tested the role of [cAMP]_i dynamics for bidirectional movement of pigment vesicles. Isolated melanophores on glass coverslips were microinjected with 60 μM (pipette concentration) of FICRhr, and fluorescence ratio images were recorded. Cells were allowed to rest for 10–40 min before challenging the cells to aggregate pigment. The basal fluorescence ratio in melanophores before stimulation was higher than in fibroblasts or smooth muscle cells suggesting [cAMP]_i was elevated compared to these cells (Adams et al., 1991). Upon the addition of epinephrine, the fluorescence ratio decreased concomitantly with melanosome aggregation to the center of the cell (Fig. 4). Aggregation typically proceeded over 30–60 s in injected cells, the same as in uninjected cells. The ratio remained constant until the addition of atropine, whereupon the ratio increased to basal levels and melanosomes redispersed. The cell presented in Fig. 4 was then reaggregated a second time and the ratio again decreased.

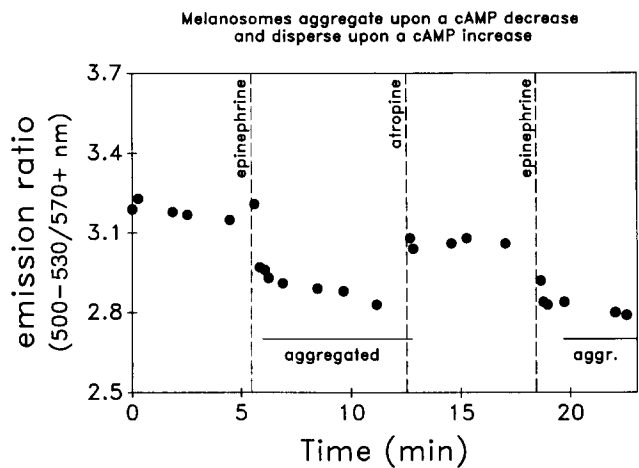


Figure 4. FICRhr fluorescence ratios show that [cAMP]_i decreases upon addition of epinephrine. Melanophores were injected with 60 μM FICRhr (pipet concentration). The ratio of emission at 500–530 nm to that >570 nm was normalized to the same ratio obtained from equimolar fluorescein and rhodamine B in buffer. The decrease in fluorescence ratio reflected a rapid decrease in [cAMP]_i at the start of vesicle movement. Redispersion and re-aggregation were reflected by a rise and subsequent fall in ratio. The reversibility of the ratio change as FICRhr dissociates and reassociates is clearly demonstrated.

This cell presented an unusual opportunity to observe dispersion in one region of the cell a few seconds before the rest of the cell. The ratio increase was initially limited to the region where pigment vesicles moved, and later increased throughout the rest of the cell. This observation suggests that control of movement is determined locally by the [cAMP]_i. However, we have not systematically repeated this observation and its generality remains to be tested. Fig. 4 also shows a slow overall decrease in the fluorescence ratio. The decrease was due to high illumination levels and the faster photobleaching of the fluorescein-labeled catalytic subunit than the rhodamine-labeled regulatory subunit. Lower illumination levels reduced photobleaching, but produced noisier ratio traces (compare Figs. 4 and 6). The average fluorescence ratio decreased by 0.27 ± 0.08 (mean ± SD, *n* = 7). For reasons stated in the Materials and Methods section, we did not calibrate the fluorescence ratio of FICRhr in melanophores. However, we can estimate the decrease of [cAMP]_i by comparing the fractional ratio decrease in melanophores to that in cuvettes (Adams et al., 1991). The fluorescence ratio decrease corresponds to at least a two to threefold decrease in [cAMP]_i.

The fluorescence ratio of a melanophore injected with 7.6 μM FICRhr is presented pictorially in Fig. 5. Fig. 5a shows the initial, unstimulated fluorescence ratio in a cell with dispersed pigment granules. Fig. 5, b and c show a progressive and uniform decrease of the ratio as epinephrine is increased from 100 to 150 μM. The degree of aggregation increased as the fluorescence ratio decreased, which showed that melanosome aggregation was proportional to the reduction in [cAMP]_i. The darkening of the center of the cell is caused by the aggregation of the light-absorbing pigment to that region. Fig. 5d shows the effect of 1 μM IBMX, a phos-

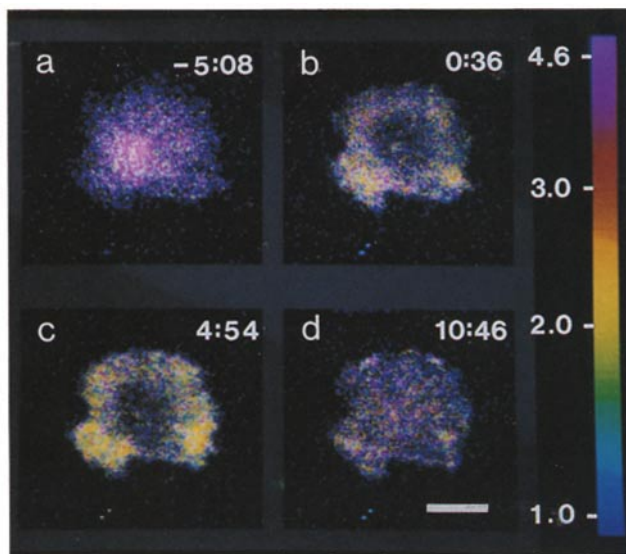


Figure 5. [cAMP]_i decreases in a single living cell during melanosome aggregation. Melanophores were injected with 7.6 μM FICRhR and fluorescence ratios were prepared as in Fig. 5 and are displayed in pseudocolor according to the color bar at the right. Cell 5:08 (min/sec) before hormone addition (a) had very high ratios compared to other cell types tested. The melanosomes were uniformly distributed throughout the cell (a) until they were progressively aggregated and [cAMP]_i was progressively lowered with 100 μM epinephrine at 0:00 (b) and 150 nM epinephrine at 2:24 (c). The dark zone in the center of the cell in b and c was produced by the aggregated pigment but otherwise the fluorescence extended to the periphery of the cell. Addition of 1 μM IBMX at 5:06 (d) increased [cAMP]_i and dispersed melanosomes. Bar, 20 μm .

phodiesterase inhibitor. The ratio increased to near basal levels and the dark central mass of pigment dispersed. The final ratio was slightly lower than the initial ratio because of disproportionate photobleaching of the fluorescein. This figure shows that increasing concentrations of epinephrine produce both progressive lowering of [cAMP]_i and increasingly aggregated melanosomes.

Agents that increase [cAMP]_i also increased the fluorescence ratio and dispersed melanosomes. Concentrations from 0.5 to 2 mM IBMX produced progressively increasing ratios and melanosome dispersion. The ratio also could be increased in cells by the addition of 1 mM 8-bromo cAMP, but not 500 μM dibutyryl cAMP. The dibutyryl derivative failed because it poorly entered melanophores, since esterifying the phosphate with an acetoxymethyl group rendered dibutyryl cAMP effective at concentration as low as 10 μM (Carsten Schultz, Paul Sammak, and Roger Tsien, unpublished results). These results directly demonstrate in single living cells that [cAMP]_i dynamics respond in a dose dependent manner to the concentration of extracellular hormone, phosphodiesterase inhibitor or cAMP analog, and that bidirectional vesicle movement in melanophores follows [cAMP]_i dynamics.

Potential Artifacts in the Measurement of [cAMP]_i

During aggregation there are dramatic changes in morphology as melanosomes, mitochondria, and other cytoplasmic

components are drawn into the central mass. The resulting thickening of the central portion of the cell causes this region of the cell, just around the central pigment mass, to appear brighter. It might be argued that the fluorescence ratio decrease is an artifact of the geometric changes. To test this possibility directly, melanophores were microinjected with 25 μM fluorescein and 25 μM rhodamine B in standard injection buffer. There was no change in slope of the fluorescence ratio upon aggregation or dispersion (not shown) in contrast to FICRhR injected cells (Fig. 3). There was a general decrease in ratio over time; this is due primarily to unequal rates of dye leakage from the cell and secondarily to unequal rates of photobleaching. We conclude that the fluorescence ratio is unaffected by the geometric changes that accompany melanosome aggregation and that the ratio change reflects fluorescence energy transfer as holoenzyme is formed *in vivo*.

To test whether the concentration of microinjected, labeled kinase might affect the response of melanophores to epinephrine, we observed cells that had been injected with a range of concentrations of FICRhR. Cells that were injected with a bolus of either 7.6 or 60 μM FICRhR that equalled 1 to 5% of the cell volume showed no morphological changes. However, six cells that were injected with larger volumes of 60 μM FICRhR (10 to 20%) aggregated melanosomes. At moderate concentrations, injection induced a transient aggregation of melanosomes. In two of the six cells, partial aggregation was induced within seconds, followed by partial dispersion in a few seconds (Fig. 6 b). This is much faster than epinephrine and atropine induced movement, which took nearly 2 min. In four of six cells injected with large amounts of FICRhR, nearly complete, permanent aggregation was induced. In Fig. 6, we present a comparison of the response of cells injected with low (Fig. 6 a) and high (Fig. 6 b) concentrations of FICRhR. In Fig. 6 a robust movement and [cAMP]_i dynamics were observed while in Fig. 6 b, melanosomes partially aggregated after injecting FICRhR and aggregated somewhat more when epinephrine was introduced 11 min later. This aggregation could not be reversed by adding atropine. Besides its effect on the movement of melanosomes, excess injected holoenzyme reduced the basal fluorescence ratio and suppressed [cAMP]_i dynamics. These perturbations were easy to avoid by reducing the intracellular concentrations of FICRhR by 20–40-fold (see Fig. 5).

Requirement of the Catalytic Subunit for Movement and Potential Buffering of [cAMP]_i by FICRhR

To further test the effects of FICRhR and to determine the requirement for the enzyme subunits for motility, the R^I and C_α subunits were separately injected into dispersed and aggregated cells. 58 μM R^I (which was saturated with cAMP) and 59 μM C_α were prepared in injection buffer and injected at approximately 1 to 20% of the cell volume. Dispersed melanosomes were unaffected by an overdose of R^I or of C_α or even sequential injection of both R^I (with cAMP) and C_α into the same cell. As described above, dispersed melanosomes were aggregated by microinjection of a comparable overdose of FICRhR (without bound cAMP). In contrast, aggregated melanosomes were dispersed by injection of C_α but not R^I (Table I). Aggregated melanosomes

**cAMP dynamics and melanosome movement are reduced
in cells with high concentrations of FICRHR**

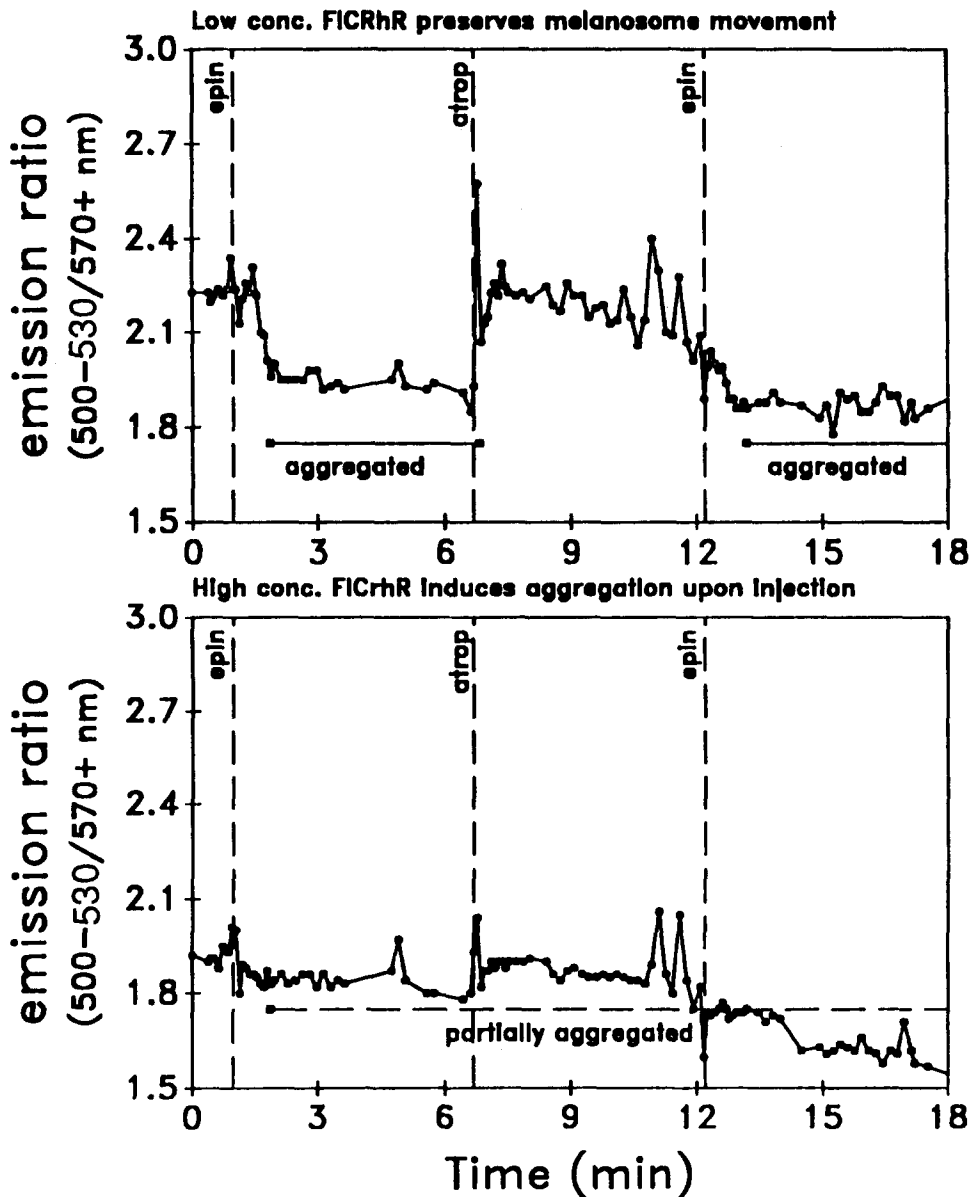


Figure 6. cAMP dynamics and melanosome movement were reduced in cells with high concentrations of FICRHR. (a) This cell was injected with low volumes of 60 μ M FICRHR. The initial ratio was high and melanosomes remained dispersed until epinephrine decreased the ratio and aggregated melanosomes. (b) An adjacent cell in the same field that had been injected with a much larger volume of 60 μ M FICRHR exhibited a lower initial ratio and negligible changes. Injection was shortly followed by the partial aggregation of melanosomes. Epinephrine aggregated melanosomes somewhat more while atropine was unable to redisperse melanosomes.

that were injected with small volumes of C_{α} (~5%) partially dispersed for a few seconds (Fig. 7, a-c). Cells injected with 20% C_{α} dispersed more fully and remained dispersed for many minutes (Fig. 7, d-f). In addition, 2 μ M (pipette concentration) of the specific peptide inhibitor of the catalytic subunit, PKI₅₋₂₄ amide, induced aggregation. At the lowest final concentrations, melanophores would aggregate partially for a few minutes (Fig. 8, a-c), but at higher concentrations, nearly complete aggregation was maintained (Fig. 8, d-f). The concentration of PKI₅₋₂₄ amide that completely blocked kinase activity in melanophores (20 to 400 nM estimated intracellular concentration) was somewhat over the IC₅₀ for the catalytic subunit, 2.5 nM (Cheng et al., 1986). Control of the activity of the catalytic subunit is required for both aggregation and dispersion since exoge-

nous C_{α} subunit dispersed melanosomes and inhibition of the endogenous C subunit aggregated melanosomes.

Okadaic Acid Blocks Aggregation and Induces Dispersion

Since kinase activity controls bidirectional vesicle transport, we tested the requirement of phosphatases for melanosome movement. Melanophores were pretreated with okadaic acid, a phosphatase inhibitor, and then challenged to aggregate pigment (Table II). The threshold for inhibition of aggregation was between 100 and 500 nM. Blocked cells were unable to aggregate melanosomes even after thorough washout and a 10 min rest without okadaic acid. However, blockage of aggregation was probably not a non-specific

Table I. Effects of cAMP Dependent Protein Kinase Injections

Agent	In pipette (μM)	Number of cells	Induced movement?
Dispersed cells (native state, high [cAMP] _i)			
R ₂ subunit with bound cAMP	58	5 of 5	No
C subunit	59	7 of 7	No
FICR _h R, ([2C]R ₂ without cAMP)	60	4 of 11	Aggregated
PKI ₅₋₂₄ amide (inhibitor of C subunit)	200	7 of 7	Aggregated
Aggregated cells (induced by 50 nM epinephrine, low [cAMP] _i)			
R ₂ subunit with bound cAMP	58	4 of 4	No
C subunit	59	8 of 8	Dispersed

The catalytic subunit of cAMP-dependent kinase controls the direction of movement. The catalytic (C) subunit of cAMP dependent protein kinase was injected (pipette concentration, 59 μM). In cells with aggregated melanosomes, dispersion was induced, and in cells with dispersed melanosomes there was no effect. The peptide inhibitor of C, PKI₅₋₂₄ amide (200 μM), aggregated melanosomes. The R₁ subunit, with bound cAMP, had no effect on cells with either dispersed or aggregated melanosomes. At high concentrations, the fluorescently labeled holoenzyme, FICR_hR (without bound cAMP) also aggregated melanosomes.

effect since okadaic acid also induced dispersion (Table II). These results suggested a specific effect on a regulator of the motile machinery. Okadaic acid inhibits the phosphatase 2A at low concentrations (IC₅₀ = 1 nM), PP-1 at intermediate concentrations (IC₅₀ = 315 nM), PP-2B at high concentrations (IC₅₀ = 4.5 μM) and affects the PP-2C over 10 μM

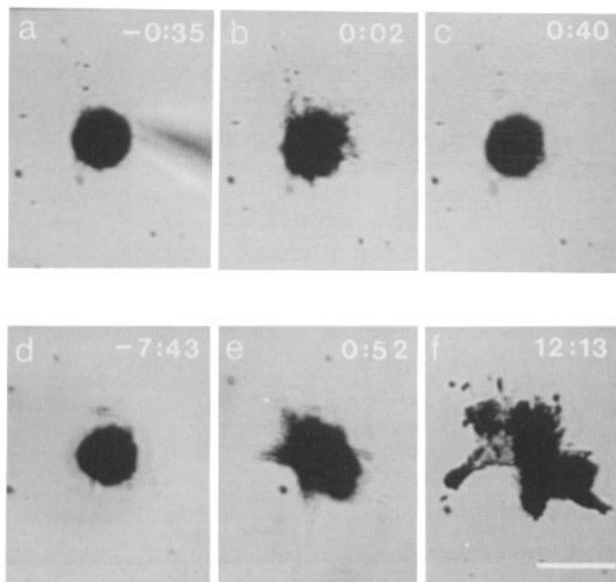


Figure 7. The catalytic subunit disperses melanosomes. C_α (59 μM) was injected into cells that were preaggregated with 50 nM epinephrine. (a-c) show a cell that underwent transient, slight dispersion by injection of 59 μM C_α at 0:00 (min/sec). A second injection in this cell produced a second, temporary dispersion (not shown). d-f show a cell that was dispersed at 0:00 by microinjection of C_α and remained dispersed. The injection volume was larger in this cell. Bar, 20 μm .

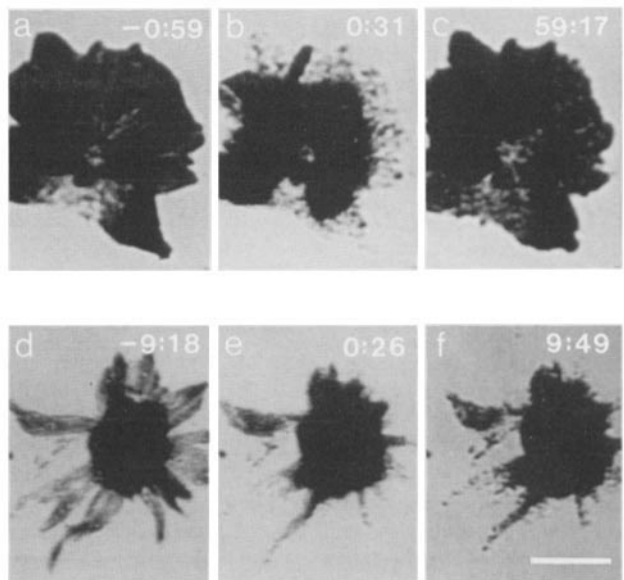


Figure 8. Protein kinase inhibitor peptide aggregated melanosomes. A synthetic peptide inhibitor of the catalytic subunit, PKI₅₋₂₄ amide was injected into dispersed cells at a pipette concentration of 200 μM at 0:00 (min/sec). a-c show a cell that underwent transient, slight aggregation (most of the redispersion had occurred within the first minute), while d-f show a cell that was injected with a larger volume and was permanently aggregated.

(Bialojan and Takai, 1988). Since okadaic acid completely blocked melanosome aggregation and induced full dispersion at concentrations that are lower than the IC₅₀ for PP-2B and PP-2C, their action is probably not required for movement, unlike *Tilapia* melanophores (Thaler and Haimo, 1990). The dose-response of okadaic acid nominally implicate PP-1, but the action of PP-2A cannot be excluded in melanophores since the endogenous concentration of PP-2A can alter the effective IC₅₀ by more than 10-fold (Cohen et

Table II. Okadaic Acid Blocks Melanosome Aggregation and Induces Dispersion

Treatment	Number of cells	Movement after wash?
Dispersed cells (okadaic acid pretreatment, aggregate with 50 nM epinephrine)		
10 nM, 30 min	0 blocked of 55	Aggregated
100 nM, 10 min	51 partial of 89	Aggregated
500 nM, 10 min	68 blocked of 68	No
1 μM , 10 min	44 blocked of 44	No
10 μM , 30 min	54 blocked of 54	No
Aggregated cells (aggregate with 50 nM epinephrine, followed by okadaic acid)		
1 μM	14 dispersed of 15	

Okadaic acid blocks aggregation and induces dispersion. Cells with dispersed melanosomes were incubated with okadaic acid, and then challenged to aggregate with 50 nM epinephrine. 100 nM okadaic acid only partially blocked aggregation in most cells, and 500 nM blocked all aggregation. Cells were washed and after 10 min were challenged with epinephrine again. Cells that were initially blocked were unable to aggregate after okadaic acid washout. Aggregated cells dispersed pigment when 1 μM okadaic acid was added showing that the effect of okadaic acid was specific to the motile machinery.

al., 1989, 1990). Okadaic acid has no effect on ATP levels, or on cAMP-dependent protein kinase (Cohen, 1990), and so it should not act on the hormonally regulated kinase pathway in melanophores. We conclude that continuous phosphate turnover is required for maintenance of both the dispersed and aggregated state.

Effect of cAMP Active Agents on Melanosome Movement

To show that [cAMP]_i dynamics are both necessary and sufficient for movement, exogenous agents that increase or decrease cAMP were used to induce or block melanosome movement. In Table III, the action of several agents on movement are described. Aggregation could be induced with an antagonist of cAMP, R_pcAMPS. A high dose, 400 μM, had to be applied for 1 h probably because the binding affinity for the regulatory subunit is low and because R_pcAMPS had to compete with the unusually high [cAMP]_i found in melanophores. Cells induced to aggregate melanosomes with R_pcAMPS would redisperse pigment when washed free of the antagonist, and could subsequently reaggregate melanosomes in response to epinephrine.

Dispersion of pigment vesicles required an elevation of the [cAMP]_i. Table III shows several agents that increased [cAMP]_i and dispersed melanosomes in the continuous presence of aggregating concentrations of epinephrine, (5–50 nM). S_pcAMPS, an isomer of the antagonist, R_pcAMPS, is a good cAMP analog and is effective in dispersing melanosomes. The analogs, 8-bromo cAMP and the AM ester of dibutyryl cAMP were also effective, but dibutyryl cAMP alone was ineffective (see above). Melanophores that were on scales also could be induced to aggregate with 1 mM 8-bromo cAMP confirming that isolation of cells did not confer cAMP sensitivity. The phosphodiesterase inhibitors IBMX (1 mM) and Ro20-1724 (500 μM) dispersed melanosomes, and the adenylate cyclase activator, forskolin (200 μM), was also

Table III. Effect of cAMP-Active Agents on Melanophores

Agent	Threshold for movement	Action
Dispersed cells (native state)		
R _p cAMPS	400 μM, 1 h, Aggregated	cAMP antagonist
Aggregated cells (induced by 5–50 nM epinephrine)		
S _p cAMPS	30 μM, 20 min, Dispersed	cAMP analog
8-Bromo cAMP	500 μM, 17 min, Dispersed	cAMP analog
Bt ₂ cAMP	1 mM, 1 min, No effect	cAMP analog
IBMX	1 mM, 1 min, Dispersed	PdE inhibitor
Ro20-1724	500 μM, 1 min, Dispersed	PdE inhibitor
Forskolin	200 μM, 1 min, Dispersed	Adenylate cyclase activator

cAMP antagonists aggregate and cAMP agonists disperse melanosomes. The cAMP antagonist R_pcAMPS competed for endogenous cAMP in dispersed cells and induced full aggregation at 400 μM. Cells aggregated with 50 μM epinephrine were induced to redisperse melanosomes with the cAMP analogs, S_pcAMPS and 8-Bromo cAMP. Phosphodiesterase inhibitors, IBMX, and Ro20-1724 completely dispersed melanosomes while forskolin partly disperses melanosomes.

able to disperse melanosomes, albeit only partially. Again, when these agents were washed away and replaced with epinephrine, the cells were still competent to reaggregate melanosomes demonstrating that movement was not induced by cell damage. These results demonstrate that [cAMP]_i dynamics are sufficient for directional control of motility.

The necessity of a decrease of [cAMP]_i for aggregation was tested by blocking the decrease thereby preventing aggregation. While 500 μM 8-bromo cAMP was sufficient to redisperse epinephrine-treated melanosomes (Table III), 4 mM 8-bromo cAMP was required to block epinephrine-induced aggregation (Table IV). We also tested the necessity of a [cAMP]_i decrease for aggregation by blocking the phosphodiesterase. 1 mM IBMX, the concentration required to disperse melanosomes, was also able to block epinephrine-induced aggregation. We conclude that high concentrations of a cAMP analog can block aggregation and that IBMX-sensitive phosphodiesterase activity and a decrease in [cAMP]_i are required for melanosome aggregation.

Receptor Pharmacology of Melanophores

Since an adrenergic agonist (epinephrine) and a muscarinic antagonist (atropine) induce movement of pigment vesicles, we performed pharmacological experiments to sort out the receptors responsible for transducing signals in angelfish melanophores. Agents were applied to isolated cells on glass coverslips at concentrations that did not produce (or block) movement, and then the concentrations were increased by 10-fold until movement was produced (or blocked). 5 nM epinephrine fully aggregated melanosomes in some cells, but reliable aggregation required 50 nM (Table V). Since dispersion could be induced simply by washing out epinephrine, all dispersing agents were tested in the presence of threshold concentrations of epinephrine (5–50 nM). Under these conditions, 100 nM atropine would induce dispersion. Epinephrine potentially acts on all α and β adrenergic receptor subtypes. However, 10–100 nM of the α₂-specific antagonist, yohimbine, blocked epinephrine-induced aggregation, and induced dispersion while the α₁-specific antagonist prazosin was without effect. The α₂-specific agonist aminoclonidine (100 nM) aggregated melanosomes as well. Stimulation of α₂-receptors decreases [cAMP]_i in all cell types, although effects on other second messengers also might occur (Michel et al., 1989). The effectiveness of α₂-adrenergic receptors in aggregating melanosomes provides additional evidence

Table IV. Blocking Epinephrine-induced Melanosome Aggregation

Preincubate dispersed cells with agents and then treat with 50 nM epinephrine			
Agent	Preincubation time	Number cells dispersed	Blocked?
8-Bromo cAMP			
250 μM	30 min	none of 12	No
4 mM	35 min	12 of 12	Yes
IBMX (PdE inhibitor)			
1 mM	1 min	15 of 15	Yes

Blocking the [cAMP]_i decrease blocks melanosome aggregation. The epinephrine induced aggregation of melanosomes could be blocked by agents that maintain high [cAMP]_i, such as high concentrations of 8-Bromo cAMP and the phosphodiesterase inhibitor, IBMX.

Table V. Receptor Pharmacology of Melanophores

Agent	Threshold for movement	Action
Dispersed cells (normal state)		
Epinephrine	50 nM, Aggregated	α and β adrenergic agonist
Aminoclonidine	100 nM, Aggregated	α_2 specific agonist
Carbachol	4 mM, No effect	muscarinic agonist
Aggregated cells (induced with 5–50 nM epinephrine)		
Yohimbine	10–100 nM, Dispersed	α_2 specific antagonist
Prazosin	100 nM, No effect	α_1 specific antagonist
Atropine	100 nM, Dispersed	Muscarinic antagonist, uncharged
Methyl-Atropinium	1 mM, Dispersed	Muscarinic antagonist, less permeable
Scopolamine	100 μ M, Dispersed	Muscarinic antagonist, uncharged
Methyl-Scopolaminium	1 mM, No effect	Muscarinic antagonist, less permeable
Isoproterenol	10 M, No effect	β_1 and β_2 agonist
Propranolol	1 μ M, Does not block dispersion	β_1 and β_2 antagonist
Alprenolol	1 μ M, Does not block dispersion	β_1 and β_2 antagonist

α_2 adrenergic receptors control bidirectional melanosome movement. Epinephrine aggregated melanosomes via an α_2 adrenergic receptor (mimicked by aminoclonidine and blocked by yohimbine but not prazosin). Atropine, a muscarinic antagonist did not act via a muscarinic receptor or a β adrenergic receptor to disperse melanosomes. β receptors are not present, unlike melanophores from other teleosts. The other muscarinic antagonists work only at unusually high concentrations suggesting atropine's action on melanophores was by an atypical mechanism.

that a decrease in $[cAMP]_i$ is responsible for pigment movement.

In some teleost melanophores, α -adrenergic receptors are responsible for aggregation while β -adrenergic receptors are responsible for dispersion. However, in *Pterophyllum scalare* melanophores, the β_1 and β_2 agonist isoproterenol was ineffective in dispersing melanosomes. In addition the β antagonists, propranolol, and alprenolol did not block atropine induced dispersion, suggesting that atropine did not act by cross-stimulating a β -adrenergic receptor.

Since the muscarinic antagonist, atropine dispersed melanosomes, we tested other muscarinic agents for activity. The muscarinic agonist, carbachol, acts on all muscarinic receptor subtypes and yet was unable to induce aggregation. Therefore, atropine did not block a muscarinic receptor when it induced dispersion. The muscarinic antagonists, scopolamine, and the less membrane permeable quaternary derivatives, methyl-atropinium, and methyl scopolaminium are all effective at the same concentration in other cell types (Brown and Goldstein, 1986). However, in melanophores, scopolamine was 1,000-fold less effective than atropine and the charged quaternary derivatives were even less effective (Table 5). A standard muscarinic receptor was not involved in pigment vesicle motility.

Discussion

We have elucidated part of the regulatory mechanism that governs vesicle movement in angelfish melanophores. There are changes in the intracellular concentration of both Ca^{2+} and cAMP during epinephrine-induced aggregation, but $[Ca^{2+}]_i$ dynamics are not required while $[cAMP]_i$ dynamics are uniquely linked to intracellular movement. The evidence for the requirement of $[cAMP]_i$ for bidirectional melanosome movement includes first, direct measurement of $[cAMP]_i$ dynamics with the indicator, FICRHR; second, cAMP agonists and the catalytic subunit of the cAMP-dependent protein kinase disperse melanosomes or block

aggregation; third, cAMP antagonists and kinase inhibitors aggregate melanosomes; fourth, the receptor pharmacology is consistent with α_2 receptors, which are known in other systems to decrease $[cAMP]_i$. In the current study, the fluorescent indicators fura-2 and FICRHR provided a direct measure of the dynamics of $[cAMP]_i$ and $[Ca^{2+}]_i$ during physiological activity in single living cells.

Direct Measurement of the Dynamics of cAMP

The measurement of $[cAMP]_i$ in single living cells with FICRHR is the most direct means of establishing the involvement of this second messenger. Fluorescence ratio imaging has the unique advantages of reporting real-time changes of the fraction of cAMP that is available to interact with its kinase. Both spatial and temporal variations within a single cell can be measured, heterogeneity among cells can be revealed, and most importantly this diversity can be correlated with cell behavior. There are several requirements of this technique. First, the best advantages require digital image microscopy and fluorescence emission ratioing, which are well known but non-trivial techniques. Second, the indicator is a protein and is not available in a membrane-permeant form. Third, the fluorophores are subject to photobleaching. With our present equipment, many tens of pictures can be obtained with only a minor shift in ratio. The fluorescence ratio change induced by energy transfer from fluorescein-catalytic to rhodamine-regulatory subunits does not report the $[cAMP]_i$ per se, but the fraction of holoenzyme formed. However, the assembly of the $C_{\alpha_2}R^1_2$ holoenzyme is not modified by autophosphorylation, as it is with the R^{II} subunit (Rangel-Aldao and Rosen, 1977), and the holoenzyme dissociation faithfully reflects $[cAMP]_i$ in vitro (Adams et al., 1991).

The basal fluorescent ratio in melanophores was much higher than in fibroblasts or smooth muscle cells (Adams et al., 1991), so $[cAMP]_i$ is higher than in other cells. In most cell types the holoenzyme is not fully activated under basal conditions, whereas in melanophores, the half maximal acti-

vation of FICRhr (90 nM cAMP; Adams et al., 1991) was exceeded in unstimulated cells. Previous radioimmunoassay measurements estimated 2 μ M total cAMP in melanophora cells (Chou et al., 1989). Chou et al. reported a moderate decrease of $[cAMP]_i$ upon stimulation, 30%, while $[cGMP]_i$ remained essentially unchanged. In our study the fluorescence ratio change (27%) suggested at least a two- to threefold change of $[cAMP]_i$ based on the dynamic range of FICRhr in vitro. The more dramatic $[cAMP]_i$ dynamics measured here could be due to the selective sensitivity of FICRhr to biologically active cAMP in the cytosol and not total cAMP in the tissue.

The major known cellular binding site of cAMP is the regulatory subunit of the kinase, which releases the active catalytic subunit. The catalytic subunit is essential since the injection of C_α is sufficient to induce dispersion of epinephrine-aggregated melanosomes and the specific peptide inhibitor of the catalytic subunit, PKI₅₋₂₄ amide, is sufficient to aggregate melanosomes in unstimulated cells. The regulatory subunit, R^I, was unable to induce movement in either direction, but we have not tested the regulatory subunit, R^{II}. R^{II} can act independently of the catalytic subunit by inhibiting the phosphatase PP-1 (Jurgensen et al., 1985). It is a strong candidate regulator since R^{II} binds specifically to microtubules (Miller et al., 1982), and its phosphorylation in sea urchin sperm alters dynein motility (Tash, 1989). A full understanding of the regulation of vesicle movement by the cAMP-dependent protein kinase would require tests of the role of R^{II}.

Experiments with the phosphatase inhibitor okadaic acid showed that either the phosphatase PP-1 or 2A was required to induce and to maintain aggregation in *Pterophyllum* melanophores. We do not know whether phosphatase activity was regulated during the directional control of pigment granules or whether a constant basal activity was required for movement. One way that epinephrine might coordinately control both the phosphatase as well as the kinase is via the inhibitor-1, which inhibits PP-1 only when it is phosphorylated by the cAMP-dependent protein kinase (Cohen, 1989). The coordination of the kinase and phosphatase would increase sensitivity to epinephrine and increase energy efficiency. Coordinated enzyme action also might promote the homogeneous movement of pigment granules and help explain why cells are usually found either completely dispersed or aggregated and not partly so. A complete understanding of the regulation of melanosome movement would require tests of the control of the phosphatase.

The labeled holoenzyme had two additional effects when injected at high concentrations: $[cAMP]_i$ dynamics were reduced and melanosomes were induced to aggregate without hormones. It is not too surprising that large amount of FICRhr tended to buffer the apparent $[cAMP]_i$ since any indicator at too high a concentration becomes a buffer for the substance being measured. For example, the highest concentrations of FICRhr attained in the cell might have reached 12 μ M, which would require 48 μ M cAMP to be saturated, an amount well above the 2 μ M estimated total cAMP in teleost melanophora cells (Chou et al., 1989) and that might well exceed the capacity of the cell to synthesize. The induced aggregation is more difficult to explain, since it implies that adding a huge excess of holoenzyme to fixed amounts of endogenous kinase and cAMP actually decreases the resulting

total kinase activity. Two possible explanations, not mutually exclusive, are as follows. First, each mole of regulatory subunit has to bind two moles of cAMP to release the active catalytic subunit. When the concentration of R subunits considerably exceeds that of total cAMP, statistics would favor single rather than double occupancy of each R, which would divert the cAMP to nonproductive complexes. (The Hill coefficient for cAMP activation for the batch of FICRhr used in these experiments was 1.1, at the low end of the normal range of variation, so that positive cooperativity was very slight.) Second, the particular batch of kinase used in these experiments was later discovered to have a lower-than-usual V_{max} towards the artificial substrate Kemptide though the V_{max} was not further reduced by the process of labeling. Such material when injected as holoenzyme in large excess might well compete with endogenous kinase for cAMP and produce less final kinase activity, whereas injection of the isolated C subunit without R could only augment kinase activity. In any case, perturbation of $[cAMP]_i$ with excess FICRhr is not an insurmountable problem since adequate fluorescent signals were obtained from FICRhr at concentrations 20- to 40-fold lower than buffering concentrations (Fig. 5).

cAMP Active Agents Can Induce or Block Melanosome Movement

Since $[cAMP]_i$ decreases upon epinephrine-induced aggregation of melanosomes, it could be expected that agents that antagonize $[cAMP]_i$ would induce aggregation. The antagonist R_p cAMPS can aggregate melanosomes. Conversely, cells with aggregated pigment should disperse melanosomes when treated with agents that increase $[cAMP]_i$. As expected, analogs of cAMP such as S_p cAMPS, dibutyryl cAMP/AM ester and 8-bromo cAMP, can induce dispersion, and agents that block the action of phosphodiesterases such as IBMX and Ro20-1724 also readily induce dispersion. Epinephrine-induced aggregation of melanosomes could be blocked by agents that maintained an elevated $[cAMP]_i$. Either high concentrations of 8-bromo cAMP or moderate concentrations of IBMX prevented aggregation. These agents demonstrate that the decrease of $[cAMP]_i$ is both necessary and sufficient for melanosome aggregation.

Since the movement of melanosomes depends on $[cAMP]_i$ in a concentration-dependent manner, simple brightfield observations of melanophores could be used as an optical sensor of $[cAMP]_i$ (Lerner, 1988). For example, activity in vivo of the new AM ester of dibutyryl cAMP was confirmed by its ability to aggregate melanosomes. The action of other uncharacterized agents on $[cAMP]_i$ could be rapidly assayed in living cells by the degree of dispersion of the melanosomes.

Intracellular Calcium Dynamics Do Not Govern Bidirectional Vesicle Movement in Angelfish Melanophores

Direct measurements of the $[Ca^{2+}]_i$ in angelfish melanophores using the calcium indicator, fura-2, indicated a change in $[Ca^{2+}]_i$ from 70 to just under 400 nM upon epinephrine-stimulated aggregation. The source of the increased $[Ca^{2+}]_i$ is probably both extracellular and intracellular. Calcium-free Ringer's solution reduced but did not eliminate the $[Ca^{2+}]_i$ rise and so a portion of the increase is

from extracellular calcium. Digitonin lysis in calcium-free Ringer's demonstrated the presence of internal calcium stores and it is possible that these stores also contribute to the rise in $[Ca^{2+}]_i$. The endogenous mechanism of inducing a $[Ca^{2+}]_i$ elevation remains unknown. It is possible that the $[Ca^{2+}]_i$ increase is produced by α_2 stimulation (Limbird, 1989), although Ca^{2+} elevation is usually associated with α_1 adrenergic stimulation and cAMP reduction is associated with α_2 stimulation (Exton, 1985). It is also possible that α_1 stimulation elevates $[Ca^{2+}]_i$ independently of α_2 -stimulated motility.

Three lines of evidence suggested that the $[Ca^{2+}]_i$ rise was not needed for melanosome aggregation, and that the return to basal levels was not needed for dispersion: the timing of $[Ca^{2+}]_i$ changes, artificial induction of changes, and blockade of endogenous changes. First, since the $[Ca^{2+}]_i$ peak did not precede movement, the initiation of movement occurred when $[Ca^{2+}]_i$ was close to basal levels throughout the cell (although submicroscopic elevations could be significant before we could detect a rise). Dispersion did not occur as the $[Ca^{2+}]_i$ transient returned to basal levels. Second, ionomycin-induced increases of $[Ca^{2+}]_i$ to physiological levels or higher did not aggregate melanosomes. Additionally, low concentrations of digitonin (5 μ M) and penetration with a microneedle promote entry of extracellular calcium into cells, but did not aggregate melanosomes. Dispersion was not produced in an aggregated cell that was exposed to calcium-free Ringer's to decrease $[Ca^{2+}]_i$ (Fig. 1). Third, aggregation was not blocked by eliminating or reversing the rise in $[Ca^{2+}]_i$ with BAPTA or with calcium-free Ringer's containing EGTA. Dispersion was not blocked when elevated $[Ca^{2+}]_i$ was maintained with ionomycin. It is still possible that low $[Ca^{2+}]_i$ is required for movement since we only tested two fold reductions of basal levels, or that Ca^{2+} might participate in ways that are not mimicked by modulating whole cell $[Ca^{2+}]_i$. Despite these qualifications, we conclude that the endogenous $[Ca^{2+}]_i$ rise is neither necessary nor sufficient for bidirectional vesicle movement in angelfish melanophores.

Aggregation in *Pterophyllum* melanophores was not blocked by calcium chelators or by the inhibitors of calmodulin activation, calmidazolium and W-7. Therefore, the calmodulin dependent phosphatase PP-2B (calcineurin) did not participate in aggregation in *Pterophyllum* melanophores as it does in *Tilapia* melanophores (Thaler and Haimo, 1990). The $[Ca^{2+}]_i$ rise could have regulated a decrease in $[cAMP]_i$ via calmodulin activation of type I phosphodiesterase. However, calmodulin inhibitor experiments do not support this possibility. Although the dispersion of melanosomes was induced by near lethal concentrations of calmidazolium and W-7, dispersion cannot be specifically ascribed to calmodulin inhibition and could instead be a result of nonspecific inhibitor effects (Inagaki et al., 1986). We conclude that neither calcium nor calmodulin are essential to the aggregation of melanosomes.

Concerted Melanosome Movement and Maintenance of the Central Pigment Mass

The aggregation of melanosomes requires centripetal movement of vesicles and subsequent maintenance of the tightly aggregated central pigment mass. Hypothetically, a tight

central mass could be maintained by persistent centripetal movement against a tightly packed pigment mass, but other mechanisms probably act to lock melanosomes together as well. The ionomycin-induced loosening of the central mass suggests a second mechanism independent of cAMP-regulated motors. Previous experiments have shown that the tightness of the central mass in angelfish melanophores can be maintained in the absence of microtubules and that the mechanical rigidity of the central mass is reduced in calcium and magnesium-free medium (Schliwa and Bereiter-Hahn, 1975). It is possible that maintenance of the central mass requires tight regulation of $[Ca^{2+}]_i$ homeostasis.

A second, microtubule-independent anchoring of pigment has yet to be elucidated, but in the absence of microtubules, epinephrine promotes melanosomes clustering (Schliwa and Euteneuer, 1978a). Additional evidence of coordinated interactions among melanosomes can be found in experiments that sought to reverse the polarity of microtubules in severed melanophore arms. In cell arms with mixed microtubule polarity, epinephrine-induced movement of melanosomes was uniform and unidirectional and did not reflect the mixed polarity (McNiven et al., 1984). A simple association of one vesicle with one motor and independent movement of each vesicle does not seem consistent with such observations. Low-voltage scanning EM has revealed that melanosomes in *Gymnocorymbus ternetzi* are physically enmeshed in a network of 10-nm intermediate filaments (S-S. Lim, Indiana University, Indianapolis, IN; and H. Ris, University of Wisconsin, Madison, WI, personal communication), and these filaments are candidates for mediating the coordination of melanosome movements. Concerted movement probably reflects interlinked filament networks and coordinated control by messengers. The rapid, global control of $[cAMP]_i$ encourages the collective movement of melanosomes (via dynein and kinesin), and the potential coordination of kinase and phosphatase also would promote concerted movement. $[Ca^{2+}]_i$ also might aid by maintaining the aggregated state.

Receptor Pharmacology of Melanophores

Previous studies on *Pterophyllum scalare* melanophores showed that epinephrine and high concentrations of histamine aggregate melanosomes and that atropine and low concentrations of histamine disperse melanosomes (Egner, 1971). Paradoxically, Egner also showed that acetylcholine induces partial dispersion, the same direction as the muscarinic antagonist, atropine. These studies were performed on intact scales, in which the melanophores were still innervated by neuronal fragments that could have been the immediate pharmacological targets. In a related freshwater angelfish, *Pterophyllum eimeke*, Reed and Finnin (1972) showed that α adrenergic receptors are responsible for aggregation and β receptors stimulate dispersion; atropine has no effect at low concentrations. This study was also performed on intact scales.

To avoid possible stimulation of presynaptic nerve fragments, we studied the effect of various ligands on single cultured melanophores. The threshold for epinephrine-induced aggregation was 5 nM with complete aggregation reliably induced by 50 nM. Epinephrine was blocked by the α_2 antagonist, yohimbine, but not by the α_1 antagonist, prazosin. Dispersion was induced with a thorough washout of epineph-

rine, or by the addition of 100 nM atropine to cells in the continued presence of epinephrine. Dispersed melanosomes and elevated $[cAMP]_i$ are the normal state of unstimulated melanophores. The action of atropine is usually taken to be diagnostic for the presence of a muscarinic receptor, but the muscarinic agonist, carbachol, did not aggregate melanosomes. Muscarinic receptors respond similarly to atropine, scopolamine, and their quaternary derivatives, but in *Pterophyllum*, scopolamine was a poor activator and the charged derivatives were almost without effect. One possibility is that atropine was acting on an intracellular site that was not accessible to the impermeant quaternary compounds (see Brown and Goldstein, 1986). Another possibility is that atropine induced dispersion by acting on an adrenergic receptor. At high concentrations, atropine has been reported to have some adrenergic effects (Weiner, 1985). Atropine might act by antagonizing the binding of epinephrine to α_2 receptors in angelfish, but this hypothesis remains untested.

We tested for the presence of β adrenergic receptors on *Pterophyllum scalare* melanophores. Multiple adrenergic receptor types can be present on the same cell (Hughes et al., 1982) and β -adrenergic receptors are present on other angelfish. In some melanophores, β ligands, (including epinephrine), are known to induce dispersion at moderate concentrations and aggregation at high concentrations (Miyashita and Fujii, 1975). However, in our hands, the β agonist, isoproterenol, had no effect. Atropine-mediated dispersion was not mediated through a β receptor since its action could not be blocked by β antagonists.

Does $[Ca^{2+}]_i$ Regulate Microtubule Dynamics?

If the $[Ca^{2+}]_i$ rise is not important for motility, could it play a role in a parallel cellular function? There are other morphological and functional changes that occur during aggregation that were not assayed by us and that could be either cAMP or Ca^{2+} dependent. For example, either the rate or frequency of microtubule shortening in vivo might be regulated (Sammak and Borisov, 1988) independently of melanosome movement. In *Pterophyllum* melanophores (but not in all chromatophores), the number of microtubules is reduced by nearly half during the process of aggregation (Schliwa, 1975; Schliwa and Euteneuer, 1978b), and the nucleating capacity of the microtubule organizing center is also reduced (Schliwa, 1979). The loss of microtubule nucleating capacity that is seen during melanosome aggregation is also apparent during mitosis where protein phosphorylation alters microtubule dynamics. In *Xenopus* egg extracts, the mitotic changes in microtubule dynamics are produced by the p34^{cdc2} kinase, are independent of the cAMP-dependent protein kinase, and are blocked by PP-1 (Verde et al., 1990). The polymerization and depolymerization rates are similar in mitosis and interphase extracts, but the frequency of shrinking is higher in mitosis (Belmont et al., 1991). The Ca^{2+} dependence of these changes is unknown, but $[Ca^{2+}]_i$ might influence microtubule dynamics (Schliwa et al., 1981; Izant, 1983) and is required for entry into mitosis (Tombs and Borisov, 1989; Kao et al., 1990). The regulation of microtubule dynamics by phosphorylation during mitotic spindle formation might be similar to that in *Pterophyllum* melanophores.

How General Is the cAMP Regulation of Microtubule-based Intracellular Motility?

Can the $[cAMP]_i$ regulated motility in angelfish be extrapolated to other teleosts? The second messenger in several chromatophores including *Holocentrus* and *Xiphophorus* is $[Ca^{2+}]_i$. However, in *Holocentrus*, motility is 10-fold faster than in *Pterophyllum* and might be mediated by other motor proteins, and in *Xiphophorus*, motility is partly actin dependent. In *Tilapia*, where rates are similar to *Pterophyllum*, $[Ca^{2+}]_i$ regulates the phosphatase, but $[cAMP]_i$ regulates the kinase. The diversity of chromatophore motility regulation reflects the variety of mechanochemical systems that are used. However, among those cells that clearly depend on microtubule motors, cAMP dependent protein kinase might be a common thread of the regulatory system of motor proteins.

What proteins are regulated by the cAMP dependent kinase? Since *Pterophyllum* motility is clearly microtubule based (Schliwa, 1975), it is likely that the proteins ultimately responsible for dispersion and aggregation are kinesin, (Rodionov, 1991), and dynein (Clark and Rosenbaum, 1982; Beckerle and Porter, 1982; Ogawa et al., 1987). The immediate substrate of the catalytic subunit is not known in *Pterophyllum*, but in *Tilapia*, a 57-kD protein is phosphorylated during dispersion (Rozdzial and Haimo, 1986). cAMP-dependent phosphorylation of a 56 kD protein (identified as R^{II}), leads to the activation of sea urchin sperm dynein and increased microtubule gliding in vitro (Tash, 1989). Perplexingly, it is a dephosphorylation that activates dynein in melanophores and in *Chlamydomonas* flagella. There are several axonemal peptides whose phosphorylation is motility dependent, including several components of dynein (Tash, 1989), and it is possible that melanophore dynein is also activated by phosphorylation of associated peptides.

Could cAMP regulate motility in mammalian cells? The anterograde movement of neuronal parcels in chick axons is enhanced in the presence of 8-bromo cAMP or forskolin, suggesting that kinesin-dependent motility in neurons might be regulated by cAMP (R. L. Morris and P. J. Hollenbeck, Harvard Medical School, personal communication; and *J. Cell Biol.* 111:414a). It is not known if phosphorylation specifically alters the ATPase activity of kinesin in vitro. Phosphorylation also could act by altering the activity of competing motor proteins or by affecting binding of motor proteins to vesicles (McIntosh and Porter, 1989). An example of competing motors is found in mitotic movements where phosphorylation regulates dynein and an unnamed plus end-directed motor on kinetochores (Hyman and Mitchison, 1991). An example of phosphorylative control of vesicle binding is found in *Xenopus* extracts where organelle motility is altered by a phosphorylation-associated reduction of vesicle binding to motors during mitosis (Allan and Vale, 1991). Although the specific mechanisms that regulate the direction and the prevalence of intracellular transport are obscure, cAMP, and not calcium, could be a component of the control of microtubule based movement in the cytoplasm.

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References

- Adams, S. R., A. T. Harootyan, Y. J. Buechler, S. S. Taylor, and R. Y. Tsien. 1991. Fluorescence ratio imaging of cyclic AMP in single cells. *Nature (Lond.)*. 349:694-697.
- Allan, V. J., and R. D. Vale. 1991. Cell cycle control of microtubule-based membrane transport and tubule formation in vitro. *J. Cell Biol.* 113:347-359.
- Allen, R. D., J. Metzels, I. Tasaki, S. Brady, and S. Gilbert. 1982. Fast axonal transport in squid giant axon. *Science (Wash. DC)*. 218:1127-1128.
- Beckerle, M., and K. R. Porter. 1982. Inhibitors of dynein activity block intracellular transport in erythrocytes. *Nature (Lond.)*. 195:701-703.
- Belmont, L. D., A. A. Hyman, E. E. Sawin, and T. J. Mitchison. 1991. Real-time visualization of cell cycle-dependent changes in microtubule dynamics in cytoplasmic extracts. *Cell*. 62:579-589.
- Bialojan, C., and A. Takai. 1988. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. *Biochem. J.* 256:283-290.
- Bomsel, M., R. Parton, S. A. Kuznetsov, T. A. Schroer, and J. Gruenberg. 1991. Microtubule- and motor-dependent fusion in vitro between apical and basolateral endocytic vesicles from MDCK cells. *Cell*. 62:719-731.
- Brown, J. H., and D. Goldstein. 1986. Analysis of cardiac muscarinic receptors recognized selectively by nonquaternary but not quaternary ligands. *J. Pharm. Exp. Ther.* 238:580-586.
- Cheng, H.-C., B. E. Kemp, R. B. Pearson, A. J. Smith, L. Misconi, S. M. Van Patten, and D. A. Walsh. 1986. A potent synthetic peptide inhibitor of the cAMP-dependent protein kinase. *J. Biol. Chem.* 261:989-992.
- Chou, S.-C., J. D. Taylor, and T. T. Tchen. 1989. Epinephrine-induced pigment aggregation in goldfish melanophore cells: apparent involvement of an unknown second messenger. *Pigment Cell Res.* 2:414-420.
- Clar, T. C., and J. L. Rosenbaum. 1982. Pigment particle translocation in detergent-permeabilized melanophores of *Fundulus heteroclitus*. *Proc. Natl. Acad. Sci. USA*. 79:4655-4659.
- Cohen, P. 1989. The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* 58:453-508.
- Cohen, P., C. F. B. Holmes, and Y. Tsukitani. 1990. Okadaic acid: a new probe for the study of cellular regulation. *Trends Biochem. Sci.* 15:98-102.
- Cooper, M. S., A. H. Cornell-Bell, A. Chernjavsky, J. W. Dani, and S. J. Smith. 1990. Tubulovesicular processes emerge from trans-Golgi cisternae, extend along microtubules, and interlink adjacent trans-golgi elements into a reticulum. *Cell*. 61:135-145.
- Egner, O. 1971. Zur physiologie der melanosomenverlagerung in den melanophoren von *Pterophyllum scalare* Cuv. u. Val. *Cytobiologie*. 4:262-292.
- Exton, J. H. 1985. Mechanisms involved in alpha-adrenergic phenomena. *Am. J. Physiol.* 248:E633-E647.
- Fujii, R., and Y. Miyashita. 1975. Receptor mechanisms in fish chromatophores-I. Alpha nature of adrenoreceptors mediating melanosome aggregation in guppy melanophores. *Comp. Biochem. Physiol.* 51C:171-178.
- Fujii, R., and N. Oshima. 1986. Control of chromatophore movements in teleost fishes. *Zool. Sci.* 3:13-47.
- Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440-3450.
- Hollenbeck, P. J., and J. A. Swanson. 1990. Radial extension of macrophage tubular lysosomes supported by kinesin. *Nature (Lond.)*. 346:864-866.
- Hughes, R. J., M. R. Boyle, R. D. Brown, P. Taylor, and P. Insel. 1982. Characterization of coexisting alpha₁ and beta₂-adrenergic receptors on a cloned muscle cell line, BC3H-1. *Mol. Pharm.* 22:258-266.
- Hyman, A. A., and T. J. Mitchison. 1991. Two different microtubule-based motor activities with opposite polarities in kinetochores. *Nature (Lond.)*. 351:206-211.
- Inagaki, M., S. Kawamoto, H. Itoh, M. Saitoh, M. Hagiwara, J. Takahashi, and H. Hidaka. 1986. Naphthalenesulfonamides as calmodulin antagonists and protein kinase inhibitors. *Mol. Pharm.* 29:577-581.
- Izant, J. 1983. The role of calcium ions during mitosis. *Chromosoma*. 88:1-10.
- Jurgensen, S. R., P. B. Chock, S. Taylor, J. R. Vanderheede, and W. Merlevede. 1985. Inhibition of the Mg(II)ATP-dependent phosphoprotein phosphatase by the regulatory subunit of cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA*. 82:7565-7569.
- Kao, J. P. Y., J. M. Alderton, R. Y. Tsien, and R. A. Steinhardt. 1990. Active involvement of Ca²⁺ in mitotic progression of Swiss 3T3 fibroblasts. *J. Cell Biol.* 111:183-196.
- Kumazawa, T., and R. Fujii. 1984. Concurrent release of norepinephrine and purines by potassium from adrenergic melanosome-aggregating nerve in tilapia. *Comp. Biochem. Physiol.* 78C:263-266.
- Lerner, M. R., J. Reagan, T. Gyorgyi, and A. Roby. 1988. Olfaction by melanophores: what does it mean? *Proc. Natl. Acad. Sci. USA*. 85:261-264.
- Limbird, L. L. 1988. Receptors linked to inhibition of adenylate cyclase: additional signaling mechanisms. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 2: 2686-2695.
- Luby-Phelps, K., and K. R. Porter. 1982. The control of pigment migration in isolated erythrocytes of *Holocentrus ascensionis* (Osbeck) II. The role of calcium. *Cell*. 29:441-450.
- Lynch, T. J., B.-y. Wu, J. D. Taylor, and T. T. Tchen. 1986. Regulation of pigment organelle translocation II. Participation of a cAMP-dependent protein kinase. *J. Biol. Chem.* 261:4212-4216.
- McIntosh, J. R., and M. E. Porter. 1989. Enzymes for microtubule-dependent motility. *J. Biol. Chem.* 264:6001-6004.
- McNiven, M. A., M. Wang, and K. R. Porter. 1984. Microtubule polarity and the direction of pigment transport reverse simultaneously in surgically severed melanophore arms. *Cell*. 37:753-765.
- McNiven, M. A., and J. B. Ward. 1988. Calcium regulation of pigment transport in vitro. *J. Cell Biol.* 106:111-125.
- Michel, M. C., L. F. Brass, A. Williams, G. M. Bokoch, V. J. LaMorte, and H. J. Motulsky. 1989. α₂-adrenergic receptor stimulation mobilizes intracellular Ca²⁺ in human erythroleukemia cells. *J. Biol. Chem.* 264:4986-4991.
- Miller, P., U. Walter, W. E. Theurkauf, R. B. Vallee, and P. De Camilli. 1982. Frozen tissue sections as an experimental system to reveal specific binding sites of the regulatory subunits of type II cAMP-dependent protein kinase in neurons. *Proc. Natl. Acad. Sci. USA*. 79:5562-5566.
- Miyashita, Y., and R. Fujii. 1975. Receptor mechanisms on fish chromatophores-II. evidence for beta adrenoreceptors mediating melanosome dispersion in guppy melanophores. *Comp. Biochem. Physiol.* 51C:179-187.
- Murphy, D. B., and L. G. Tilney. 1974. The role of microtubules in the movement of pigment granules in teleost melanophores. *J. Cell Biol.* 61:757-779.
- Novales, R. R., and R. Fujii. 1970. A melanin-dispersing effect of cyclic adenosine monophosphate on *Fundulus melanophores*. *J. Cell. Physiol.* 75:133-136.
- Ogawa, K., H. Hosoya, E. Yokota, T. Kobayashi, Y. Wakamatsu, and K. Ozato. 1987. Melanoma dynein: evidence that dynein is a general "motor" for microtubule-associated cell motilities. *Eur. J. Cell Biol.* 43:3-9.
- Oshima, N., M. Suzuki, N. Yamaji, and R. Fujii. 1988. Pigment aggregation is triggered by an increase in free calcium ions within fish chromatophores. *Comp. Biochem. Physiol.* 91A:27-32.
- Palazzo, R. E., T. J. Lynch, J. D. Taylor, and T. T. Tchen. 1989. cAMP-independent and cAMP-dependent protein phosphorylations by isolated goldfish xanthophore cytoskeletons: evidence for the association of cytoskeleton with a carotenoid droplet protein. *Cell Motil. Cytoskeleton*. 13:21-29.
- Paschal, B. M., S. M. King, A. G. Moss, C. A. Collins, R. B. Vallee, and G. B. Witman. 1987. Isolated flagellar outer arm dynein translocates brain microtubules in vitro. *Nature (Lond.)*. 330:672-674.
- Poenie, M. 1990. Alteration of intracellular fura-2 fluorescence by viscosity: a simple correction. *Cell Calcium*. 11:85-91.
- Rangel-Aldao, R., and O. M. Rosen. 1977. Effect of cAMP and ATP on the reassociation of phosphorylated and nonphosphorylated subunits of the cAMP-dependent protein kinase from bovine cardiac muscle. *J. Biol. Chem.* 252:7140-7145.
- Reed, B. L., and B. C. Finnin. 1972. Adrenergic innervation of melanophores in a teleost fish. In *Pigmentation: Its Genesis and Biologic Control*. V. Riley, editor. Appleton-Century-Crofts, New York. 285-294.
- Rivas, R. J., and H.-P. H. Moore. 1989. Spatial segregation of the regulated and constitutive secretory pathways. *J. Cell Biol.* 109:51-60.
- Rodionov, V. I., F. K. Gyoeva, and V. I. Gelfand. 1991. Kinesin is responsible for centrifugal movement of pigment granules in melanophores. *Proc. Natl. Acad. Sci. USA*. 88:4956-4960.
- Rozdzial, M. M., and L. T. Haimo. 1986. Bidirectional pigment granule movements of melanophores are regulated by protein phosphorylation and dephosphorylation. *Cell*. 47:1061-1070.
- Sammak, P. J., and G. G. Borisy. 1988. Direct observation of microtubule dynamics in living cells. *Nature (Lond.)*. 332:724-726.
- Scheline, R. R. 1963. Adrenergic mechanisms on fish: chromatophore pigment concentration in the cuckoo wrasse, *Labrus ossifragus* L. *Comp. Biochem. Physiol.* 9:215-227.
- Schliwa, M. 1975. Microtubule distribution and melanosome movements in fish melanophores. In *Microtubules and Microtubule Inhibitors*. M. Borgers and M. de Brabender, editors. Elsevier Science Publishers, Amsterdam. 215-228.
- Schliwa, M., and J. Bereiter-Hahn. 1973. Pigment movements in fish melanophores: morphological and physiological studies. III. The effects of colchicine and vinblastine. *Z. Zellforsch. Mikrosk. Anat.* 147:127-147.
- Schliwa, M., and J. Bereiter-Hahn. 1975. Pigment movements in fish melanophores: morphological and physiological studies. V. Evidence for a microtubule-independent contractile system. *Cell Tiss. Res.* 158:61-73.

- Schliwa, M., and U. Euteneuer. 1978a. A microtubule-independent component may be involved in granule transport in pigment cells. *Nature (Lond.)*. 273:556-558.
- Schliwa, M., and U. Euteneuer. 1978b. Quantitative analysis of the microtubule system in isolated fish melanophores. *J. Supramol. Struct.* 8:177-190.
- Schliwa, M., U. Euteneuer, W. Herzog, and K. Weber. 1979. Evidence for rapid structural and functional changes of the melanophore microtubule-organizing center upon pigment movements. *J. Cell Biol.* 83:623-632.
- Schliwa, M., U. Euteneuer, J. C. Bulinski, and J. G. Izant. 1981. Calcium lability of cytoplasmic microtubules and its modulation by microtubule-associated proteins. *Proc. Natl. Acad. Sci. USA*. 78:1037-1041.
- Schliwa, M., K. Weber, and K. R. Porter. 1981. Localization and organization of actin in melanophores. *J. Cell Biol.* 8:267-275.
- Tash, J. S. 1989. Protein phosphorylation: the second messenger signal transducer of flagellar motility. *Cell Motil. & Cytoskeleton*. 14:332-339.
- Terasaki, M. 1990. Recent progress on structural interactions of the endoplasmic reticulum. *Cell Motil. & Cytoskeleton*. 15:71-75.
- Thaler, C. D., and L. T. Haimo. 1990. The regulation of organelle transport in melanophores by calcineurin. *J. Cell Biol.* 111:1939-1948.
- Tombes, R. M., and G. G. Borisy. 1989. Intracellular free calcium and mitosis in mammalian cells: anaphase onset is calcium modulated, but is not triggered by a brief transient. *J. Cell Biol.* 109:627-636.
- Tsien, R. Y., and T. Rink. 1983. Measurement of free Ca^{2+} in cytoplasm. In *Current Methods in Cellular Neurobiology*. J. L. Barker and J. McKelvy, editors. John Wiley & Sons Inc., New York. 249-312.
- Tsien, R., and T. Pozzan. 1989. Measurement of cytosolic free Ca^{2+} with quin2. *Methods Enzymol.* 172:230-262.
- Tsien, R. Y., and A. T. Harootunian. 1990. Practical design criteria for a dynamic ratio imaging system. *Cell Calcium*. 11:93-109.
- Vale, R. D., T. S. Reese, and M. P. Sheetz. 1985. Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell*. 41:39-50.
- Verde, F., J-c. Labbé, M. Dorée, and E. Karcenti. 1990. Regulation of microtubule dynamics by cdc2 protein kinase in cell-free extracts of *Xenopus* eggs. *Nature (Lond.)*. 343:233-238.
- Weiner, N. 1985. Atropine, scopolamine and related anti-muscarinic drugs. In *Goodman and Gilman's The Pharmacological Basis of Therapeutics*. 7th ed. A. Goodman Gilman, L. S. Goodman, T. W. Rall, and F. Murad, editors. Macmillan Publishing Co. New York. 130-144.
- Winchester, J. D., F. Ngo, T. T. Tchen, and J. D. Taylor. 1976. Hormone-induced dispersion or aggregation of carotenoid-containing smooth endoplasmic reticulum in cultured xanthophores from the goldfish, *Carrasius auratus* L. *Endocr. Res. Commun.* 3:335-342.